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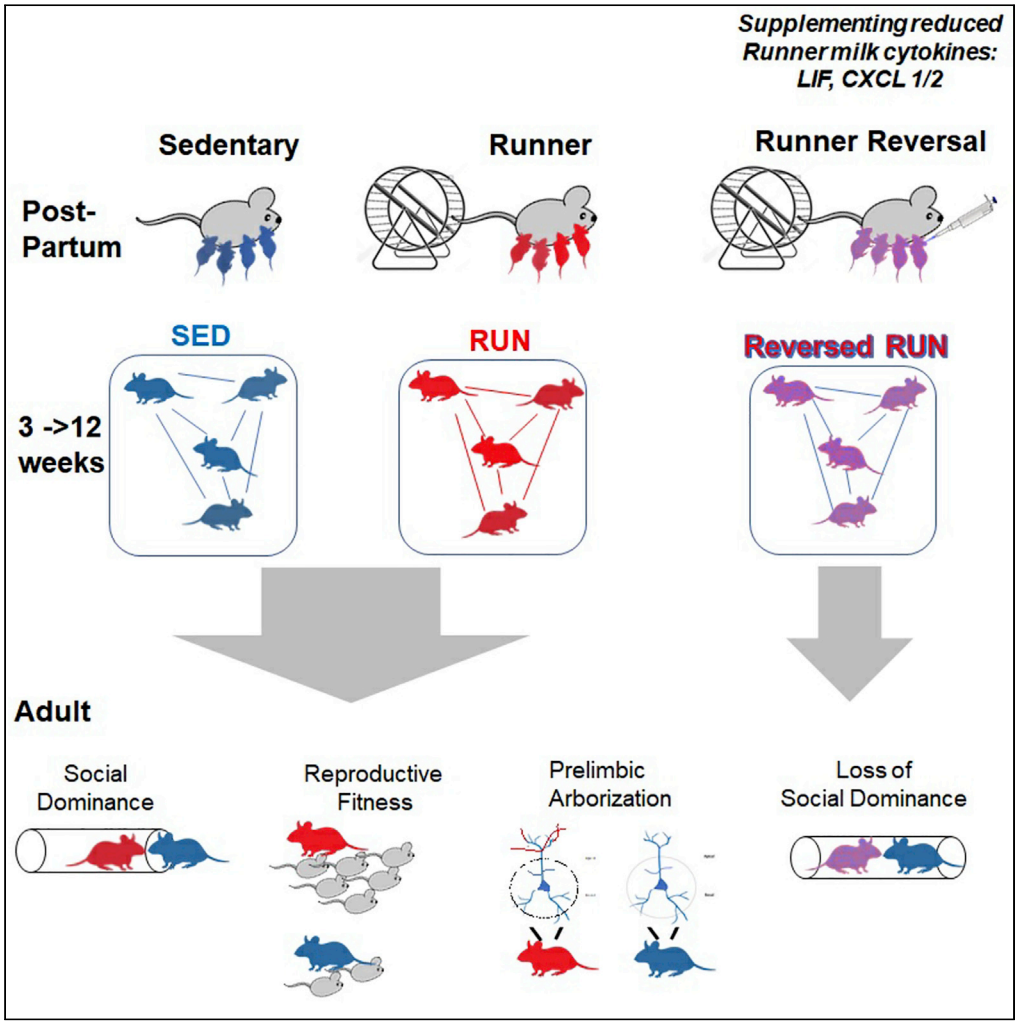

Faten Taki, Katherine Lopez, Bojana Zupan, ..., David E. Cohen, Marcel R.M. van den Brink, Miklos Toth

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HIGHLIGHTS

Maternal postpartum wheel running enhances male offspring social dominance

Maternal postpartum running promotes offspring reproductive fitness

Maternal postpartum running has no effect on maternal care and gut microbiota

Maternal running programs offspring social dominance via specific milk cytokines

Taki et al., iScience *23*, 101357
August 21, 2020
https://doi.org/10.1016/j.isci.2020.101357

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Maternal Programming of Social Dominance via Milk Cytokines

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SUMMARY

Regular physical activity improves physical and mental health. Here we found that the effect of physical activity extends to the next generation. Voluntary wheel running of dams, from postpartum day 2 to weaning, increased the social dominance and reproductive success, but not the physical/metabolic health, of their otherwise sedentary offspring. The individual's own physical activity did not improve dominance status. Maternal exercise did not disrupt maternal care or the maternal and offspring microbiota. Rather, the development of dominance behavior in the offspring of running mothers could be explained by the reduction of LIF, CXCL1, and CXCL2 cytokines in breast milk. These data reveal a cytokine-mediated lactocrine pathway that responds to the mother's postpartum physical activity and programs offspring social dominance. As dominance behaviors are highly relevant to the individual's survival and reproduction, lactocrine programming could be an evolutionary mechanism by which a mother promotes the social rank of her offspring.

INTRODUCTION

Maternal gestational physiology and environment have a major influence on offspring brain development and function (Toth, 2014). It is well established that stress, infection, and malnutrition during pregnancy contribute, via transplacental mechanisms, to the development of cognitive, emotional, reward, and social abnormalities in the progeny, including anxiety, depression, schizophrenia, and autism spectrum disorders (Painter et al., 2005; Hoek et al., 1998; Gangi et al., 2009; Scharf, 2007; Abdallah et al., 2013; Brown, 2012; Zerbo et al., 2013; Atladóttir et al., 2010; Malkova et al., 2012; Patterson, 2009; Smith et al., 2007; Reynaert et al., 2016; Cordero et al., 2012). Maternal effects during the postpartum period, given the length and intricacy of mother-infant interaction, are equally important in both human and other mammals. In particular, maternal influences are important for functions that develop during the early postnatal period, such as attention, executive functions, and social behavior (Chen and Baram, 2016).

Maternal programming of offspring behavior during the postpartum period in rodents has primarily been focused on the effect of maternal care. Over two decades ago, maternal care was identified as a major influence on the cognitive performance and overall anxiety of the adult offspring (Liu et al., 1997, 2000; Meaney, 2001; Weaver et al., 2006). However, maternal care may not be the only postnatal mechanism influencing adult offspring behavior. For example, breastmilk provides not only nutrients but also a plethora of biologically active compounds, including cytokines and growth factors (Dvorak, 2010; Garofalo, 2010; Zhang et al., 2016) that, via gut-brain communication (Powell et al., 2017; Foster et al., 2017; Fung et al., 2017), may influence brain development and behavior (Walfisch et al., 2013; Lucas et al., 1992, 1994). Indeed, some studies suggested the beneficial effect of breast-feeding over formula on the cognitive and emotional development of full and preterm infants (Lucas et al., 1992; Der et al., 2006; Kramer et al., 2008; Yang et al., 2018). However, given the design limitations of human studies, disentangling possible behavioral effects of milk bioactive compounds from socioeconomic and demographic confounds is difficult, if not impossible. Animal experiments allow causative studies, and we previously reported that reducing the levels of tumor necrosis factor (TNF)-α-regulated proinflammatory cytokines in the breast milk of dams improves the cognitive performance of their offspring (Liu et al., 2014). We now asked if the concept of milk cytokine-mediated

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https://doi.org/10.1016/j.isci.2020.101357

(lactocrine) programming of behavior can be extended to more physiological situations and beyond a TNF-α-specific mechanism.

As voluntary wheel running approximates activity in the real world and is known to suppress the production of proinflammatory cytokines (Gleeson et al., 2011), here we tested if voluntary exercise of mothers during the lactation period results in improved adult cognitive, emotional, and social behaviors in the otherwise sedentary offspring. Although the beneficial effects of exercise on fitness and brain health have been extensively studied (Cotman and Berchtold, 2002; Kempermann et al., 2010), only a few studies have investigated the intergenerational effect of maternal exercise. Voluntary wheel running during gestation was reported to increase offspring neurogenesis at birth and activity during adulthood (Eclarinal et al., 2016; Bick-Sander et al., 2006). However, it is not known if exercise, strictly during the postpartum period, has an influence on offspring behavior. A further motivation for selecting exercise as a maternal factor to study offspring outcomes was that physical activity is no longer a necessity in daily life and thus sedentary lifestyle of mothers may affect a substantial proportion of the population. Here we report that maternal exercise during the postpartum period, by altering the cytokine composition of the breastmilk, affects offspring social dominance, a behavior highly relevant to the individual's survival and reproduction.

RESULTS

Voluntary Wheel Running of Mothers during the Lactating Period Increases the Social Dominance of the Male Offspring

C57BL/6Tac females, raised and kept in standard (sedentary) cages, were mated, and 2 days after delivery, they were randomly transferred with their pups to cages with running wheels or to standard (sedentary) cages (Figure 1A). Maternal exercise was limited to the lactating period (i.e., no running wheel during gestation). Offspring of running mothers (Run) and offspring of sedentary mothers (Sed) were weaned at day 21 and housed in standard cages separately in groups of 3–5 until behavioral testing at 12–15 weeks of age. Thus all offspring, whether Run or Sed, were kept under sedentary conditions. Mothers readily used the wheels, primarily during the dark period, running an average of 3,164.48 m per dark period and only 98.73 m during the light period, consistent with the nocturnal circadian rhythm of rodents. During the "low activity" light period, maternal care, as assessed by several endpoints that included the two major postures arched back nursing (ABN) and licking/grooming (LG) (Figure 1B), was high and comparable between running and sedentary mothers. ABN and LG are fundamentally important in the proper behavioral and neuroendocrine development of murine offspring (Liu et al., 1997; Fenoglio et al., 2006). During the dark period, maternal care, in particular ABN, of both runner and sedentary mothers was significantly reduced and was comparable between the two groups. In addition, we assessed possible changes in the quantity of maternal care (Baram et al., 2012) but found no difference in "maternal absence" (OP = off the pups, Figure 1B) between runner and sedentary mothers. This suggests that the time sedentary mothers normally spend away from the pups is used for exercise by the running mothers. Furthermore, maternal running may cause fragmented care, resulting in later life anxiety and stress sensitivity (Baram et al., 2012). However, anxiety-like behavior of the Run offspring was comparable to that of the Sed offspring (Figure 2I), and maternal running did not increase the vulnerability of the offspring to chronic social defeat stress (CSDS) (Figure S1). Finally, average litter size and pup survival (from the beginning of maternal running at P2 to weaning at P21), and postnatal weight gain (between P2 and P56) of the Run and Sed offspring were comparable (Figures 1C and 1D), indicating that nourishment of pups and their development were not disrupted by maternal running and/or the presence of the running wheel.

