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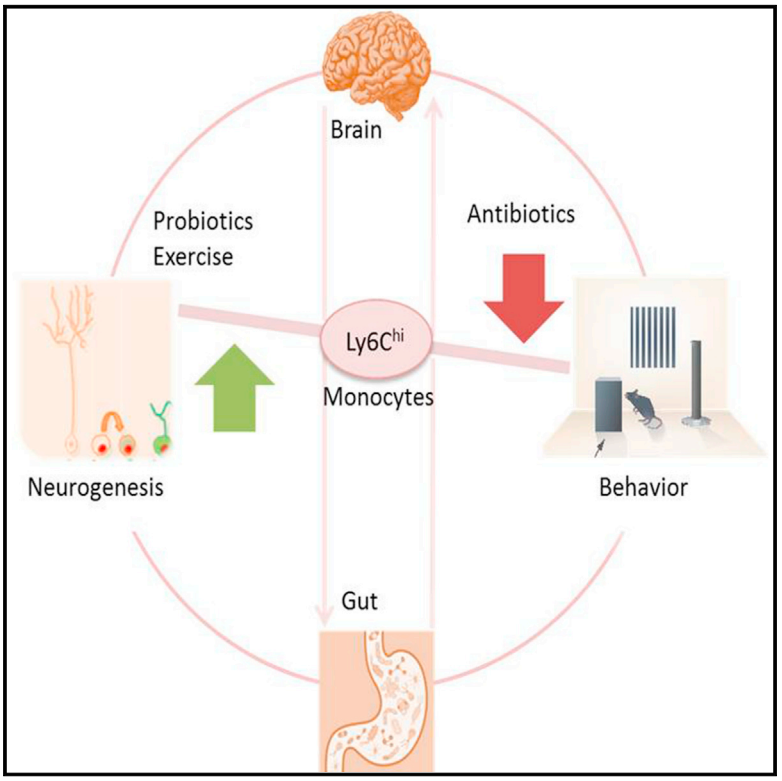
Ly6C^{hi} Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis

Graphical Abstract



Authors

Luisa Möhle, Daniele Mattei, Markus M. Heimesaat, ..., Polly Matzinger, Ildiko R. Dunay, Susanne A. Wolf

Correspondence

susanne.wolf@mdc-berlin.de

In Brief

Möhle et al. show the impact of prolonged antibiotic treatment on brain cell plasticity and cognitive function. They were able to rescue the decrease in neurogenesis by probiotic treatment, physical exercise, or transfer of Ly6C^{pos} monocytes. They propose that the Ly6C^{hi} population is crucial for brain homeostasis and plasticity.

Highlights

- Antibiotics decrease neurogenesis and cognitive function
- Probiotics or exercise rescues neurogenesis and cognitive function
- Ly6C^{hi} monocytes are crucial for brain homeostasis

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Ly6C^{hi} Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis

Luisa Möhle,[1,7] Daniele Mattei,[2,7] Markus M. Heimesaat,[3,7] Stefan Bereswill,[3] André Fischer,[3] Marie Alutis,[3] Timothy French,[1] Dolores Hambardzumyan,[4,5] Polly Matzinger,[6] Ildiko R. Dunay,[1,8] and Susanne A. Wolf[2,8,*]

[1]Institute of Medical Microbiology, University of Magdeburg, 39106 Magdeburg, Germany
[2]Department of Cellular Neuroscience, Max-Delbrueck-Center for Molecular Medicine, 13125 Berlin, Germany
[3]Charité - University Medicine Berlin, Department of Microbiology and Hygiene, 14195 Berlin, Germany
[4]Department of Neurosciences at the Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA
[5]Department of Pediatrics, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Emory University School of Medicine, Atlanta, GA 30322, USA
[6]Ghost Lab, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, MD 20892-9760, USA
[7]Co-first author
[8]Co-senior author
*Correspondence: susanne.wolf@mdc-berlin.de
http://dx.doi.org/10.1016/j.celrep.2016.04.074

SUMMARY

Antibiotics, though remarkably useful, can also cause certain adverse effects. We detected that treatment of adult mice with antibiotics decreases hippocampal neurogenesis and memory retention. Reconstitution with normal gut flora (SPF) did not completely reverse the deficits in neurogenesis unless the mice also had access to a running wheel or received probiotics. In parallel to an increase in neurogenesis and memory retention, both SPF-reconstituted mice that ran and mice supplemented with probiotics exhibited higher numbers of Ly6C^{hi} monocytes in the brain than antibiotic-treated mice. Elimination of Ly6C^{hi} monocytes by antibody depletion or the use of knockout mice resulted in decreased neurogenesis, whereas adoptive transfer of Ly6C^{hi} monocytes rescued neurogenesis after antibiotic treatment. We propose that the rescue of neurogenesis and behavior deficits in antibiotic-treated mice by exercise and probiotics is partially mediated by Ly6C^{hi} monocytes.

INTRODUCTION

Antibiotics (Abxs) are applied by millions of people and billions of farm animals worldwide every day. Although their use has saved countless lives, Abxs also can negatively impact the physiology and psychology of the patients (Bercik and Collins, 2014; Mostafa and Miller, 2014). Some of these changes have been ascribed to the impact of Abxs on the gut-brain axis, as the microbiota colonizing the intestinal lumen are thought to affect their host's vegetative and cognitive functions (Diaz Heijtz et al., 2011; Mayer, 2011). Clinical and experimental evidence indicates that the microbiota-brain relationship plays a crucial role in the development of metabolic and mental diseases (Bested et al., 2013).

The lifelong production of new neurons in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus is a cell-based process of neuronal plasticity in adult mammals (Kempermann et al., 2008). Previous studies have shown that adult hippocampal neurogenesis occurs in rodents (Altman and Das, 1965; Cameron et al., 1993; Kaplan and Bell, 1983, 1984; Kaplan and Hinds, 1977; Kempermann et al., 1997; Kuhn et al., 1996; Seki and Arai, 1993), non-human primates (Gould et al., 2001; Kornack and Rakic, 1999), and humans (Eriksson et al., 1998; Spalding et al., 2013). Adult neurogenesis has been linked to hippocampus-dependent cognitive function (Bruel-Jungerman et al., 2007; Deng et al., 2010), and it is required for memory resolution and for proper pattern separation in the DG of the hippocampus (Aimone et al., 2011; Jessberger et al., 2008; Sahay et al., 2011; Stringer et al., 2015). The hippocampus has long been studied for its critical involvement in many mental diseases (Bloom, 1975; Holsboer, 1988).