To assess the consequences of maternal postpartum physical activity on offspring behavioral fitness, 12- to 15-week-old Run and Sed offspring were assessed in a behavioral test battery. We tested two to three randomly selected offspring from each mother and performed both offspring and litter-based comparisons. We found no effect of maternal postpartum exercise on offspring locomotor activity and cognitive (spatial memory) and emotional (anxiety-like) behavior (Figures 2I, 2J, 5C, and 5D), but noticed differences in social dominance, assessed as "winning" or "losing" in direct competition in a narrow tube, i.e., in tube test (Lindzey et al., 1961). As Figure 1A shows, social dominance has two basic forms, "hierarchical dominance," when mice kept together in a cage establish social dominance hierarchy, and "situational dominance," when two individuals with no prior experience of each other's ability to dominate compete (Tibbetts and Dale, 2007; Wang et al., 2014). Rank in hierarchical dominance reflects prior intra-cage winning and losing (Zhou et al., 2017), whereas dominance between strangers is based on signals that correlate with an individual's fighting ability (Hurst et al., 2001). Although most research focused on

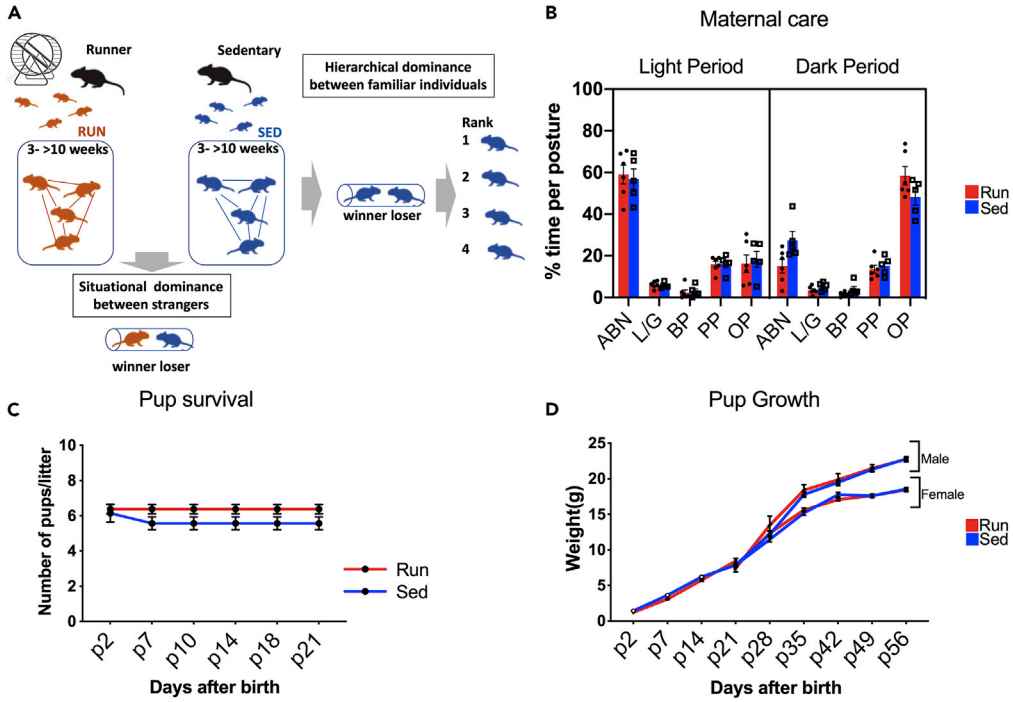

Figure 1. Females Extensively Use the Running Wheel Postpartum, but Postpartum Running Does Not Affect Maternal Care and the Survival and Physical Development of the Offspring

(A) Experimental design to generate Run and Sed offspring and to assess situational and hierarchical dominance in the tube test.

(B) High maternal care, in particular ABN, during the light period. Averages of various maternal care-related behaviors, measured at postnatal day (P) 3, 5, 7, 9, 11, 13, and 15 are displayed. L/G, licking/grooming; ABN, arched back nursing; BP, blanket posture; PP, passive posture; OP, off the pups. No overall effect of maternal activity (Run versus Sed) and no difference between runner and sedentary mother's individual care behaviors. Three-way ANOVA. Main effect of maternal activity, $F(1,45) = 0.416$, $p = 0.522$, Interaction between maternal activity × maternal care behavior, $F(4,45) = 1.325$, $p = 0.275$, $N = 5$ (Run mother), 6 (Sed mother). No significant difference in individual care behaviors after correction for multiple testing; e.g., dark phase ABN runner versus ABN sedentary $p = 0.2118$.

(C) Pup survival was not impacted by maternal running. Two-way ANOVA. Repeated measures. Main effect of groups: $F(1,13) = 2.452$, $p = 0.141$. $N = 8$ (Run), 7 (Sed).

(D) Weight gain during the postnatal (days 3–21) and juvenile/adolescent (days 21–56) periods was comparable in the Run and Sed groups. Two-way ANOVA. Main effect of groups: Females, $F(1,157) = 0.205$, $p = 0.652$. $N = 14$ (Run), 15 (Sed). Males, $F(1,163) = 0.963$, $p = 0.328$. $N = 14$ (Run), 20 (Sed). Data are represented as mean ± SEM.

hierarchical dominance between cage mates, situational dominance between stranger mice is ecologically more relevant. Wild mice live in territories inhabited by one adult male, several females, and their offspring (Kappel et al., 2017). Although laboratory mice have been bred for many generations, studies indicate that they continue to exhibit wild-type behaviors under naturalistic conditions (Kappel et al., 2017). As the outcome of tube test competition between strangers could be influenced by the individuals' hierarchical rank within their own group, we randomly selected two to three individuals from Run and Sed cages for testing (Figure 1A). To minimize prior experience of winning or losing in the tube test, all mice were naive. As Figure 2A shows, Run offspring exhibited increased male-to-male social dominance in direct pairwise competitions with unfamiliar and age- and weight-matched Sed offspring (Run versus Sed offspring $t(41) = 4.693$, $^*p < 0.0001$). Of note, individuals participated in only two to three competitions and their wins were weighed according to the wins of their contestants (Clutton-Brock et al., 1979). Tube test dominance of Run offspring was still present when litter (instead of offspring) dominance scores were compared (Run versus Sed litters $t(14) = 2.994$, $p = 0.001$), indicating no "litter effects," such as differences in prenatal environment and litter size, on behavior. In contrast to males, dominance of females in tube test was not influenced by maternal postpartum activity (Run versus Sed offspring $t(14) = 0.544$, $p = 0.595$; Run versus Sed litters $t(11) = 0.774$, $p = 0.455$), indicating that maternal programming of offspring tube test dominance is male specific (Figure 2B). Male offspring dominance was not enhanced by the mother when the running

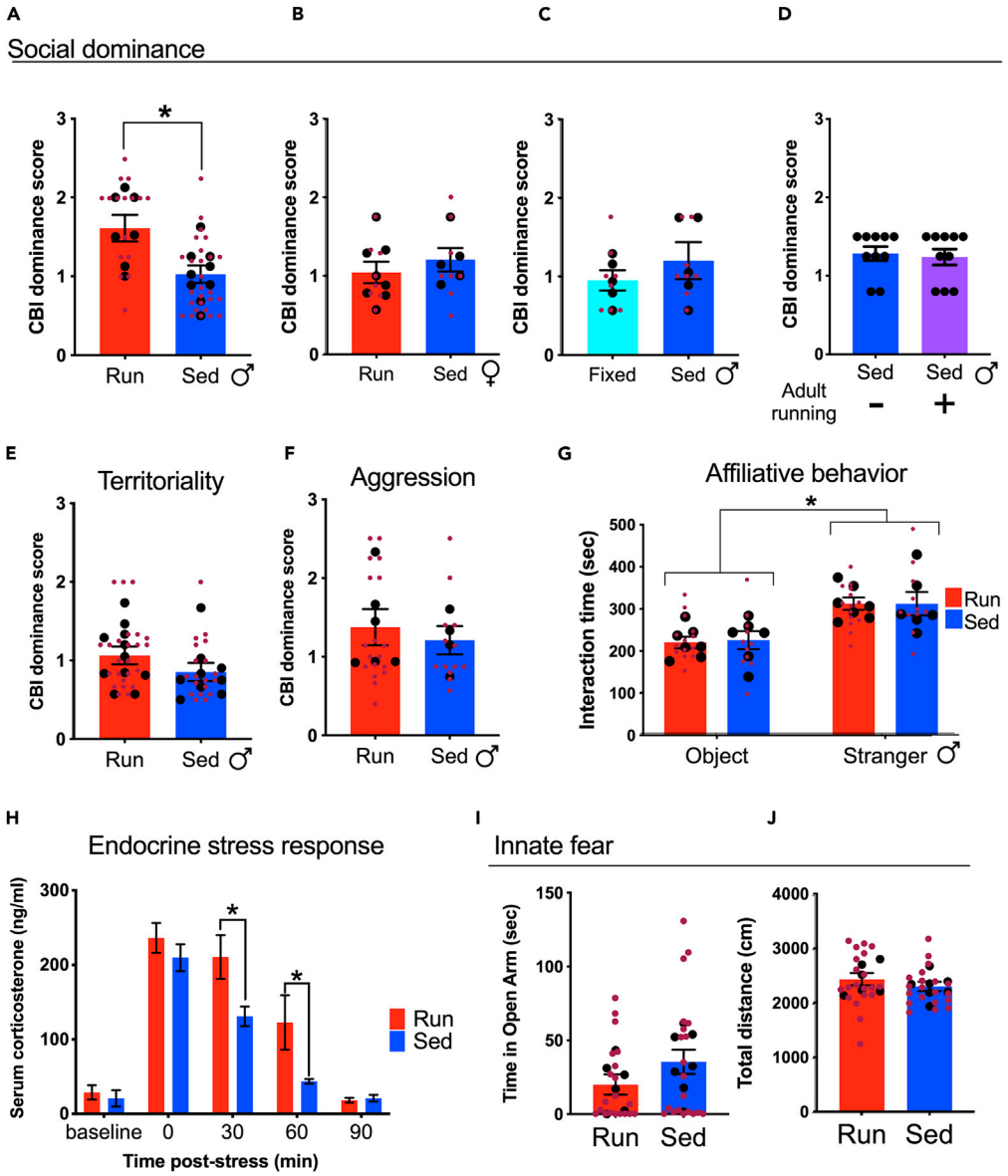

Figure 2. Maternal Postpartum Running Increases the Tube Test Dominance of Their Offspring

(A) Run male offspring had higher Clutton Brock index (CBI) in the tube test, reflecting their dominance over Sed mice. Small circles signify individual offspring values. t test, t(41) = 4.693, *p < 0.0001, N = 16 (Run), 27 (Sed). Large black circles denote litter averages, t(14) = 2.994, *p = 0.0097, N = 7 (Run), 9 (Sed).

(B) Run female offspring had similar CBI as their Sed counterparts, suggesting no effect of maternal postpartum running on female social dominance in the tube test. Small circles signify individual offspring values, t test, t(14) = 0.544, p = 0.5947, N = 8 (Run), 8 (Sed). Large black circles denote litter averages, t(11) = 0.774, p = 0.4555, N = 8 (Run), 5 (Sed).

(C) Male offspring raised by mothers with access to a locked wheel showed CBI dominance score similar to that of Sed offspring. Small circles signify individual offspring values, t test, t(14) = 0.812, p = 0.4303, N = 8 (Fixed wheel), 8 (Sed). Large black circles denote litter averages, t(8) = 0.936, p = 0.3768, N = 5 (Fixed), 5 (Sed).

(D) Adult running for 4 weeks does not increase social dominance in the tube test (based on the number of offspring because running applies to individual adult animals), t test, t(18) = 0.335, p = 0.7415, N = 10 (Sed), 10 (Sed with running wheels).

(E and F) Run male offspring were not different from Sed mice in (E) territorial marking and (F) aggression. Territorial behavior: small circles signify individual offspring values, t test, t(41) = 1.629, p = 0.111, N = 24 (Run), 19 (Sed); large black circles denote litter averages, t(18) = 1.3, p = 0.2101, N = 11 (Run), 9 (Sed). Aggression: offspring, t test, t(29) = 0.7589, p = 0.4541, N = 18 (Run), 13 (Sed); litter, t(8) = 0.5164, p = 0.6195, N = 6 (Run), 4 (Sed).