Decreases in hippocampal neurogenesis can be induced by lack of maternal care (Mirescu et al., 2004) or social isolation (Lu et al., 2003), both paradigms leading to behavioral aberrances. It was reported that chronic stress (Pham et al., 2003) and alcohol (Nixon and Crews, 2002) have a negative impact on hippocampal neurogenesis (Schoenfeld and Gould, 2013) and also lead to mental disorders, such as major depression, posttraumatic stress disorder, and Alzheimer's disease (Jacobs et al., 2000; Reif et al., 2007; Sahay and Hen, 2007; Santarelli et al., 2003). In all three disorders, clinical evidence suggests that the individual level of overall activity, either physical or cognitive, partly counteracts the impairment (Daley, 2008; Pajonk et al., 2010; Sagatun et al., 2007; Scarmeas et al., 2009; Wilson et al., 2002). These findings provide indirect evidence that, in humans, activity can improve neurogenesis comparable to the effects of a voluntary exercise or an enriched environment in rodents (Kempermann et al., 1997; van Praag et al., 1999).

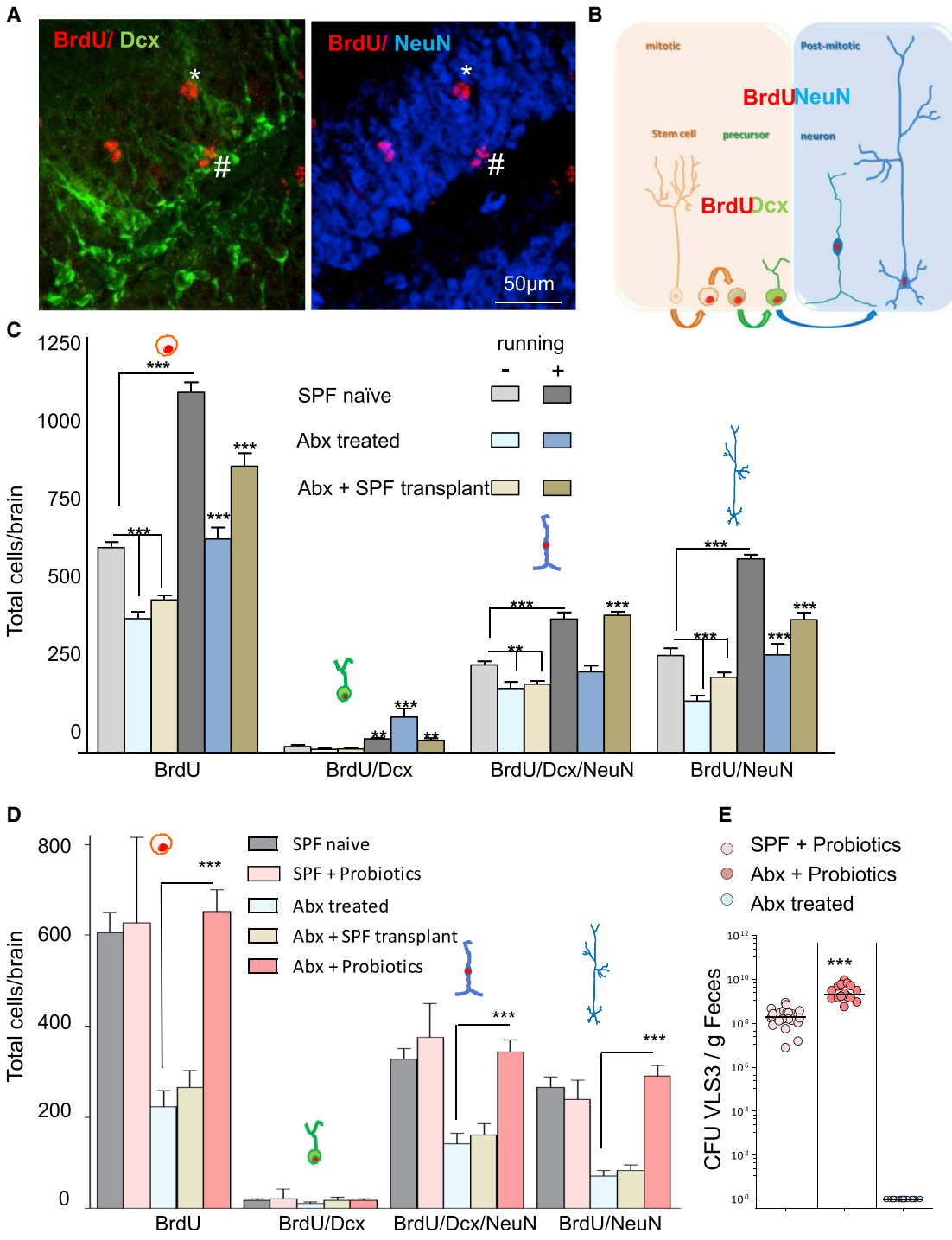

Figure 1. Reduced Neurogenesis in Abx-Treated Mice Can Be Restored by Exercise and Probiotic Treatment

(A) The first panel shows representative micrographs of the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Proliferating cells labeled with BrdU (red) arise in the SGZ of the DG, mature, and integrate into the granular cell layer labeled with NeuN (blue). Doublecortin (Dcx, green) is a marker for mitotic neuronal progenitor cells (NPCs) and NeuN (blue) is a marker for mature neurons (*cell expressing BrdU and NeuN and #cell expressing BrdU/NeuN and Dcx).

(B) The cartoon visualizes the steps in neuronal maturation in the DG, and the colors match with the fluorochromes in (A).

(C) The total number of BrdU-positive cells is quantified along with the number of BrdU-labeled cells at certain maturation stages from SPF mice treated (Abx treated) or not (SPF naive) with antibiotics (Abxs). Some mice were then given a fecal transplant (Abx + SPF transplant), and half the mice in each group was given access to a running wheel (darker bars, running +) for 4 weeks prior to analysis. The asterisk on the Abx + SPF transplant and Abx + running + SPF transplant show

(legend continued on next page)

Indeed, it has been shown that physical exercise can increase hippocampal volume in schizophrenic patients (Pajonk et al., 2010) and adult hippocampal neurogenesis in a rodent model of schizophrenia (Wolf et al., 2011). Whether and how the gut microbiota can impact the process of neurogenesis are not yet known.

Here we studied Abx-treated adult C57BL/6 mice to investigate the impact of gut flora dysbiosis on hippocampal neurogenesis. In our study we included voluntary exercise and treatment with probiotics to counteract antibiosis.

Intestinal microbiota are able to influence the number, migration, and functions of certain immune cell subsets (Dorrestein et al., 2014), and thus they modulate immune responses at mucosal surfaces during infection, inflammation, and autoimmunity (Kamada et al., 2013; Round and Mazmanian, 2009). Gut microbes also can regulate the immune response in other organ systems as reported in animal models for diabetes and rheumatoid arthritis (Markle et al., 2013; Wu et al., 2010). Growing evidence suggests that gut microbial composition may direct the character and level of an immune response in the brain (Berer et al., 2011). Very recently it was shown that gut microbiota shape the maturation and function of microglia, the intrinsic immune cells of the CNS (Erny et al., 2015). Moreover, a link among neuropsychiatric disorders, neuroinflammatory components, and gut microbiota dysregulation was discussed (Petra et al., 2015). One way to balance gut microbiota composition is to administer probiotic treatment (for review see Sanders, 2011). Here we used VSL#3, which is a mixture of eight different strains of bacteria. In the clinical setting, studies have confirmed that VSL#3 is effective in reducing inflammation and symptom severity (Kim et al., 2015; Mimura et al., 2004). VSL#3 administration to mice rendered the inflammatory response in colitis and a pain model (Dai et al., 2013; Reiff et al., 2009). Moreover, application of VSL#3 modulated the expression of a large group of genes in brain tissue, and it attenuated an age-related deficit in long-term potentiation in aged rats (Distrutti et al., 2014) and sickness behavior development in mice (D'Mello et al., 2015).

While the crosstalk between peripheral immune cells and the CNS has been established over the past decade (Schwartz et al., 2013; Schwartz and Shechter, 2010), it is unclear whether the gut microbiota can shape the maintenance of the peripheral immune system in the steady state. The selective gateway for leukocyte entry to the CNS is the choroid plexus, which mediates important neuro-immunological processes also under steady-state conditions (Baruch and Schwartz, 2013). Among other groups, we have proved previously that T cells are required to maintain neurogenesis (Wolf et al., 2009; Ziv et al., 2006). Here we focused on the potential of monocytes to serve as a messenger between gut and brain and to shape adult hippocampal neurogenesis. Monocytes are a fundamental leukocyte subset of the innate immune system, and they contribute to the immune surveillance and host defense upon infections and inflammation (Biswas et al., 2015; Dunay et al., 2008; Shi and Pamer, 2011). However, they also can promote disease progression in certain disorders (Hammond et al., 2014; Karlmark et al., 2012; Schumak et al., 2015). How monocytes are affected by Abx-induced dysbiosis of the gut flora and subsequently shape neurogenesis is the focus of this study.

RESULTS

Abx Treatment Decreases Adult Hippocampal Neurogenesis

We started by treating adult animals with broad-spectrum Abxs for 7 weeks. In a previous study we have shown that application of these Abxs severely depleted the intestinal microbiota of mice (Heimesaat et al., 2006). To determine the phenotype of the proliferating cells in the hippocampus, we stained with antibodies against NeuN (which labels mature neurons) and doublecortin (Dcx, which labels the majority of transient proliferating mitotic neuronal progenitor cells [NPCs]; Figures 1A and 1B). The progenitor cells expressing Dcx represent the most plastic population of progenitors. Moreover, the commitment to the neuronal lineage starts with the expression of Dcx (Kempermann et al., 2004, 2015). We analyzed hippocampal sections by immunofluorescence and detected that the number of BrdU-positive cells in the SGZ of the DG was significantly lower in the brains of Abx-treated animals (387 ± 60; Figure 1C, light blue bar) compared to naive ones (592 ± 48; Figure 1C, light gray bar). This effect was apparent in both neuronal progenitors (BrdU/Dcx/NeuN) and mature neurons (BrdU/NeuN). These data suggest that Abx treatment has a long-lasting effect on neurogenesis.

Voluntary Exercise Rescues Neurogenesis Levels Despite Antibiosis

To test whether exercise enhances neurogenesis in Abx-treated mice, we supplied the mice with running wheels for 10 days during the treatment, injected BrdU for the last 3 days with running wheels, and assayed neurogenesis 4 weeks later. Mice voluntarily ran ~0.28 ± 0.05 km/day (6 p.m. to 8 a.m.) across all groups. Figure 1C shows that access to running wheels (running +) significantly increased neurogenesis by 44% in naive specific pathogen-free (SPF) mice and by far less (28%) in Abx-treated animals. In Abx-treated mice that had received a fecal transplant, neurogenesis was increased by 47%, equivalent to its effect on control mice. Ten days of running did not change the composition of the gut flora in naive SPF mice, Abx-treated mice, or Abx mice that received an SPF fecal transplant (Figures S1A and S1B). We show representative images of the BrdU,

the significant differences compared to the Abx-treated group revealed by Bonferroni's post hoc test (n = 10 per group; three independent experiments; two-way ANOVA, Bonferroni's post hoc test, $^{**}p < 0.01$ and $^{***}p < 0.001$; $F_{(5,576)} = 185.67$).

(D) The total numbers of BrdU-positive cells and NPCs (BrdU/Dxc/NeuN) and the resulting mature neurons (BrdU/NeuN) normalized after probiotic treatment are shown (n = 10 per group; two independent experiments; two-way ANOVA, Bonferroni post hoc test, $^{***}p < 0.001$; $F_{(3, 304)} = 164.25$).

(E) The total amount of colony-forming units (CFUs) in samples derived from feces of Abx-treated mice after probiotic treatment is significantly higher compared to SPF naive mice treated with probiotics (one-way ANOVA, Bonferroni post hoc test,$^{***}p < 0.001$; $F_{(3,63)} = 35.81$). Please refer to Figure S1 for the analysis of fecal samples of the other groups.

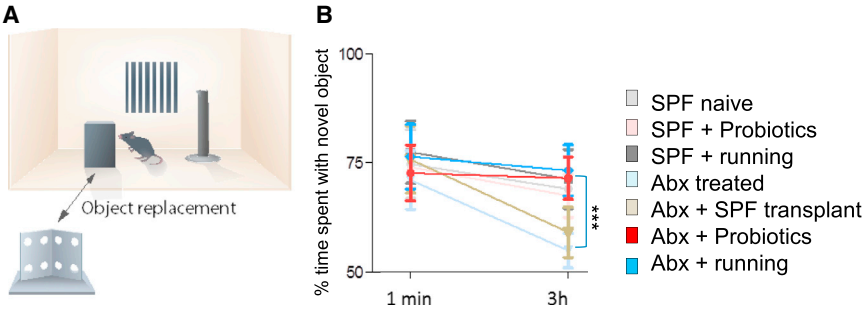

Figure 2. Novel Object Recognition Performance in Abx-Treated Mice Is Restored by Exercise and Probiotics

(A) Test procedure and apparatus are depicted in the graphic. After a familiarization phase of 10 min with two objects (e.g., cylinder and cube), one object is exchanged (e.g., bracket) and the mouse is tested after 1 min and again after 3 hr.

(B) We calculated the percentage of time spent with the novel object within a total of 1 min of contact with both objects. For the 1-min delay interval, all groups performed above chance and there were no group differences. For the 3-hr delay interval, the Abx-treated group and the Abx + SPF transplant group failed to perform as well as SPF naive (n = 12 per group; one-way ANOVA; $F_{(7,84)} = 27.95$ $p < 0.001$). The Abx + run and Abx + probiotics performed similarly like the SPF naive group, and all groups performed above chance. However, there was no effect of voluntary exercise or probiotics when applied to the SPF naive group.

NeuN, and Dxc staining from the naive SPF animals with access to a running wheel versus the mice treated with Abxs to give an overview of the two extremes of the spectrum for BrdU cell numbers detectable per hippocampal section (Figure S2).