Figure 2. *Continued*

(G) Maternal postpartum running has no effect on offspring affiliative behavior in the three-chamber test. Run and Sed male offspring had a comparable preference for a caged male stranger over an empty cage. Small circles signify individual offspring values, two-way ANOVA, effect of stranger, $F(1,46) = 23.630$, *$p < 0.0001$, N = 14 (Run), 11 (Sed). Large black circles denote litter averages, effect of stranger, $F(1,22) = 20.82$, *$p = 0.0002$, N = 7 (Run), 6 (Sed).

(H) Increased restraint stress-induced serum corticosterone levels in Run male offspring at 30 and 60 min after a 10-min restraint stress. Two-way ANOVA, N = 5 (Run), 5 (Sed), post-hoc, Sidak's multiple comparison correction, *$p = 0.0184$, time 60 min *$p = 0.0198$.

(I) Maternal postpartum running does not alter the innate fear/anxiety-like behavior of male offspring. Run and Sed offspring spent comparable time in the open arm of the elevated plus maze indicating that Run males do not perceive the open arm more aversive and anxiety-inducing than Sed males. t test; small circles denote offspring values, $t(38) = 1.4$, $p = 0.3105$ (p-adjusted based on Holm-Sidak method), N = 20 (Run), 20 (Sed); large black circles denote litter averages, $t(11) = 1.411$, $p = 0.4606$ (p-adjusted based on Holm-Sidak method), N = 6 (Run), 7 (Sed).

(J) Run and Sed offspring covered similar total distances in the elevated plus maze. t test; offspring $t(38) = 0.868$, $p = 0.391$ (p-adjusted based on Holm-Sidak method), N = 20 (Run), 20 (Sed); litter, $t(11) = 0.9546$, $p = 0.4606$ (p-adjusted based on Holm-Sidak method), N = 6 (Run), 7 (Sed). Data represented as mean ± SEM.

wheel was locked during the entire postpartum period (offspring $t(14) = 0.812$, $p = 0.430$; litter $t(8) = 0.936$, $p = 0.377$), demonstrating that achieving higher dominance is due to maternal exercise, rather than due to the more complex environment in the presence of a wheel (Figure 2C). Finally, wheel running for 4 weeks in adulthood did not increase the dominance of Sed males in the tube test ($t(18) = 0.335$, $p = 0.740$), indicating that the low social rank of Sed males, relative to Run males, cannot be improved by physical activity later in life (Figure 2D).

Another expression of dominance is territorial marking, a strategy to protect critical resources in particular when population density is reduced (Bronson, 1979; Desjardins et al., 1973). Territorial dominance was assessed in the urine scent marking test (Wang et al., 2011; Arakawa et al., 2008; Desjardins et al., 1973). Run and Sed males did not differ in territorial marking behavior (offspring $t(41) = 1.629$, $p = 0.111$; litter $t(18) = 1.300$, $p = 0.210$) (Figure 2E). Although intuitively inconsistent, lack of territorial dominance of tube test dominant C57BL/6 males was also reported by others (Hou et al., 2016). Furthermore, Run males exhibited no more aggression and agonistic behavior than Sed males in direct pairwise interactions in a cage novel for both and which allowed more contacts and space to attack and communicate social status than the tube test (offspring $t(29) = 0.759$, $p = 0.454$; litter $t(8) = 0.516$, $p = 0.620$) (Figure 2F). Although the concepts of dominance and aggression are often used interchangeably, aggression is just one form of dominance (Sapolsky, 2005; Vermande et al., 2018; Schaal et al., 1996; Jeon et al., 2010). Indeed, lack of correlation between tube test dominance, urine marking, and aggression was previously reported in laboratory mice (Benton et al., 1980). Finally, Run and Sed males had similar affiliative behavior in the three-chamber social test (Moy et al., 2004), indicated by their comparable preference for a caged male stranger over an empty cage (two-way ANOVA, Run versus Sed offspring $F(1,46) = 0.0025$, $p = 0.960$; Run versus Sed litter $F(1,22) = 0.0180$, $p = 0.895$) (Figure 2G). Taken together, these data are consistent with the multidimensional nature of dominance and indicate that the influence of maternal running on male social behavior is specific for direct interactions in the tube test.

Although social dominance is considered beneficial in animal societies, attaining high dominance rank may entail cost. Multiple studies, especially those conducted in the wild, demonstrate higher stress hormone levels in dominant individuals; albeit many other studies indicate increased endocrine stress response in subordinates (Creel, 2001; Sapolsky, 2005). We found a more sustained elevation of plasma corticosterone levels in Run males to a 10-min restraint stress at 30 and 60 min poststress (two-way ANOVA, main effect of maternal running, $F(1,40) = 10.84$, $p = 0.0021$; Sidak's correction for multiple comparison, time 30 min *$p = 0.018$, time 60 min *$p = 0.020$) (Figure 2H). The increased neuroendocrine stress response of Run offspring, however, was not associated with increased innate fear/anxiety-like behavior in the elevated plus maze (EPM, offspring $t(38) = 1.400$, $p = 0.310$; litter $t(11) = 1.411$, $p = 0.460$) (Figure 2I). Their resilience to chronic stress was also not impaired, indicated by Sed-like open arm behavior (in the EPM), affiliative behavior, and sucrose consumption, following CSDS (Berton et al., 2006) (Figures S1A, S1B, and S2). Of note, CSDS, as expected, increased anxiety-like behavior and reduced affiliative behavior in both Run and Sed mice, but had no effect on sucrose consumption presumably because we did not separate the groups of stress-sensitive and resilient individuals (the goal was to detect an overall effect on Run and Sed mice). Others reported similar results when animals were not segregated based on their vulnerability to stress

(Matikainen-Ankney et al., 2018). Overall, the increased endocrine stress response of the Run offspring correlated with their tube test dominance and raised the possibility that it might support the dominance phenotype by promoting alertness and responsivity toward the environment.

Voluntary Wheel Running of Mothers during the Lactating Period Increases Offspring Reproductive Success

Dominant males typically enjoy greater reproductive success/fitness (Dewsbury, 1982). Indeed, Run males sired more progeny than Sed males when co-housed in triads with Run females (chi-square test: Run *p = 0.046, Sed: p = 0.670) (Figure 3A). However, when the female in the triad was a Sed offspring (rather than Run), comparable numbers of pups were sired by Run and Sed males (Figure 3A), indicating that the increased reproductive success of Run males is manifested only when the females are also programmed by maternal running. The increase in reproductive success of Run males was not due to a post-copulatory paternity bias (i.e., sperm competition) (Dean et al., 2006) because polyandrous females (31.25% of 32 females that mated with both Run and Sed males) had a similar number of pups sired by Run and Sed fathers (two-way ANOVA. main effect of father, $F(3,32) = 0.520$, $p = 0.671$) (Figure 3B). Additional evidence supporting the higher reproductive "quality" of Run males included the higher preference of females (either Run or Sed) for Run, relative to Sed, male urine odor in an olfactory discrimination test (Jones and Nowell, 1974); two-way ANOVA; time spent by females in compartments previously occupied by males; small dots individual offspring, $F(1,42) = 4.377$, *p = 0.0425, N = 11 (Run), 12 (Sed); large dots litter averages, $F(1,12) = 5.601$, *p = 0.0356, N = 4 (Run), 4 (Sed) (Figure 3C). Furthermore, in large arenas (4 m in diameter) Run males interacted with females (either Run or Sed) more frequently than Sed males, from ~day 5, in parallel with the onset of estrus in the initially virgin females (two-way ANOVA, group effect, $F(1,154) = 9.872$, *p = 0.002; Figure 3D). In standard cages, because of their relatively small size, it was not possible to separate deliberate interactions from chance encounters, explaining the high proportion of time both Run and Sed males spent in close proximity to females (two-way ANOVA, group effect, $F(1,105) = 0.777$, $p = 0.380$) (Figure 3D).

Voluntary Wheel Running of Mothers during the Lactating Period Does Not Alter the Metabolic Fitness of the Offspring

As certain attributes such as larger body size, physical fitness, and overall health have been proposed to contribute to male social dominance and reproductive success in several (but not all) studies (Clinchy et al., 2004; Clutton-Brock et al., 1976; Sapolsky, 2005; Hiadlovská et al., 2015), we measured a variety of physical and metabolic parameters in Run and Sed animals. We found no significant difference between adult Run and Sed males in total body mass, total body composition, food intake, heat generation, energy expenditure, O_2 consumption, and CO_2 production (Table S1). These data indicate no overt reprogramming of offspring growth and metabolism by postpartum maternal running, which is in stark contrast with the changes in tube test dominance and reproductive success.

Social Dominance of the Run Offspring Is Associated with Increased Dendritic Arborization in the Prelimbic Region of the Prefrontal Cortex

As individuals with the highest and lowest rank within a social group have been reported to differ in synaptic strength in the area of the prelimbic (PL) region of the medial prefrontal cortex (mPFC)/anterior cingulate cortex (Wang et al., 2011), and because connectivity in the mPFC undergoes significant changes during the early postnatal period, we tested if postnatal maternal running results in long-term structural changes in the offspring PL. We measured dendritic length and complexity in the PL of Run and Sed mice, selected randomly from groups of naive Run and Sed mice (i.e., not tested against each other in tube test). Mice were also not ranked for home cage social status in tube test because winning in a competition may alter synaptic strength in the mPFC (Zhou et al., 2017). Golgi-stained PL sections of Run and Sed mice showed layer-specific and rostrocaudal differences in dendritic length. Apical dendrites (Figure 4A) of layer II/III neurons had increased length at the rostral (dorsal) but not caudal (ventral) PL of Run mice (two-way ANOVA, group × length effect, $F(9,60) = 14.99$, $p < 0.0001$; Tukey's correction for multiple comparisons, rostral 0–150 μm: *p < 0.0001) (Figure 4B). In contrast, apical dendrites of layer V neurons had increased length at the caudal but not rostral PL of Run mice (two-way ANOVA, group × length effect, $F(9,60) = 14.99$, $p < 0.0001$; caudal 330–450 μm: *p = 0.010) (Figure 4C). Maternal running had no effect on basal dendrites (not shown). The layer and subregion specificity of dendritic alterations in Run mice may reflect the sensitivity of these neurons and/or the involvement of their connected network to maternal programming.