Probiotics Fully Restore Neurogenesis

To see if reconstitution with a complex gut flora rescues neurogenesis, we gave a fecal transplant from untreated SPF mice 2 days after the final Abx treatment. Then 7 days later, we injected BrdU on 3 consecutive days, and we analyzed hippocampal neurogenesis 4 weeks later. Figure 1C shows that the fecal transplant (Abx + SPF transplant versus Abx treated: 265 ± 114 versus 223 ± 109) had little effect on hippocampal neurogenesis, even though it seemed to normalize the overall species distribution of the gut flora (Figure S1A). These results indicate that treatment with Abxs impairs adult neurogenesis and that reconstitution with normal intestinal flora does not promote regeneration. When mice received probiotics instead of a fecal transplant 2 and 4 days after we discontinued the Abx treatment, we were able to fully restore neurogenesis to the control level (probiotics versus SPF naive: 652 ± 143 versus 607 ± 133; Figure 1D). Probiotic treatment of naive SPF mice did not increase neurogenesis levels above baseline. The total amount of colony-forming units (CFUs) in samples derived from feces of Abx-treated mice after probiotic treatment is even higher compared to naive SPF mice treated with probiotics, confirming a successful recolonization of probiotics within the gut of Abx-treated mice (Figure 1E). Of note, exercise and probiotic treatment rescued the number of progenitor cells at a late maturation stage (BrdU/Dcx/NeuN) and mature neurons (BrdU/NeuN) within the analysis time window of 4 weeks. Additionally, wheel running had a measurable effect on the younger $NeuN^{neg}$ progenitor cell population. This is consistent with the known robust effect of exercise on progenitor cell proliferation (Fabel et al., 2009). Probiotic treatment seems to have less impact on proliferation and predominantly promotes the survival of progenitor cells.

Probiotic Treatment and Exercise Rescue the Behavioral Deficits of Abx-Treated Mice in a Novel Object Recognition Test

It was shown by Jessberger et al. (2009) that the DG-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. Here we adapted the protocol for mice. For the 1-min delay interval, all groups performed above chance and there were no group differences. This indicates that all of the groups exhibited substantial memory retention at the 1-min delay (Figure 2B). For the 3-hr delay interval, the Abx-treated group (55% ± 4%) and the Abx + SPF transplant group (58% ± 6%) failed to perform as well as the naive SPF group (69% ± 4%, $p < 0.05$). The Abx + running (74% ± 6%) and Abx + probiotics (72% ± 5%) groups performed similarly to the naive SPF group, and all groups performed above chance. However, there was no effect of voluntary exercise (72% ± 7%) or probiotics (68% ± 5%) on the naive SPF group. Both groups performed better than chance levels and on a similar level to the naive SPF group. This is in line with findings where an increase of adult hippocampal neurogenesis, induced by genetic expansion of adult-born neurons, above baseline control levels shows normal object recognition and spatial learning (Sahay et al., 2011). Thus, probiotics and voluntary exercise are potent interventions to rescue the impaired performance of the Abx-treated mice, but they do not enhance the baseline control level. The rescue of the hippocampus-dependent behavior in the Abx mice by voluntary exercise and probiotics corresponds to the neurogenesis levels, which were returned to normal by the same interventions (Figure 1C).

$Ly6C^{hi}$ Monocytes Provide a Missing Link among Brain, Gut, and Treatment Paradigms

Although it is well known that there are neural associations between the gut and the brain (Mayer, 2011), there is also evidence that the innate immune system may serve as an additional link. Previous studies have indicated that cells of the immune system help to maintain neurogenesis (Wolf et al., 2009; Ziv et al., 2006), can be influenced by Abxs (Morgun et al., 2015), and can respond to exercise (Shantsila et al., 2012). Therefore, we asked whether innate immune cells in the brain may act as a link among the brain, gut, and exercise. Analyzing the infiltrating immune cell populations in single-cell preparations from one brain hemisphere of Abx-treated mice (Figure 3A), we detected that the proportions of $Ly6C^{hi}$ monocytes had significantly decreased compared to the naive SPF group (0.16% ± 0.05% versus 0.26% ± 0.1%; Figure 3B). The decrease in $Ly6C^{hi}$ cells also was evident in bone marrow

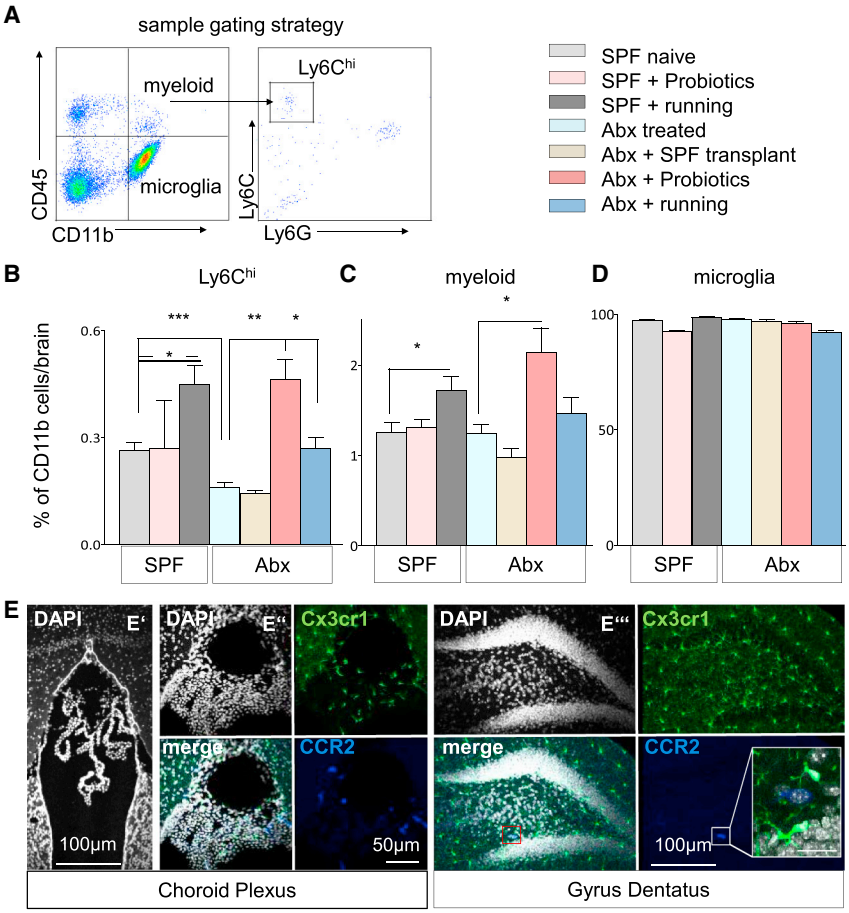

Figure 3. Ly6C^{hi} Monocyte Number Drops in the Brain of Abx-Treated Mice and Is Rescued by Exercise and Probiotics, but Not by Fecal Transplant

(A) Cells isolated from brains were stained for CD45, CD11b, Ly6G, and Ly6C for flow cytometric analysis. Representative pseudocolor plots demonstrate the gating strategy used to identify resident CD11b^{pos}CD45^{lo} microglia (lower right quadrant) and recruited CD11b^{pos}CD45^{hi} myeloid cells in the upper right quadrant, from which the Ly6C^{hi} monocytes were further separated (right plot) from Ly6G^{pos} neutrophil granulocytes.