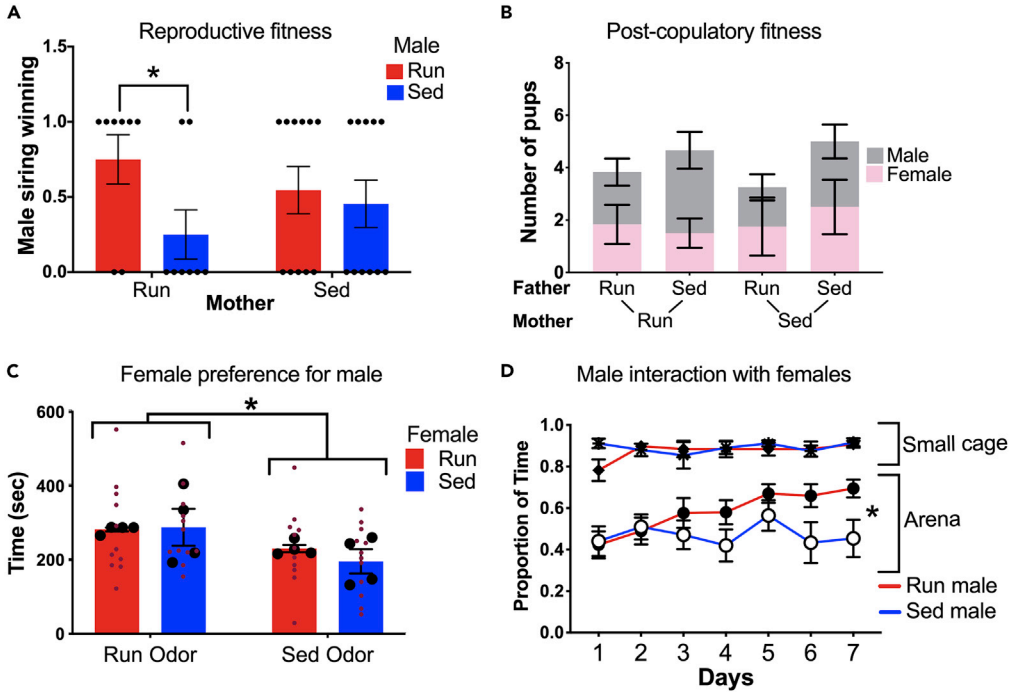

Figure 3. Maternal Postpartum Running Increases Offspring Reproductive Fitness

(A) Reproductive success of Run and Sed males (1 = father, 0 = not father) in triads with either Run (left; chi-squared test: *p = 0.046, N = 8 triads) or Sed females (right, p = 0.670, N = 11 triads). In a counterbalanced design, half of the males and females was Jackson, whereas the other half was Taconic, which allowed determining the Run or Sed paternity of offspring of Run and Sed mothers.

(B) Maternal postpartum running does not influence postcopulatory male fitness. Polyandrous Run and Sed females (10 of 32 females) had a similar number of male and female pups sired by Run and Sed fathers. N = 6 (Run mother), 4 (Sed mother) triads, two-way ANOVA, group effect, $F(3,32) = 0.5202$, $p = 0.6714$.

(C) Run and Sed females preferred Run, relative to Sed, male urine odor. Time spent by females in compartments previously occupied by males is measured. Two-way ANOVA. Small circles signify individual offspring, $F(1,42) = 4.377$, *p = 0.0425, N = 11 (Run), 12 (Sed). Large black circles denote litter averages, $F(1,12) = 5.601$, *p = 0.0356, N = 4 (Run), 4 (Sed).

(D) Proportion of time spent by Run and Sed males in interaction with females across 7 days in large arenas and standard cages. Because of no statistical difference, data with Run and Sed females were combined. Two-way ANOVA; large arena, main group effect, $F(1,154) = 9.872$, *p = 0.002, N = 13 (Run), 14 (Sed). Two-way ANOVA; small cage, $F(1,105) = 0.7773$, $p = 0.38$, N = 9 (Run), 8 (Sed). Data represented as mean ± SEM.

Cytokines in Milk Contribute to the Maternal Programming of Social Dominance

Next, we worked toward specifying the mechanism underlying the maternal physical activity-dependent programming of offspring dominance. As we found no significant change in daily overall maternal care during postpartum running (Figure 1B), we explored the possibility that increased postpartum physical activity alters the maternal, and then the offspring, microbiota, which in turn programs the offspring brain. However, analysis of maternal fecal microbiota at the time of weaning showed that postpartum running had no major effect on the relative abundance of gut microbial genera (Figure S3A). The Run fecal microbiota at P14, P21, and in adulthood were not different from those of Sed either (Figures S3B–S3D). These data indicate that programming of social dominance is unlikely mediated by the maternal or offspring microbiota.

Another way of mother-offspring communication during the postnatal period is via milk bioactive substances. Because production of gastric acid and pancreatic proteases is delayed in neonates (Blais et al., 2006), maternal cytokines, like maternal IgG, lactoferrin, and soluble Cd14 (Rodewald and Abrahamson, 1982; Prentice et al., 1987; Blais et al., 2006), could reach the offspring's upper digestive system in biologically relevant concentrations. As the programming effect of milk of runner mothers could not be tested

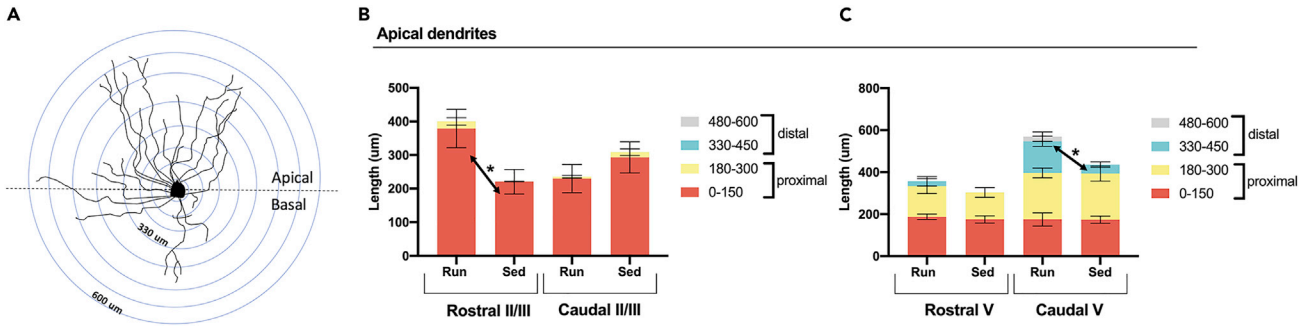

Figure 4. Social Dominance of Run Offspring Is Associated with Increased Dendritic Arborization in the PL

(A) Schematic for neuronal tracing by Neurolucida to determine dendritic length in concentric areas from the cell body.

(B and C) Increased length of apical dendrites of rostral layer II/III (B) (two-way ANOVA, post-hoc, Tukey's correction for multiple comparison, *p < 0.0001) and caudal layer V (C) (two-way ANOVA, post-hoc, Tukey's correction for multiple comparison, *p = 0.010) neurons in Run, relative to Sed male mice. N = 6 (Run), 6 (Sed). Data represented as mean ± SEM.

directly because of the very high mortality rate of pups fed with milk collected from running and sedentary mothers, we first assayed milk samples for possible running-induced changes in biologically active substances. We tested postpartum day 10 milk because the volume of milk collected at earlier time points (e.g., postpartum day 5–6) was insufficient for immunoassays. Milk samples collected at later time points (e.g., postpartum day 14) contain reduced levels of cytokines as pups gradually switch from milk to solid food. By measuring 38 bioactive molecules, mostly cytokines, by Luminex multiplex immunoassay (Mouse InflammationMAP, Myriad RBM), we detected significantly lower levels of LIF (leukocyte inhibitory factor, 3.6 fold reduction), CXCL1 (KC/GRO, 7.6 fold), and CXCL2 (MIP2 alpha, 9.0 fold) in running, relative to sedentary, mother's milk (multiple t tests with FDR [Q = 1%] LIF $q = 9.04 \times 10^{-6}$; CXCL1 $q = 4.8 \times 10^{-5}$; CXCL2: $q = 2.2 \times 10^{-6}$) (Figure 5A). These data suggest that maternal running modulates the expression of specific cytokines in milk immune cells and/or mammary epithelial cells.

Next, we asked if running-induced milk cytokine changes are directly linked to the maternally programmed dominance behavior. We counteracted the running-induced reductions in milk LIF, CXCL1, and CXCL2 by cytokine supplementation of Run pups, using a recombinant cytokine cocktail (i.e., Run^{CC} offspring). The cytokine cocktail was delivered via daily oral gavage from postnatal day 2–14. The delivered amounts of cytokines were calculated daily from the volume of milk consumed by the offspring (~0.1 mL milk per g pup weight) and the concentration of the cytokine in the milk (Figure 5A). Control Run offspring received BSA by gavage (Run^{BSA} offspring). Pup weights in the cytokine and control groups were comparable through the gavage period. Supplementation of Run offspring with LIF and CXCL1/2 during the postpartum period resulted in loss of dominance (i.e., subordination of Run^{CC} mice) in competition with control offspring (Run^{BSA} mice) in tube test (one-way ANOVA, offspring $F(3,32) = 3.363$, $p = 0.031$; litter $F(3,13) = 6.154$, $p = 0.0078$, Tukey's multiple comparison Run^{BSA} versus Run^{CC} litter *p = 0.0087) (Figure 5B). Sed mice were not used in these experiments because subordinate (Sed) mice cannot be programmed more subordinate and because the goal was to reverse running-induced low levels, rather than to increase already high cytokine levels to a non-physiological range. Supplementation with LIF and CXCL1/2 separately was not sufficient to reverse the Run's dominance phenotype (Figure 5B), indicating that milk LIF and CXCL1/2 changes together drive maternal programming of social dominance. Locomotor activity, spatial memory as measured in the Morris water maze, and anxiety-like behavior in the EPM were not altered by the administration of BSA and cytokine cocktail, indicating that the gavage procedure and the administered proteins did not impact adult behavior in cognitive and emotional domains tested in our experiments (Figures 5C–5E). Overall, these experiments linked postpartum running-induced changes in milk LIF and CXCL1/2 levels to alterations in offspring tube test social dominance (Figure 5F). We concluded that the social dominance of the Run offspring is due, at least partly, to activity/running-induced alterations in specific maternal milk cytokines.

DISCUSSION

Although the structure and dynamics of social hierarchies have been extensively studied across many species (Wang et al., 2014; Shemesh et al., 2013), it is unknown why a particular individual, even in a genetically

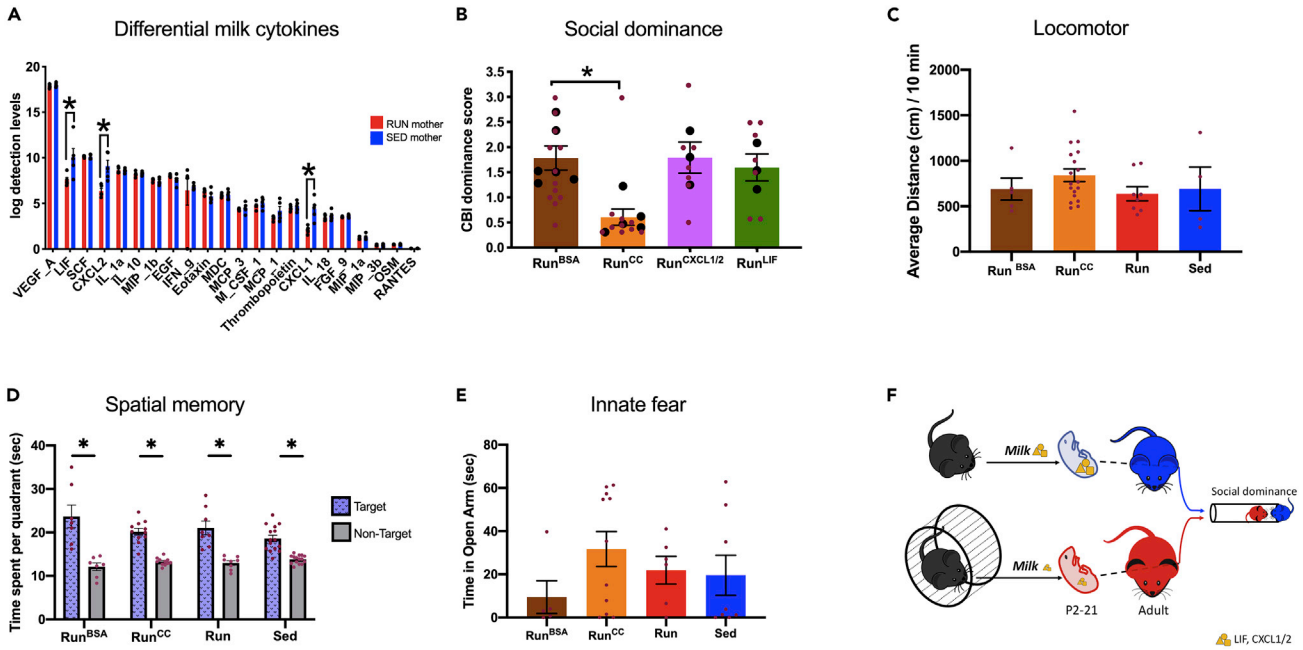

Figure 5. Milk Cytokines Program Tube Test Dominance

(A) Select cytokines in maternal milk contribute to programming offspring tube test dominance. Maternal postpartum running resulted in changes in milk cytokine levels. Twenty-three bioactive compounds detected in all 5 samples per group (Run IFNg in 4 samples), of the total 38 tested, were included in the analysis (multiple t tests with FDR Q = 1% based on 2-stage step-up method of Benjamini, Krieger, and Yekutieli, N = 5 running mothers, 5 sedentary mothers; LIF *q = 9.04×10^{-6}; CXCL1 *q = 4.8×10^{-5}; CXCL2: *q = 2.2×10^{-6}).