(B–D) Bar graphs display the results of flow cytometric analysis of (B) Ly6C^{hi} monocytes, (C) total myeloid cells, and (D) microglia in brain homogenates of mice from the respective groups (one-way ANOVA; $F_{(7,79)} = 7.27$, $p < 0.001$). We detected a significant decrease in Ly6C^{hi} monocytes in the brains of Abx-treated mice compared to the SPF naive group (n = 38 from five independent experiments; unpaired two-tailed t test; $t_{(31)} = 3.884$, $p = 0.0005$). The SPF fecal transplant did not rescue the Ly6C^{hi} numbers in the brains of Abx-treated mice (Abx treated versus Abx + SPF transplant: n = 25 from three independent experiments; $t_{(22)} = 1.17$, $p = 0.25$). Access to a running wheel brought brain Ly6C^{hi} numbers back to control level (SPF naive versus Abx + running: n = 31 from three independent experiments; $t_{(29)} = 0.11$, $p = 0.9$), while running increased Ly6C^{hi} numbers in brains of SPF naive mice (n = 27 from two independent experiments; $t_{(25)} = 3.57$, $p = 0.0015$). Probiotic treatment of Abx mice increased the brain Ly6C^{hi} numbers even above SPF naive levels (n = 29 from two independent experiments; $t_{(27)} = 3.99$, $p = 0.0005$), while it had no effect in SPF naive mice per se (n = 29 from two independent experiments; $t_{(24)} = 0.077$, $p = 0.94$). (C) In the myeloid population similar trends are visible, and the effects of exercise in the SPF naive group and probiotics in the Abx-treated group were significant ($p < 0.05$). (D) There was no change detectable in the resident microglia population.

(E) To visualize the location of the Ly6C^{hi} monocytes, we utilized a reporter for *Ccr2* (RFP) and *Cx3cr1* (EGFP). (E′) Localization of the choroid plexus within the third ventricle, cell nuclei stained with DAPI (white) is shown (objective 10×). (E″) Representative images of CCR2^{+} cells (blue) in the choroid plexus (DAPI white) are distinct from CX3CR1-expressing cells (green) (objective 20×). (E‴) Representative picture shows a CCR2^{+} cell (blue) in the hippocampus (DAPI white, CX3CR1 green) (objective 20×). In the inset within the CCR2 single-channel image, we zoomed in on the single CCR2-expressing cell with a 63× objective (merge of DAPI in white, CCR2 in blue, and CX3CR1 in green).

and blood 1 week after Abx treatment but normalized 4 weeks later (Figures S3A–S3F).

We further tested the impact of fecal and probiotic reconstitution and exercise on the population of Ly6C^{hi} monocytes in the brain. We detected that reconstitution with SPF gut flora did not reverse the drop in brain-infiltrating Ly6C^{hi} monocytes, pointing toward a long-lasting effect of Abxs on brain homeostasis (0.14% ± 0.02%; Figure 3B, light brown bar). However, access to running wheels (0.27% ± 0.06%) and probiotic treatment (0.46% ± 0.15%) significantly elevated the Ly6C^{hi} monocyte proportions in the brain (Figure 3B). Similar trends as in Ly6C^{hi} monocytes could be seen in the myeloid population (Figure 3C). In comparison, the number of the intrinsic microglia population was not changed in any of the conditions (Figure 3D). Thus, neurogenesis rescue by running or probiotic treatment, as depicted in Figures 1C and 1D, was paralleled by an increase in the brain's CD11b^{pos}CD45^{hi}Ly6G^{neg}Ly6C^{hi} monocyte population, while the number of the brain's intrinsic CD11b^{pos}CD45^{int} population was unchanged. To visualize the location of the Ly6C^{hi} monocytes, we utilized reporters for *Ccr2* (red fluorescent protein [RFP]) and *Cx3cr1* (EGFP). CCR2 has a substantial overlap with the Ly6C^{hi} population and labels infiltrating monocytes (Biswas et al., 2015; Quintar et al., 2015). We detected CCR2^{+} cells in the choroid plexus, as shown in the representative images (Figure 3E″). In our previous study, we located CCR2^{+}Ly6C^{hi} cells in the inflamed brain parenchyma of mice infected with the neurotropic parasite *Toxoplasma gondii*. In the current sudy in the non-infected brain, we could observe only few CCR2^{+} cells in the hippocampus (Figure 3E‴). Due to the low number of immune cells in the brain parenchyma, we decided to use flow cytometric analysis of one hemisphere for quantification.

Lack of Monocytes Decreases Neurogenesis

To determine whether the influx of Ly6C^{hi} monocytes was a cause, effect, or simply correlated with the rate of hippocampal neurogenesis, we used the following three different strategies to

alter monocyte numbers: genetic depletion, antibody depletion, and adoptive transfer. In the first case, we used *Ccr2*-deficient (*Ccr2*$^{-/-}$) mice, in which the lack of the chemokine receptor CCR2 deters Ly6C^{hi} monocytes from exiting the bone marrow, consequently reducing their number in the periphery (Figure S4; Serbina and Pamer, 2006). Figure 4A shows that mice lacking *Ccr2* exhibited significantly decreased levels of neurogenesis (488 ± 134 BrdU^{+} cells) when compared to wild-type mice (1,014 ± 83), as well as a 40% drop in Ly6C^{hi} monocytes in their brains (Figure 4B). Since Ccr2 is absent throughout development in *Ccr2*-deficient mice and may affect brain development from the early ages, we utilized a second model in which we targeted Ly6C^{hi} monocytes in adult mice using the anti-CCR2 monoclonal antibody MC-21, which specifically ablated Ly6C^{hi} monocytes in the brain (Figure 4D). One day after the first antibody treatment, we injected BrdU and 3 weeks later evaluated the effect on neurogenesis. Treatment of naive SPF mice with MC-21 (Figure 4C) led to a 25% decrease in total BrdU-positive cells (1,111 ± 32) when compared to IgG-treated controls (1,496 ± 119). The depletion of another myeloid-derived mononuclear cell subset, namely Ly6G^{pos} neutrophils, with the specific monoclonal antibody 1A8 did not alter the number of Ly6C^{hi} monocytes in the brain (Figure 4D) or modify neurogenesis levels (Figure 4C). Together, these two depletion studies suggest that CCR2^{pos} cells, particularly Ly6C^{hi} monocytes, are necessary for neurogenesis in the adult brain.

Adoptive Transfer of Ly6C^{hi} Monocytes Rescues Neurogenesis in Abx-Treated Mice

To resolve whether the transfer of Ly6C^{hi} monocytes can rescue neurogenesis in Abx-treated mice, we intravenously injected 10^6 CD11b^{pos} Ly6C^{hi} monocytes sorted from the bone marrow into Abx-treated mice at days 2 and 7 after discontinuing the Abx treatment. Next we injected BrdU for 3 consecutive days starting 24 hr after the first cell transfer, and we analyzed the numbers of Ly6C^{hi} monocytes and proliferating neuronal precursor cells 3 weeks after the BrdU injections. Figure 4F displays that the adoptive transfer of Ly6C^{hi} monocytes led to a doubling of the proportion of Ly6C^{hi} monocytes in the brains and completely restored neurogenesis in Abx-treated mice (Abx treated versus Abx + monocytes Ly6C^{hi}: 648 ± 84 versus 1,381 ± 149; Figure 5E). Naive SPF mice that received Ly6C^{hi} monocytes showed neither an increase in Ly6C^{hi} monocytes in the brain (Figure 4F) nor a change in neurogenesis (Figure 4E). These results confirm that Ly6C^{hi} monocytes are important messengers and they signal between the periphery and the brain to restore homeostatis.

Ly6C^{hi} Monocytes Promote Sphere Formation of NPCs In Vitro

To determine if the Ly6C^{hi} monocytes could have a direct effect on neurogenesis, we co-cultured various numbers of bone marrow-derived CD11b^{pos}Ly6C^{hi} monocytes with primary NPCs derived from the hippocampi of postnatal day (P)16 C57BL/6 mice. Due to the trans-well system, NPCs and Ly6C^{hi} monocytes could not interact via direct cell-cell contact. Figure 5 displays that, at a ratio of one Ly6C^{hi} monocyte per 1,000 NPCs, the Ly6C^{hi} monocytes significantly increased sphere formation (96 ± 5.6) compared to medium alone (64 ± 8.9), whereas the addition of CD11b^{pos}Ly6C^{neg} cells at the same ratios did not change sphere formation or even decreased the number of spheres (25 ± 6). Thus, it appears that CD11b^{pos}Ly6C^{hi} monocytes can support primary NPCs to enhance neurogenesis via soluble factors.

DISCUSSION

Here we examined the complex interaction among Abx treatment, gut microbiota, and the brain's NPC pool. Colonization with probiotics, as well as voluntary exercise, rescued neurogenesis in Abx-treated mice. Previous studies have shown that probiotics also were able to rescue neurogenesis in a murine model of stress (Ait-Belgnaoui et al., 2014). Voluntary exercise is a widely used strategy that was applied by us and others to increase neurogenesis in various disease models including endophenotypes of psychiatric disorders (Kempermann et al., 2010; van Praag et al., 2014; Wolf et al., 2011). Here we demonstrate that both treatment paradigms were accompanied by an increase in Ly6C^{hi} monocytes in the brain. Lack of the monocyte cell population, either by antibody treatment or in *Ccr2* knockout mice, led to a decrease in neurogenesis, while adoptive transfer of Ly6C^{hi} monocytes rescued neurogenesis in Abx-treated mice. This observation was compelling, as disparate effects of Ly6C^{hi}CCR2^{pos} monocytes on brain-related functions were reported previously (Gordon and Taylor, 2005). Bone marrow-derived Ly6C^{hi}CCR2^{pos} monocytes are essential for recovery after spinal cord injury (Shechter and Schwartz, 2013), and they are involved in restricting cerebral amyloidosis in patients with Alzheimer's disease (Baruch et al., 2016; Naert and Rivest, 2013). In contrast, these cells also serve as key mediators of inflammation and pathology upon chronic cerebral infection, stroke, and other diseases (Biswas et al., 2015; Hammond et al., 2014; Karlmark et al., 2012). Thus, as it has been reported for T cells (Schwartz et al., 1999), Ly6C^{hi} monocytes can perform complex sets of beneficial as well as detrimental functions in the CNS.

The impact of Abxs on neurogenesis was not rescued by the SPF fecal transplant, which implicates a direct effect of Abxs on the host. Indeed, it was shown previously that changes in gene expression in the gut of Abx-treated mice were driven by at least two factors: depletion of the microbiota and direct effects of Abxs on host tissues (Bercik and Collins, 2014). Direct effects of Abx treatment were demonstrated in our previous study showing that Abxs can even influence host mitochondria, which retain many features of the bacteria from which they descended (Morgun et al., 2015). Abx treatment as well as probiotic treatment could have a direct effect on cell metabolism of all body cells, including neuronal stem cells and neuronal precursors. Although antibiosis can possibly have a direct effect on neurogenesis, we bypassed the Abx effect by an adoptive transfer of Ly6C^{hi} monocytes into Abx-treated animals. Moreover, under Abx treatment, voluntary exercise alone was able to counteract the lack of a complex gut flora and partially restore neurogenesis. This led us to the conclusion that not exclusively the lack of gut flora determines neurogenesis levels, and additional factors also play an important role. Therefore, we hypothesized the presence of a common mediator, which signals from the