(B) Supplementation of low milk LIF and CXCL1/2 levels of running mothers by the direct administration of a cytokine cocktail to P2 to P14 pups (i.e., Run^{CC} offspring) reversed the social dominance of Run mice in the tube test, apparent by their subordination in direct competition with control RUN^{BSA} offspring. The three cytokines were delivered by daily oral gavage (0.8 ng LIF/g pup weight, 7.8 ng CXCL1/g, and 0.25 ng CXCL2/g). CXCL1/2 or LIF alone was insufficient to reverse dominance. Small circles signify individual offspring, One-way ANOVA, Tukey's multiple comparison, Run^{BSA} versus Run^{CC} p = 0.060, Run^{BSA} versus $Run^{CXCL1/2}$ p = 0.993, Run^{BSA} versus Run^{LIF} p = 0.999. N = 12 (Run^{BSA}), 10 (Run^{CC}), 7 ($Run^{CXCL1/2}$), 7 (Run^{LIF}). Large black circles denote litter averages; Run^{BSA} versus Run^{CC} *p = 0.0087, Run^{BSA} versus $Run^{CXCL1/2}$ p > 0.990, Run^{BSA} versus Run^{LIF} p > 0.950. N = 6 (Run^{BSA}), 5 (Run^{CC}), 3 ($Run^{CXCL1/2}$), 3 (Run^{LIF}).

(C–E) No effect of gavage and gavaged cytokine cocktail on general behaviors in adults including locomotor activity (one-way ANOVA, F(3,31) = 1.050, p = 0.384) (C); spatial memory in the Morris water maze (two-way ANOVA Target versus Nontarget, F(1,37) = 78.87, *p < 0.0001; Sidak's multiple comparison test Target versus Nontarget; *p < 0.0001, p = 0.0006, p = 0.0006, and p = 0.0061 for Run^{BSA}, Run^{CC}, Run, and Sed, respectively); (D) and anxiety-like behavior in the elevated plus maze (ANOVA F(3,26) = 1.107, p = 0.364). (E) In the anxiety assay, the single high value in the Run^{BSA} group met exclusion criteria, but even after exclusion the difference between groups remained not significant. Data represented as mean ± SEM.

(F) Model of cytokine-mediated lactocrine programming of offspring behavior.

homogeneous group of animals and under controlled laboratory conditions, reaches the top of social hierarchy, whereas others become subordinates (Wang et al., 2011; Lindzey et al., 1961). Intuitively, larger body size and physical fitness may predispose an individual for dominance, but this correlation is not consistent across studies and species (Clinchy et al., 2004; Clutton-Brock et al., 1976; Sapolsky, 2005; Hiadlovská et al., 2015). Here we report that an individual can be programmed during early postnatal life to become dominant over competitors, which is not associated with higher body mass or other physical and metabolic traits. Most Run males are dominant over Sed males, regardless of their social rank in their own group, indicating that maternally programmed dominance is more robust than that established during group housing in a hierarchical system.

The effect of maternal postpartum running was specific to tube test dominance, as territorial dominance and direct agonistic/aggressive interactions were not affected. Similar to these data, tube test dominant C57BL/6 males exhibited no territorial dominance over paired-housed (familiar) subordinates (Hou et al., 2016). However, exposure to female odor, right before the urine test, unraveled the correlation between tube and urine marking dominance (Hou et al., 2016). Neither of these conditions was present in our experiment as we used stranger mice with no prior experience of the other's rank and used no female odor before the urine test. In contrast to these data, Wang et al. reported correlation between tube and urine

marking dominance (without prior exposure to female odor) in the same strain of mice (Wang et al., 2011). This discrepancy could be explained by differences in experimental design. Although both the Hou et al. and Wang et al. articles reported data with familiar mice, the former tested individuals once, whereas the latter tested them multiple times in a round-robin design. The significance of this difference is that prior winning has a strong positive influence on the outcome of consecutive competitions (van den Berg et al., 2015). Furthermore, whereas the Hou (as well as our) analysis included all pairs, the Wang report excluded pairs with no obvious difference in urine marks between the two competitors. Overall, these data suggest that territorial marking behavior is relatively insensitive to tube test dominance rank and that either prior female odor exposure or repeated tests are required to increase its sensitivity. This interpretation is also consistent with additional reports that found no correlation between tube test and territorial marking (Benton et al., 1980).

Although instinctively dominance is a categorical concept, it is more likely that it is a multidimensional phenomenon, controlled by different, although interacting, circuits within the large and complex social decision/behavioral network (O'Connell and Hofmann, 2012). Indeed, largely separate neuronal circuits are associated with the various forms of dominance behaviors. Tube test dominance has been linked to the activation of layer V pyramidal neurons in the mPFC projecting to the limbic system, and specifically, to increased glutamatergic transmission within this circuit (Wang et al., 2011). Indeed, we found increased dendritic length not only in layer V pyramidal neurons but also in layer II/III cells, a possible indication for increased connectivity in both layers. Layer II pyramidal neurons have cortico-cortico connections and receive long-range excitatory inputs from the midline thalamus, contralateral mPFC, basolateral amygdala, and ventral hippocampus (Little and Carter, 2012). In contrast to the involvement of mPFC in tube test dominance, territorial micturition is mediated by a cluster of neurons expressing corticotropin-releasing hormone in the pontine micturition center that send glutamatergic projections to the spinal cord (Hou et al., 2016). Finally, the ventrolateral part of the ventromedial hypothalamus is a key region driving inter-male aggression (Anderson, 2016). In contrast to the brainstem and hypothalamus, mPFC undergoes significant postnatal developmental changes and its synaptic organization can be disrupted by environmental manipulations during the suckling period (Tada et al., 2016); thus the postnatal plasticity of mPFC might explain why maternal running during lactation primarily programs tube test dominance.

In addition to the tube test dominance, Run males exhibited increased reproductive fitness in seminatural environment and in direct competition with Sed males. Although reproductive fitness, due to its complexity, is less frequently measured in laboratory dominance studies, it is considered to be the most ecologically relevant measure of dominance in mice. The tube test and reproductive fitness paradigms complement each other and together strengthen the notion that maternal postpartum physical activity increases male dominance.

Our data link a set of breastmilk cytokines to increased offspring social dominance. Although no human manipulation can match the natural "delivery" of maternal cytokines via milk, our approach of cytokine supplementation during early postnatal life achieved the reversal of a specific behavior (tube test dominance) that was programmed by maternal exercise, with the combined, but not individual cytokines, while causing no overt changes in development and behaviors (that were not altered by maternal exercise either).

The effect of maternal postpartum running on offspring dominance behavior was male specific because we found no difference in tube test dominance between unfamiliar Run and Sed females. However, Run females may be "dominant" over familiar Sed females in more naturalistic environment as Run males achieved increased reproductive fitness only with Run females. Although we found no difference in tube test dominance between unfamiliar females, group-housed females establish dominance hierarchy as measured by repeated tube tests (i.e., round-robin design) (van den Berg et al., 2015). Nevertheless, males and females attain dominance status via different mechanisms (van den Berg et al., 2015). Male dominance is strongly influenced by the outcomes of prior competitive encounters, whereas female dominance is based upon stable differences in the intrinsic attributes of individuals within a social group (Zhou et al., 2017). The male-specific mechanism is testosterone dependent as males null for the sex-determining region Y gene (*Sry*) or castrated males are female-like, whereas females with transgenic expression of SRY or females supplemented with testosterone are male-like in attaining dominance (van den Berg et al.,

2015). Therefore, one possibility is that maternal cytokines and their downstream pathway interact with the effect of male-specific sex hormones on programming tube test dominance. Alternatively, males may be more responsive to maternal cytokines (i.e., to increased cytokine levels in sedentary mother's milk) during the early postnatal period as more pronounced immune reactivity was reported in developing males than females (Cai et al., 2016; Sharma et al., 2018).

An ecological interpretation of these findings is that physical activity and competition for limited resources are part of life in the wild, and that evolution adapted milk-borne cytokines as a maternal signaling mechanism to optimize, in the given environment, the social dominance and reproductive success of the offspring. However, programming dominance is dependent on maternal physical activity (i.e., fitness), and lack of physical activity in a laboratory setting, or possibly in the wild because of disease or an adverse environment, disrupts this programming mechanism, resulting in submissiveness and reduced reproductive success of the offspring (i.e., a negative selection for the maternal lineage). It remains to be determined if variability in certain milk cytokines in human, due to maternal genetics or lifestyle, leads to variability in social dominance (i.e., prosocial behavior, coalition building, confidence and boldness) in their children.

Limitations of the Study

We do not currently know how the effects of milk cytokines reach the developing brain and program adult social dominance. However, CXCR2, the receptor for CXCL1/2 was reported to be expressed in the epithelium of the esophagus (Luan et al., 2001) and in neuroendocrine cells in the stomach and small intestine (Tecimer et al., 2000). These cells are in direct contact at their apical surface with milk and could transmit milk-borne cytokine signals to vagal afferents that terminate near the epithelia. Indeed, vagal afferents readily respond to luminal stimuli (Bravo et al., 2011; Bertrand et al., 1997). Alternatively, mucosal immune cells may respond to milk cytokines and reach the brain. Various peripheral immune cells, including CD4/8 T cells, monocytes, macrophages, and dendritic cells, have been shown to infiltrate the meninges, choroid plexus, and parenchyma in physiological conditions (Prinz and Priller, 2017; Korin et al., 2017). These or other gut-brain mechanisms should be tested in future work.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Miklos Toth (mtoth@med.cornell.edu).

Materials Availability

This study did not generate any unique reagents.

Data and Code Availability

All data used in this manuscript are available upon request from the lead author. No custom code was used in the analysis of the data.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101357.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health grants 1RO1MH080194 to M.T.; PO1HD67244 and RO1NS093872 to S.G.; 4R37DK048873, 5R01DK056626, and 5R01DK103046 to D.E.C.; and P30 CA008748 MSK Cancer Center Support Grant/Core Grant, The Lymphoma Foundation, The Susan and Peter Solomon Divisional Genomics Program, and the Parker Institute for Cancer Immunotherapy at Memorial Sloan Kettering Cancer Center to M.R.M.v.d.B.. We thank Bing Fang Liu for his involvement at the initiation of the project and Daniel Shaver in the metabolism studies.