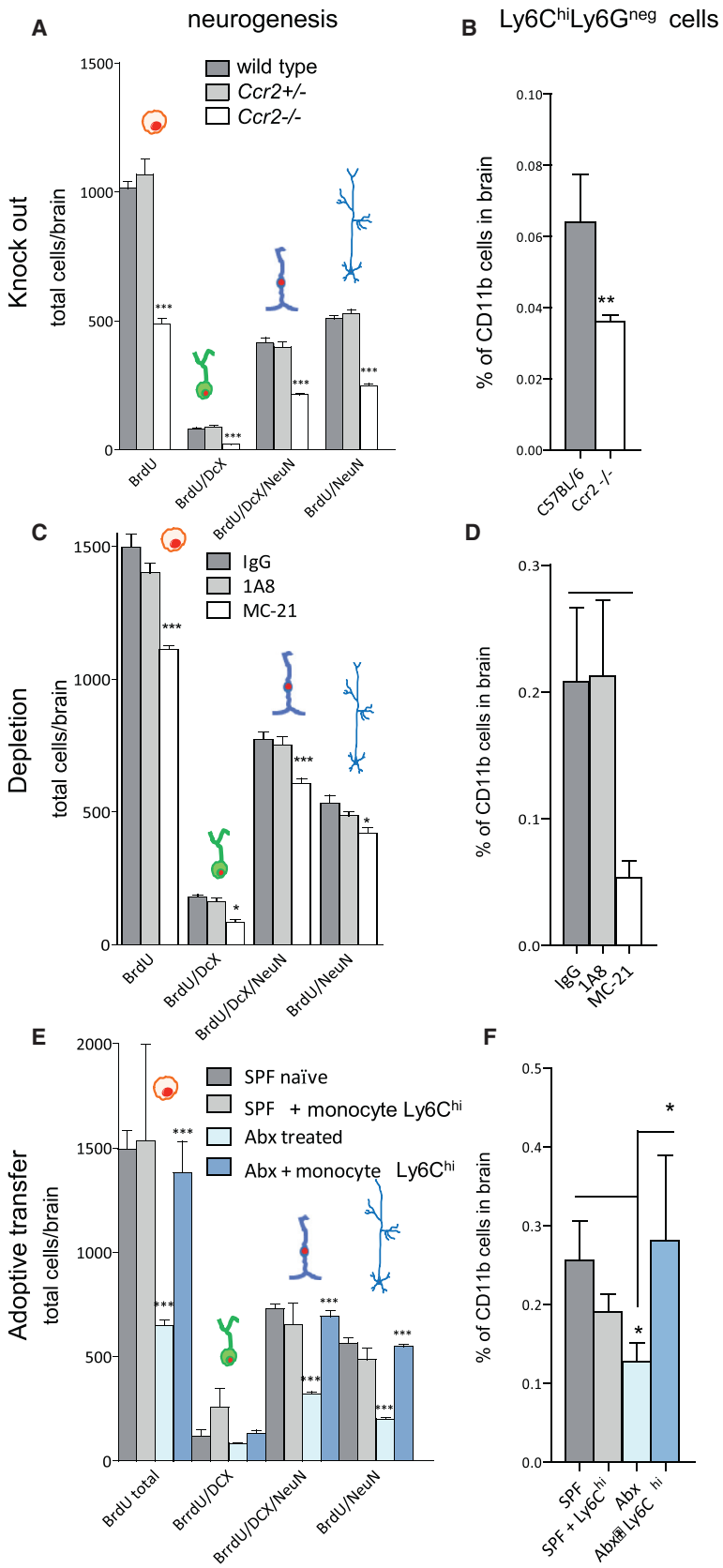

Figure 4. Genetic Absence or Antibody-Induced Loss of CCR2$^+$ Ly6C^{hi} Monocytes Results in Decreased Neurogenesis, while Transfer of Ly6C^{hi} Monocytes into Abx-Treated Animals Rescues Neurogenesis

(A and B) Adult female *Ccr2*$^{+/-}$, *Ccr2*$^{-/-}$, and wild-type littermates were injected with BrdU i.p. Four weeks later (A), neurogenesis was evaluated by phenotyping BrdU$^+$ cells with Dcx and NeuN as described in Figure 1 (one-way ANOVA, Bonferroni post hoc test, ***$p < 0.001$; $F_{(2,84)} = 193.74$). (B) The decrease of Ly6C^{hi} monocytes as a proportion of CD11b$^+$ cells in the brains of *Ccr2*$^{-/-}$ animals compared to wild-type littermates was measured using the gating strategy displayed in Figure 3 (n = 8 animals/group; unpaired two-way t test, $p = 0.046$).

(C and D) Adult C57BL/6 females were injected with an anti-CCR2 antibody (MC-21) to deplete Ly6C^{hi} monocytes or an anti-Ly6G antibody (1A8) to deplete neutrophils. (C) One day after the initial injection of the depleting antibody, they received BrdU; 4 weeks later, neurogenesis was evaluated by phenotyping BrdU$^+$ cells with Dcx and NeuN. BrdU$^+$ cells decreased in the brains of the mice treated with MC-21 compared to IgG and 1A8 controls (n = 6 animals/group; one-way ANOVA, Bonferroni post hoc, ***$p < 0.001$ and *$p < 0.05$; $F_{(2,60)} = 59.97$). (D) Ly6C^{hi} monocytes decreased in the brains of mice treated with the MC-21 antibody compared to IgG and 1A8 controls (n = 6 animals/group; one-way ANOVA, $p < 0.03$; $F_{(2,17)} = 4.44$; Bonferroni post hoc MC-21 versus IgG, *$p < 0.05$).

(E and F) Abx-treated mice were injected with freshly isolated FACS-sorted Ly6C^{hi} monocytes from the bone marrow. We repeated the adoptive transfer twice, five days apart, and labeled proliferating cells by BrdU injection 1 day after the first transfer. (E) Similar to the experiments shown in Figure 1, neurogenesis was decreased in Abx-treated mice compared to SPF naive mice (n = 6–8 per group; two independent experiments; two-way ANOVA, Bonferroni post hoc test, **$p < 0.001$; $F_{(3,68)} = 31.10$). Transfer of Ly6C^{hi} monocytes returned neurogenesis levels of Abx-treated mice back to normal (SPF naive versus Abx + Ly6C^{hi}, Bonferroni post hoc, $p > 0.05$). (F) While the Ly6C^{hi} monocytes in the brains of Abx mice decreased significantly compared to SPF naive, there was no difference between Abx mice that received Ly6C^{hi} monocytes and SPF naive animals. Transfer of Ly6C^{hi} monocytes into SPF naive animals did not change the number of Ly6C^{hi} monocytes in the brains (n = 5–7 animals/group; two independent experiments; unpaired two-way t test, SPF naive versus Abx treated: $p = 0.049$, SPF naive versus Abx treated + Ly6C^{hi} $p = 0.95$, and SPF naive versus SPF + Ly6C$^{hi:}$ $p = 0.18$).

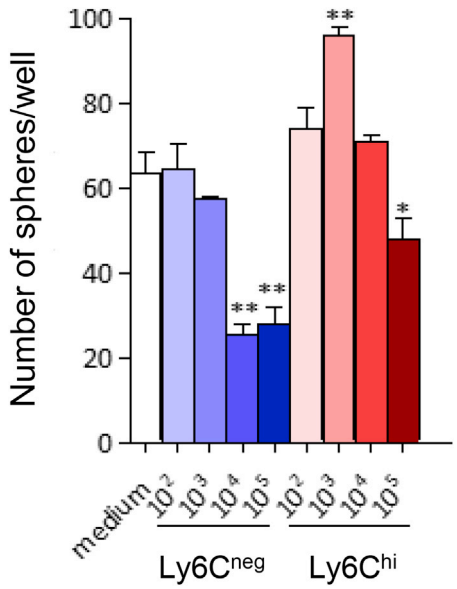

Figure 5. Co-culture of Primary Hippocampal NPCs with Bone Marrow-Derived Ly6C^{hi} Monocytes Results in an Increase of Neurosphere Number

Primary NPCs from C57BL/6 female donor mice were plated at a density of 10^5 cells/ml into the bottom of 96-well trans-well plates at a volume of 200 μl of proliferation medium. Neurospheres formed by the NPCs in each well (12 wells per group) were counted using an inverted light microscope after 72 hr of co-culture. On top of the trans-wells, we plated different numbers of freshly isolated, FACS-sorted, CD11b^{pos}Ly6C^{hi} (red bars) or CD11b^{pos}Ly6C^{neg} cells (blue bars) from naive bone marrow. Co-cultures were compared with NPCs cultured in medium only (white bar) (n = 12; two-way ANOVA, Bonferroni post hoc test; three independent experiments; *$p < 0.05$ and **$p < 0.01$; $F_{(5,420)} = 412.81$).

periphery to the brain, and that this messenger is affected by antibiosis and can be restored by exercise and probiotic treatment. Based on our data from the depletion and in vitro experiments, we propose that Ly6C^{hi} monocytes serve as a link between the gut and the brain, contributing to the modulation of neurogenesis in health and disease.