AUTHOR CONTRIBUTIONS

Conceived, designed and analyzed the experiments: F.T., B.Z., Q.C., M.D.D., M.A.-B., K.V.A., L.B., D.E.C., M.R.M.v.d.B., S.G., and M.T. Performed the experiments: F.T., P.B., B.Z., K.L., J.G.T., Q.C., M.D., M.A.B., K.V.A., L.B., E.P., N.F., and A.H. M.T. and F.T. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

Received: February 11, 2020
Revised: May 21, 2020
Accepted: July 8, 2020
Published: August 21, 2020

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iScience, Volume *23*

Supplemental Information

Maternal Programming of Social Dominance via Milk Cytokines

Faten Taki, Katherine Lopez, Bojana Zupan, Paul Bergin, Melissa D. Docampo, Michele Alves-Bezerra, Judit Gal Toth, Qiuying Chen, Kimon V. Argyropoulos, Luendreo Barboza, Emily Pickup, Nicholas Fancher, Abbi Hiller, Steven Gross, David E. Cohen, Marcel R.M. van den Brink, and Miklos Toth

Supplemental Figures

A **B**

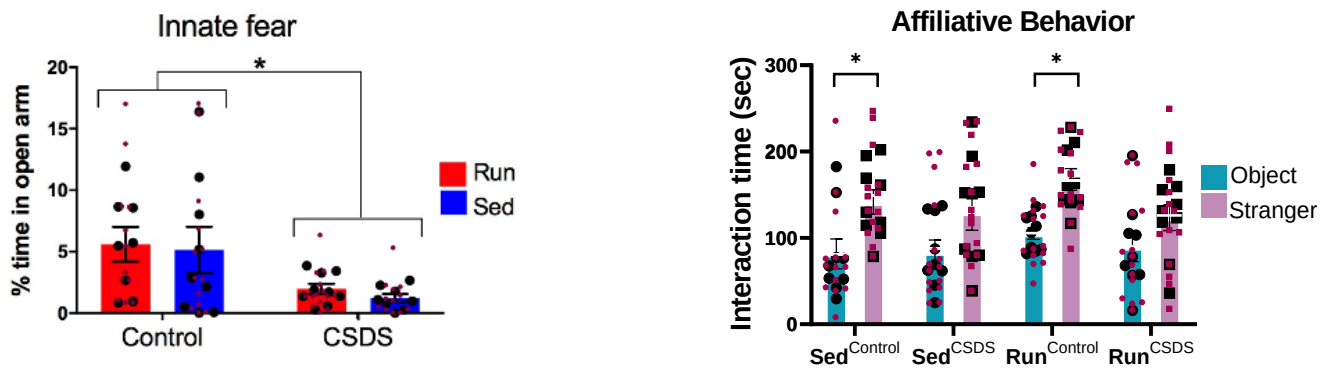

Figure S1. Maternal postpartum running does not increase the male offspring's sensitivity to chronic social defeat stress (CSDS). Related to Figure 2.

A. Run and Sed male offspring responded similarly to CSDS, measured as % time spent in the anxiety-inducing open arm of EPM. Two-way ANOVA, Main effect of group, F(1,47)=0.3049, P=0.5834. Main effect of CSDS treatment, F(1,47)=12.45, *P=0.0009. Control N=12 (Run), 13 (Sed). CSDS N=14 (Run), 12 (Sed). Large black circles denote litter averages. Main effect of group, F(1,30)=0.238, P=0.6292. Main effect of CSDS treatment, F(1,30)=9.292, *P=0.0048. Control N=8 (Run), 9 (Sed), CSDS N=9 (Run), 8 (Sed). **B**. Run and Sed male offspring responded similarly to CSDS, measured as interaction time with an unfamiliar mouse vs. an object. Small circles denote offspring values. Multiple t-test, corrected for multiple comparisons using Holm-Sidak method, Sed control, t(16)=2.699, *p=0.04666. Sed CSDS, t(16)=1.833, p=0.163763. Run control, t(16)=4.410, *p=0.001752. Run CSDS, t(16)=1.482, p=0.163763. Data are presented as mean +/- SEM.

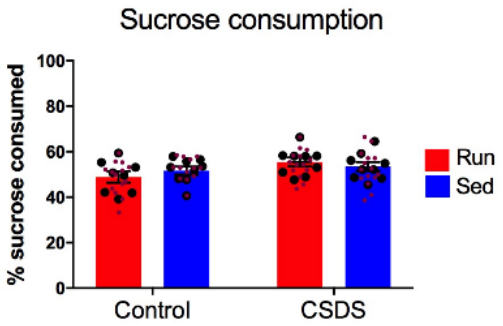

Figure S2. Run and Sed male offspring respond similarly to CSDS as measured by sucrose consumption that reflects the degree of anhedonia. **Related to Figure 2**.

Small circles denote offspring values. Two-way ANOVA. Main effect of group, $F(1,52)=0.1377$, $P=7120$. Main effect of CSDS treatment, $F(1,52)=3.246$, $P=0.0774$. Control N=14 (Run), 13 (Sed). CSDS N=14 (Run), 15 (Sed). Large black circles denote litter averages. Main effect of group, $F(1,31)=0.03591$, $P=0.8509$. Main effect of CSDS treatment, $F(1,31)=4.126$, $P=0.0509$. Control N=8 (Run), 9 (Sed). CSDS N=9 (Run), 9 (Sed). Data are presented as mean +/- SEM.

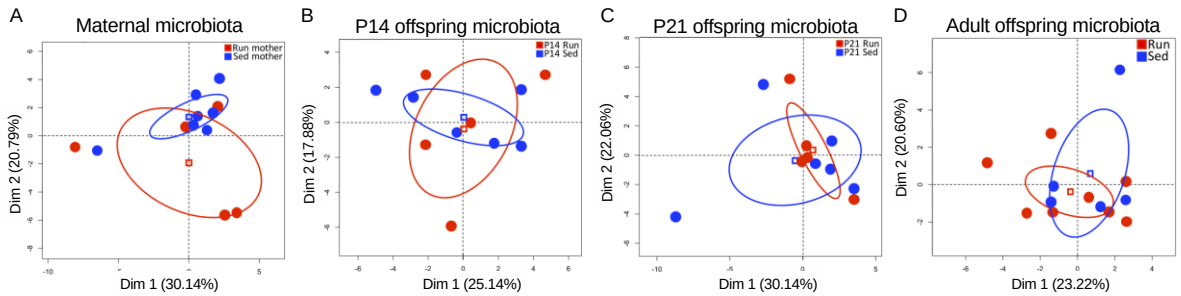

Figure S3. Similar microbiota of running and sedentary mothers and Run and Sed offspring. Related to Figure 5 and Supplementary Tables 2, 3, 4, and 5.

A. Maternal postpartum running does not alter the maternal fecal microbiota. Principal component analyses of the log-ratio transformed relative abundances of maternal and offspring microbial genera, based on 16S rRNA gene amplification and sequencing. Overlaps of 95% confidence ellipses indicate no postpartum running-induced changes in mothers (at weaning). Relative abundances of 39 genera present in more than 25% of the samples per group were similar between Run and Sed mothers. N=5 (Run), 7 (Sed). Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison (see also **Supplementary Table 2**). **B-D**. Maternal postpartum running does not alter the offspring fecal microbiota at P14 (B), P21 (C), and adult (D) male offspring. PERMANOVA test (adonis function, vegan package, R). Maternal microbiota, R2=0.07358, F(1,10)=0.7942, P=0.63; N=5 (Run mothers), 7 (Sed mothers). P14 microbiota, R2=0.07642, F(1,9)=0.74471, P=0.75; Litter N=5 (Run), 6 (Sed). P21 microbiota, R2=0.06415, F(1,9)=0.61692, P=0.94. Litter N=5 (Run), 6 (Sed). Adult microbiota, R2=0.09027, F(1,11)=1.0916, P=0.35; Litter N=8 (Run), 5 (Sed). At P14 and P21, relative abundances of 33 genera present in more than 25% of the samples per group were similar between Run and Sed offspring. Litter N=5 (Run), 6 (Sed). Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison **(**see **Supplementary Tables 3-4**). In adults, relative abundances of 23 genera present in more than 25% of the samples per group were similar between Run and Sed offspring. Litter N=8 (Run), 5 (Sed); Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison **(**see **Supplementary Table 5**).

Supplemental Tables

Table S1. Metabolic parameters using FLIR Thermal Imaging. Related to Figure 1.				
	Metabolic endpoint	Run male (N=5)	Sed male (N=6)	Run/Sed
Total Body Mass (g)		29.48; SEM=1.77	27.98; SEM=0.74,	NS
Total Body Composition	Fat	4.05; SEM=0.81	3.65; SEM=0.62	NS
	Lean	23.99; SEM=1.42	22.50; SEM=0.27	NS
Avg. O2 consumption (ml/min)	Total	1.44; SEM=0.12	1.42; SEM=0.047	NS
	Light phase	1.27; SEM=0.12	1.31; SEM=0.05	NS
	Dark phase	1.61; SEM=0.13	1.55; SEM=0.05	NS
Avg. CO2 production (ml/min)	Total	1.30; SEM=0.11	1.29; SEM=0.04	NS
	Light	1.07; SEM=0.10	1.13; SEM=0.04	NS
	Dark	1.53; SEM=0.12	1.45; SEM=0.049	NS
Avg. Energy expenditure kcal/hr	Total	10.23; SEM=0.86	10.18; SEM=0.3423	NS
	Light	4.46; SEM=0.42	4.62; SEM=0.17	NS
	Dark	5.78; SEM=0.45	5.56; SEM=0.17	NS
Intercapsular FLIR (Celsius)		33.50; SEM=0.17,	33.81; SEM=0.20	NS
iWAT FLIR (Celsius)		32.89; SEM=0.33	33.57; SEM=0.23,	NS
Avg. Food intake (g)	Total	3.48; SEM=0.21	3.72; SEM=0.43	NS
	Light	1.02; SEM=0.18	1.08; SEM=0.22	NS
	Dark	2.87; SEM=0.42	2.63; SEM=0.31	NS

Table S2. Run and Sed mothers have similar microbiota at weaning. Relative abundances of 39 genera present in more than 25% of the samples per group were similar between Run and Sed mothers. Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison. Related to Figure 5 and Figure S3.

Genus	chi-squared	df	p-value	FDR
g_Adlercreutzia	0.0065934	1	0.9353	0.9353
g_Akkermansia	2.3802	1	0.1229	0.6309333
g_Allobaculum	1.4835	1	0.2232	0.6309333
g_Bacteroides	0.16484	1	0.6847	0.9208034
g_Blautia	1.1143	1	0.2912	0.6309333
g_Butyrivibrio	1.1143	1	0.2912	0.6309333
g_Clostridium	1.1143	1	0.2912	0.6309333
g_Coprobacillus	0.059341	1	0.8075	0.9353
g_Coprococcus	0.0065934	1	0.9353	0.9353
g_Enterococcus	3.4879	1	0.06182	0.6309333
g_Escherichia	1.9055	1	0.1675	0.6309333
g_Lactobacillus	1.9055	1	0.1675	0.6309333
g_Oscillospira	0.16484	1	0.6847	0.9208034
g_Pediococcus	4.1209	1	0.04236	0.6309333
g_Prevotella	0.059341	1	0.8075	0.9353
g_rc4-4	2.9077	1	0.08816	0.6309333
g_Ruminococcus	0.16484	1	0.6847	0.9208034
g_Streptococcus	1.1143	1	0.2912	0.6309333
g_Turicibacter	3.4879	1	0.06182	0.6309333
g_Anaerostipes	0.0065934	1	0.9353	0.9353
g_Barnesiella	1.9055	1	0.1675	0.6309333
g_Butyricimonas	2.9077	1	0.08816	0.6309333
g_Dehalobacterium	0.7978	1	0.3718	0.752895
g_Lactococcus	1.4835	1	0.2232	0.6309333
g_Mucispirillum	0.32308	1	0.5698	0.888888
g_Staphylococcus	0.059341	1	0.8075	0.9353
g_x_Peptostreptococcaceae	0.16484	1	0.6847	0.9208034
g_Anaeroplasma	0.026466	1	0.8708	0.9353
g_Dorea	1.1182	1	0.2903	0.6309333
g_Proteus	0.53594	1	0.4641	0.8619
g_Anaerofustis	0.32766	1	0.567	0.888888
g_Anaerotruncus	0.32766	1	0.567	0.888888
g_Shuttleworthia	1.7491	1	0.186	0.6309333
g_Candidatus_Arthromitus	0.0070892	1	0.9329	0.9353

g_Odoribacter	0.1202	1	0.7288	0.9353
g_Roseburia	0.0075128	1	0.9309	0.9353
g_Shigella	0.75128	1	0.3861	0.752895
g_Anaerofilum	1.3856	1	0.2392	0.6309333
g_Mycoplasma	0.33607	1	0.5621	0.888888

Table S3. Run and Sed offspring have similar microbiota at P14. Relative abundances of 33 genera present in more than 25% of the samples per group were similar between Run and Sed offspring. Litter N=5 (Run), 6 (Sed). Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison. Related to Figure 5 and Figure S3.

Genus	chi-squared	df	p-value	FDR
g_Adlercreutzia	0	1	1	1
g_Akkermansia	0.3	1	0.5839	0.9102677
g_Bacteroides	0.13333	1	0.715	0.9102677
g_Blautia	0.033333	1	0.8551	0.9102677
g_Clostridium	0.53333	1	0.4652	0.9102677
g_Coprococcus	0.53333	1	0.4652	0.9102677
g_Enterococcus	0.3	1	0.5839	0.9102677
g_Escherichia	1.2	1	0.2733	0.9102677
g_Lactobacillus	0.033333	1	0.8551	0.9102677
g_Oscillospira	1.6333	1	0.2012	0.9102677
g_Prevotella	0.033333	1	0.8551	0.9102677
g_Proteus	0.3	1	0.5839	0.9102677
g_Ruminococcus	0.3	1	0.5839	0.9102677
g_Streptococcus	0.53333	1	0.4652	0.9102677
g_Turicibacter	0.033333	1	0.8551	0.9102677
g_Allobaculum	0.3	1	0.5839	0.9102677
g_Coprobacillus	0.13333	1	0.715	0.9102677
g_Staphylococcus	0.13333	1	0.715	0.9102677
g_Pediococcus	0.3	1	0.5839	0.9102677
g_unclassified_Peptostreptococcaceae	1.6408	1	0.2002	0.9102677
g_Lactococcus	0.0083714	1	0.9271	0.9560719
g_Mucispirillum	0.67808	1	0.4102	0.9102677
g_rc4-4	0.67808	1	0.4102	0.9102677
g_Butyricimonas	0.033951	1	0.8538	0.9102677
g_Butyrivibrio	1.2571	1	0.2622	0.9102677
g_Odoribacter	0.034921	1	0.8518	0.9102677
g_Anaeroplasma	0.33	1	0.5657	0.9102677
g_Anaerotruncus	0.036667	1	0.8481	0.9102677
g_Anaerostipes	2.3467	1	0.1256	0.9102677
g_Shigella	0.58667	1	0.4437	0.9102677
g_Dorea	0.99099	1	0.3195	0.9102677
g_Dehalobacterium	0.089189	1	0.7652	0.9102677
g_Shuttleworthia	0.089189	1	0.7652	0.9102677

Table S4. Run and Sed offspring have similar microbiota at P21. Relative abundances of 35 genera present in more than 25% of the samples per group were similar between Run and Sed offspring. Litter N=5 (Run), 6 (Sed). Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison. Related to Figure 5 and Figure S3.

Genus	chi-squared	df	p-value	FDR
g_Akkermansia	4.0333	1	0.04461	0.8551
g_Blautia	2.1333	1	0.1441	0.8551
g_Clostridium	2.1333	1	0.1441	0.8551
g_Lactobacillus	0.3	1	0.5839	0.8551
g_Adlercreutzia	1.6333	1	0.2012	0.8551
g_Allobaculum	0.13333	1	0.715	0.8551
g_Anaerotruncus	0.033333	1	0.8551	0.8551
g_Bacteroides	0.53333	1	0.4652	0.8551
g_Barnesiella	0.13333	1	0.715	0.8551
g_Butyricimonas	1.6333	1	0.2012	0.8551
g_Butyrivibrio	0.53333	1	0.4652	0.8551
g_Coprobacillus	0.13333	1	0.715	0.8551
g_Coprococcus	0.13333	1	0.715	0.8551
g_Dehalobacterium	0.13333	1	0.715	0.8551
g_Enterococcus	0.13333	1	0.715	0.8551
g_Escherichia	0.83333	1	0.3613	0.8551
g_Lactococcus	0.033333	1	0.8551	0.8551
g_Oscillospira	0.83333	1	0.3613	0.8551
g_Prevotella	0.033333	1	0.8551	0.8551
g_Ruminococcus	0.3	1	0.5839	0.8551
g_Staphylococcus	1.2	1	0.2733	0.8551
g_Streptococcus	0.83333	1	0.3613	0.8551
g_Turicibacter	0.3	1	0.5839	0.8551
g_unclassified_Peptostreptococcaceae	0.13394	1	0.7144	0.8551
g_Mucispirillum	0.075342	1	0.7837	0.8551
g_Pediococcus	3.3951	1	0.06539	0.8551
g_Anaeroplasma	0.033951	1	0.8538	0.8551
g_Proteus	0.1358	1	0.7125	0.8551
g_rc4-4	0.54321	1	0.4611	0.8551
g_Anaerostipes	0.42778	1	0.5131	0.8551
g_Dorea	0.034921	1	0.8518	0.8551
g_Shuttleworthia	0.14667	1	0.7017	0.8551
g_Candidatus_Arthromitus	1.9423	1	0.1634	0.8551
g_Lachnobacterium	0.044715	1	0.8325	0.8551
g_Weissella	0.044715	1	0.8325	0.8551

Table S5. Adult Run and Sed offspring have similar microbiota. Relative abundances of 23 genera present in more than 25% of the samples per group were similar between Run and Sed offspring. Litter N=8 (Run), 5 (Sed). Kruskal-Wallis test for pairwise comparison of relative abundances, with FDR adjusted for multiple comparison. Related to Figure 5 and Figure S3.

Genus	chi-squared	df	p-value	FDR
g_Adlercreutzia	0.34286	1	0.5582	0.8430048
g_Allobaculum	0.34286	1	0.5582	0.8430048
g_Anaerofustis	0.34286	1	0.5582	0.8430048
g_Anaerostipes	0.19286	1	0.6605	0.8430048
g_Anaerotruncus	0.53571	1	0.4642	0.8430048
g_Blautia	0.085714	1	0.7697	0.8430048
g_Butyrivibrio	0.53571	1	0.4642	0.8430048
g_Candidatus_Arthromitus	1.3714	1	0.2416	0.8430048
g_Clostridium	0.19286	1	0.6605	0.8430048
g_Coprobacillus	0.021429	1	0.8836	0.8836
g_Coprococcus	0.19286	1	0.6605	0.8430048
g_Dehalobacterium	2.1429	1	0.1432	0.8430048
g_Dorea	0.34286	1	0.5582	0.8430048
g_Enterococcus	1.05	1	0.3055	0.8430048
g_Faecalibacterium	0.77143	1	0.3798	0.8430048
g_Lactobacillus	0.085714	1	0.7697	0.8430048
g_Roseburia	1.3714	1	0.2416	0.8430048
g_Ruminococcus	0.77355	1	0.3791	0.8430048
g_Staphylococcus	0.086667	1	0.7685	0.8430048
g_Akkermansia	0.021488	1	0.8835	0.8836
g_Bacteroides	0.54167	1	0.4617	0.8430048
g_Mucispirillum	0.13771	1	0.7106	0.8430048
g_Parabacteroides	2.369	1	0.1238	0.8430048

Transparent Methods

Animal handling

Animal experiments were carried out in accordance with the Weill Cornell Medical College Institutional Animal Care and Use Committee guidelines. All mice were group-housed up to five per cage throughout a 12-h light/dark cycle with lights on at 6 a.m. Food and water were available ad libitum. All experiments used C57BL/6 mice from Taconic, except the reproductive fitness experiments that also used mice from The Jackson Laboratory, to be able to ascertain Run and Sed paternity (based on polymorphic differences between the two strains).

Running during the lactation period

Males and females were received at 7 weeks of age. They were habituated for 2 weeks before breeding started. The first litter was not used as maternal care can be variable. At P2, mothers and pups were randomly divided into 2 groups. One group was housed in cages equipped with running wheels, while the other was housed in standard cages without running wheels. At P21, pups were weaned and transferred to standard, “sedentary” cages (without running wheels) until adulthood (>12 weeks of age) after which they were subjected to behavioral and metabolic testing (**Fig. 1A**). Mothers were used only once in running experiments and were sacked afterwards to avoid the effect of repeated running on offspring behavior.

Adult male running

Adult mice raised in standard laboratory conditions in groups were assigned randomly and individually to new standard cages or to cages equipped with running wheels for four weeks.

Maternal Care

Maternal care observations were adapted from (van Velzen and Toth, 2010) and were performed at P3, P5, P7, P9, P11, P13, P15. Behavior was scored during two 60-minute observation periods, one in the light (at noon) and another in the dark (7-8pm, performed under infrared light). Within each observation period, ABN (arched back nursing), BP (blanket posture), LG (licking/grooming), OP (off pups), and PP (passive posture) were scored every minute, leading to 120 observations per female per test day. Proportion of time engaged in each behavior was averaged across all days per phase (light or dark).

Offspring behavioral assays

All offspring behavioral tests were done during the light cycle between 1–5 p.m. unless noted otherwise. Offspring were randomly selected from litters and blindly tested for cognitive, social, and emotional behaviors. We tested 2-3 offspring from each mother and performed both offspring and litter-based comparisons (i.e. sample size was the number of offspring and the litters, respectively). Offspring and litter based analyses yielded similar results indicating no apparent litter effects in our experiments. We used at least 3 independent cohorts in most experiments. Different behavioral tests were performed at least three days apart, starting with the least stressful such as activity in an open field, progressing to the elevated plus maze test, and then to dominance tests, including the tube test, urine marking test, and aggression test. The Morris water maze spatial memory task was performed with independent cohorts of animals. Similarly, cohorts exposed to chronic social defeat were not utilized in any other behavioral assays.

Social dominance: Tube test. Mice were single housed for at least seven days and were trained for three days prior to testing. During training days, each mouse was put into one end of the tube (with divider in the middle of the tube removed) and allowed to walk through the tube. This was repeated by putting the mouse on the other end of the tube. If a mouse tried to back out of the tube or exhibited prolonged freezing, it was gently prodded by a rod from behind. The tube was cleaned with ethanol between individual animals. After training, mice went through one test per day, with each trial lasting up to five minutes. Opposing mice were placed into each end of the tube and allowed to reach the divider in the middle. Once opposing mice reached equal distance from the center, the divider was removed. The mouse that was pushed out and had a front paw touch the surface outside of the tube first was considered the loser. The tube was cleaned with ethanol and the pair of mice was retested after switching the starting locations. The tube was cleaned with ethanol before and after each trial.

Territoriality: Urine marking test. Territorial marking test was adapted from (Wang et al., 2011). Briefly, mice were single housed for at least seven days prior testing. Mice went through one test per day, with each trial lasting up to two hours. Two mice were placed on opposite sides of a 26 cm by 26cm box divided by a mesh screen. A filter paper was placed below the mesh floor to collect urine. After two hours, dried filter papers were blindly analyzed in a UV transillumniator to determine the winner in each pair. Each side was analyzed by three criteria; the total area, location, and pattern of the urine marks. Larger area, closer to the front of the mesh and further from the back corners, and a sprinkle over pooled pattern indicated more territoriality. The side that won by at least two of these criteria was designated the winner. If there were no marks on either side the trial was discarded. The chamber was cleaned with ethanol between each trial.

Aggression test. Mice went through one test per day, with each trial lasting twenty minutes. Two mice were placed into a novel cage with 50ml of dirty bedding from each home cage mixed in with the new bedding. Videos of the mouse interactions were recorded over the next twenty minutes. Videos were blindly scored by tallying aggressive actions of each mouse and awarding a win to the mouse that performed the higher number of aggressive actions. Aggressive moves included lateral attacks, boxing, mounting, and chasing. Trials with no aggressive behavior were discarded.

Affiliative social behavior: Three Chamber Social interaction. Mice were tested using a 1-day social interaction procedure. Mice were allowed to explore the rectangular arena (39cmWx54cmLx37cmH) containing 2 steel holding pens (15.5cmLx5.5cmWx17cmH) for 10 minute sessions. The arena and the holding pens were cleaned with 70% ethanol solution between each subject. On the second trial, an age-matched stranger mouse was placed in one of the steel pen holders before introducing the experimental mouse into the arena for another 10-min session. Movements were tracked by a ceiling mounted camera and analyzed using (Ethovision) for time spent in contact with the stranger mouse vs the empty pen holder.

Innate fear/anxiety-like behavior: Elevated Plus Maze (EPM). The elevated plus maze was performed using a cross maze with 12 × 2 inch arms (Gleason et al., 2010). Briefly, animals were introduced to the middle portion of the maze facing the closed arm and were left to explore for 10 minutes. Time spent in the open and closed arms were measured by a video-tracking system (Ethovision).

Chronic social defeat stress. A 10-days chronic social defeat stress protocol was modified from (Berton et al., 2006). Run and Sed (intruder) mice were subjected to daily 20-minute social defeat episodes for 10 days. The intruder animal was placed inside of the aggressive (prescreened for attack latency of <30 seconds) resident's home cage. A wire mesh partition was placed into the cage to separate the resident and intruder during the initial 5 min. of the exposure. The partition was removed during the second 5 minutes and the confrontation was allowed to persist until the attacks by the resident resulted in a defeat posture by the intruder (supine posture display of no less then 4 sec). Following defeat, the wire mesh partition was reinserted, separating the mice for the remainder of the 20 min period. Subjects were exposed to a novel resident at each social defeat episode.

EPM Post-CSDS (Day 11, 40). Mice were placed into the center of the EPM and allowed to freely explore it for 10 min while recording their movements. Distance and time in the open and closed arms were assessed using automated tracking software (Smart v 3.0, Panlab, Harvard Apparatus). The apparatus was cleaned with 15% ethanol between each subject.

Sucrose Preference Post-CSDS (Day 13-15, 42-44). This assay was performed in the animal's home cage. During the first 24 hrs, mice were shaped to drink from two spouted bottles containing tap water. During the next 24-96 hrs, one of the bottles was replaced with a bottle containing 1% sucrose, the position of which was switched every 24 hours. Fluid intake (ml by weight) was measured every 24 hours.

Endocrine stress response: Serum corticosterone levels

Baseline and restraint induced serum corticosterone levels were quantified using ELISA (Enzo Life Sciences, Cat. No. ADI-900-097) following the manufacturer's protocol. Single housed adult (8-12 weeks old) male and female mice were restrained in a wire mesh cone for 10 minutes, and were then briefly anesthetized at 0, 30, 60, or 90 minutes post-restraint using isoflurane (Isothesia, Henry Schein Animal Health) for blood collection using submandibular puncture (n=5/group/time point). Baseline (control) mice remained in their home cage until anesthesia and blood collection. All restraint and blood sampling took place between 10AM-12PM to reduce circadian rhythm-dependent corticosterone variability. Blood samples were kept at RT for 30 minutes, then centrifuged at 1,500g for 10 minutes at +4°C. The supernatant was stored at -80°C until testing.

Reproductive fitness

Breeding. Sexually naïve adult (8-10wk) Run and Sed males, counterbalanced on the C57BL/6Tn and C57BL/6J substrains, were co-housed in triads with a Run or Sed female on the C57BL/6Tn or C57BL/6J substrain. Two types of breeding enclosures were used, one modeling a large enriched territory (4 ft diameter enclosure) with bedding, food, and water as well as crawl tunnels and an elevated nest, and the other represented by a small simple territory (standard mouse cage). The following 7 days, behavior was recorded daily during the light and dark cycle for subsequent scoring (8-9 AM and 8-9 PM; lights on/off at 6 AM/PM). The shoulders or hind haunches of the males were shaved in order to differentiate them during subsequent behavioral observations. All variables, including enclosure type and shave pattern, were counterbalanced across groups. After one week, mice were separated and females single housed in standard shoebox cages until parturition.

Paternity test. Run or Sed paternity of the offspring was ascertained by PCR at the Nnt locus (a known SNP variation between the Jackson and Taconic substrains). Genotyping was adapted from (Nicholson et al., 2010). Briefly, the two strains differ in the Nnt gene (nicotinamide nucleotide transhydrogenase), with Jax mice missing exons 7-11. PCR was performed with primers: Nnt-COM (GTAGGGCCAACTGTTTCTGCATGA), Nnt-WT (GGGCATAGGAAGCAAATACCAAGTT G), Nnt-MUT (GTGGAATTCCGCTGAGAGAACTCTT). PCR reactions began with hot start (95°C), followed by 35 cycles at 95°C for 45 seconds, 58°C for 30 seconds for annealing, and 72°C for 45 seconds for extension. Offspring sired by both males were only used for inferring post-copulatory competition (sperm competition).
Female preference for male odor test. Following a 1 hr habituation to the testing room, adult (8-10 weeks old) sexually naïve Run or Sed females were allowed to explore two ceramic dishes filled with dirty bedding from Run and Sed offspring males and placed in opposing corners of the same side of the enclosure for 10 min in a 20 x 20 x 20 cm arena. Time spent in direct contact with the side or on the top of each dish was video recorded and quantified using automated tracking software (SMART v.3, Panlab, Harvard Apparatus).

Male interaction with female in small cage and arena. Interaction between males and females during the week-long breeding design was assessed from video recordings by an experimenter blinded to the subjects' condition. Specifically, the occurrence of direct contact between a male and female mouse was recorded in twelve 1-min intervals across the hr-long observation period.

Metabolism

Body composition. Body composition was measured by Echo-MRI (Echo Medical Systems, Houston, TX, USA).

Indirect Calorimetry. Each mouse was placed in Columbus Instruments' Comprehensive Lab Animal Monitoring System (CLAMS) home cage (Columbus Instruments, OH, USA) for 24 hour monitoring of animal activity, body mass, water and food intake, and indirect calorimetry measurements of O2 consumption, CO2 production, and temperature. Data were exported and analyzed in Prism 7.

16S rRNA gene sequencing

For each stool specimen, DNA was purified using a phenol-chloroform extraction technique with mechanical disruption (bead beating) based on a previously described protocol (Turnbaugh et al., 2009). Specimens were analyzed using the Illumina MiSeq platform to sequence the V4-V5 region of the 16S rRNA gene. Sequence data were compiled and processed using mothur version 1.34 (Schloss et al., 2009) and screened and filtered for quality (Schloss et al., 2011).

Measuring cytokine levels from milk

Mothers were separated from their pups at least 2 hours before milk collection. An average of 50 μl of milk was collected at postpartum day 10 from the mammary glands of each mouse by a vacuum operated system, 1 minute after the administration of 2 IU oxytocin in 0.1 ml (Liu et al., 2014). Right after milk collection, both the mothers and pups were euthanized. Milk samples from Runner and Sedentary mothers were diluted 1:1 in ice-cold protease buffer (0.15 mM spermine, 0.5 mM spermidine, 1 mM PMSF, 1× complete protease inhibitor in phosphate-buffered saline) and were centrifuged for 10 minutes (2,300g, 4°C). Supernatant was used to determine the levels

of cytokines and other bioactive substances via multiplex Luminex immunoassay by Myriad RBM Mouse InflammationMAP® v. 1.0 (A, B and C panels) (Liu et al., 2014).

Reversal of programmed behavior by the oral gavage of a cytokine cocktail

LIF (Cat #: CYT-645-B, ProSpec, Israel), CXCL1 (Catalogue #: CHM-335, ProSpec, Israel), CXCL2 (Cat #: 452-M2, Bio-Techne Corporation, USA) were reconstituted based on manufacturer's instructions in 1 mg/ml BSA solution. A cocktail of the three recombinant cytokines was given by daily gavage (0.8 ng LIF/g mouse, 7.8 ng CXCL1/g, and 0.25 ng CXCL2/g) between P2 and P14 (a period during pups rely entirely on maternal milk), using animal feeding needles (24 gauge, Harvard Apparatus). Daily doses of cytokines were calculated based on their milk concentration and milk consumption and accounting for loss and inactivation as described in the legend of Fig. 5F.

Data analysis

Social dominance (i.e., in Tube test, Territorial urine marking, Aggression) was determined according to the method described in (Clutton-Brock et al., 1979) and expressed as Clutton Brock index (CBI). It was computed using (B+b+k)/(L+l+k) where B: number of wins for that individual; b: sum of wins of the losers against that individual; L: number of losses for that individual; l: sum of losses of the winners against that individual; k=4. The output values were then analyzed via t-tests or one-way ANOVA.

Beta diversity (unweighted unifrac total) was visualized using Principal coordinate analysis based on dissimilarity distance matrices. Scripts for principle coordinate plots with confidence interval ellipses included functions from the following R packages: vegan, stats, ellipse, ggplot2. Proportion data of genera that were present in at least 25% of the samples were centered log-ratio transformation using 'robCompositions' package in R. Zero values were replaced by 0.000001 before transformation. PCA ellipses were created using 'FactoMineR' package in R. Differences in the proportion of taxonomic groups were analyzed using non-parametric Kruskal-Wallis test and p-value was adjusted for multiple comparisons using False Discovery Rate (FDR) in R. PERMANOVA analyses were performed using the adonis function in 'vegan' package in R.

Prism (7.0c) and SPSS(20) were used to perform t-tests, one-way ANOVA, two-way ANOVA, three-way ANOVA, Repeated-measures of ANOVA, and chi-square tests and are specified in the legends of each figure.

Supplemental References

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