The host microbiota maintain systemic populations of myeloid cells in circulation by modulating hematopoietic stem cell and myeloid precursors in the bone marrow (Deshmukh et al., 2014). Consistent with these observations, bone marrow-derived splenic macrophage and monocyte populations were found to be reduced under germ-free conditions (Khosravi et al., 2014). In line with these findings, we demonstrated that, 1 week after the discontinuation of Abx treatment, bone marrow-derived myeloid populations decreased in bone marrow and blood. Then 4 weeks after the discontinuation of Abx treatment, the myeloid cells in the bone marrow and blood were back to control levels, while the drop in numbers of Ly6C^{hi} monocytes was still evident in the brain. This could be explained by a constant but slow turnover of peripheral immune cells in the brain, similar to what was described for leukocyte surveillance in the brain (Ousman and Kubes, 2012). Whether the prolonged use of Abxs would result in long-lasting effects in neurogenesis and brain function remains to be investigated. Consistent with recent findings (Erny et al., 2015), the number of the brain's resident macrophage population, the microglia, was not changed by Abx treatment. Under germ-free conditions, microglia and other immune cell populations seem to be more severely affected, since germ-free mice have never been exposed to a complex microbial flora and, therefore, development of the immune system is affected in those mice (Cerf-Bensussan and Gaboriau-Routhiau, 2010).

The study by Erny et al. (2015) investigated only microglia, missing the important contribution of myeloid-derived cells; thus, making a direct comparison of the studies is rather difficult. It is likely that some of the changes reported in germ-free mice could be attributed to alterations in the circulating immune cell composition and function. With respect to brain function and behavior, germ-free mice exhibit reduced anxiety-like behavior (Neufeld et al., 2011) and altered social development (Arentsen et al., 2015). In parallel to the behavioral alterations, germ-free mice display an increase in BDNF mRNA levels in the hippocampus (Neufeld et al., 2011). Moreover, neurogenesis is increased in germ-free mice (Ogbonnaya et al., 2015), which corresponds to the increase in hippocampal BDNF in this mouse model. Germ-free mice have been shown to display higher motor activity (Diaz Heijtz et al., 2011), and this also may account for an increase in neurogenesis.

Since adult neurogenesis is a complex procedure that is orchestrated by several factors, such as neurotransmitters, metabolites, as well as hormones (Kempermann et al., 2015), the effect in mice treated with Abxs could be driven by multiple aspects besides the levels of Ly6C^{hi} monocytes. Depletion of the gut microbiota with Abxs from weaning onward reduced anxiety and induced cognitive deficits, while BDNF was significantly reduced in the adult brain (Desbonnet et al., 2015). This is in line with our findings of reduced neurogenesis and cognitive deficits in mice treated with Abxs during adulthood. The opposite effects in behavior, BDNF levels, and neurogenesis between the germ-free mouse model and the Abx-treated mouse model show that a direct comparison of these models is rather difficult. Both models harbor aspects besides the lack of gut microbiota that should be taken into account, either developmental deficits in the germ-free mouse model or possible direct effects of the Abxs on host tissue in the Abx-treated mice.

We here demonstrate that Abx treatment results in a long-lasting impairment of neurogenesis that can be restored by probiotics and voluntary exercise. Cells of the innate immune system, in particular Ly6C^{hi} monocytes, are critical for the restorative effect. We cannot thoroughly rule out that other CNS-resident cells, such as microglia, neurons, astrocytes, or endothelial cells, are affected as well, and NPCs may receive additional signals involving other mechanisms. However, our analysis of Ly6C^{hi} monocytes in different treatment paradigms in conjunction with the depletion and repopulation experiments points toward their crucial involvement in adult hippocampal neurogenesis. With Ly6C^{hi} monocytes, here we present one distinct messenger that communicates among the gut-immune-brain axis. Finally, our results provide a rationale for probiotic supplementation and exercise to restore monocyte homeostasis and brain plasticity to counteract the devastating side effects of prolonged Abx treatment.

EXPERIMENTAL PROCEDURES

Animals

In each independent experiment for Figures 1, 2, and 3, we used 70 female 6- to 8-week-old C57BL/6 wild-type mice. We performed three independent experiments and used the data points of 210 individual mice. We need to emphasize that not all the groups mentioned below were included in each of the independent experiments. The actual experimental repeats and numbers (n) are stated in the figure legends. Mice that had access to a running wheel were housed in pairs to avoid single housing, which can result in social deprivation that has an impact on neurogenesis. A small magnet was attached to the bottom rim of the wheels to monitor the rotations by a cycling computer during the active phase from 6 p.m. to 8 a.m. Knowing the diameter of the wheel (17 cm), we were able to calculate the distance the two mice ran per active phase. Running groups were monitored over 10 days and an average distance was calculated for each group. The animals were handled according to governmental (LaGeSo) and internal (MDC/Charité/University of Magdeburg) rules and regulations. All mice were kept under a 12-hr light/dark cycle and had access to food and water ad libitum with two to five mice per cage. The experimental design is shown in Figure S5.

Abx and Probiotic Treatments

Since commensals are required for normal development especially of the immune and nervous systems, we used a model of Abx-induced dysbiosis according to the protocol published previously (Heimesaat et al., 2006). The Abx compounds were applied via the drinking water for 7 weeks and consisted of ampicillin plus sulbactam (1.5 g/l; Pfizer), vancomycin (500 mg/l; Cell Pharm), ciprofloxacin (200 mg/l; Bayer Vital), imipenem plus cilastatin (250 mg/l; MSD), and metronidazol (1 g/l; Fresenius). Then 72 hr before gut flora reconstitution, the Abx treatment was discontinued and replaced by sterile tap water. Then Abx-treated mice were orally challenged with fecal flora by 300-μl gavage on 2 consecutive days. The fecal transplant consisted of a mixture from 1 mg feces of five different SPF mice dissolved in 15 ml sterile PBS. In parallel to the fecal transplants, the Abx plus probiotics group received VSL#3 (Sigma-Tau Pharmaceuticals) probiotics orally by gavage. VSL#3 is a commercially available probiotic mixture consisting of the following eight bacterial strains: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus delbrueckii* subsp. *Bulgaricus*. We dissolved a total of 4.5×10^9 probiotic bacteria into 10 ml PBS. By gavaging 300 μl, each mouse received approximately 1×10^7 viable probiotic bacteria.

BrdU Treatment

For the analysis of cell proliferation and survival, the animals received an intraperitoneal (i.p.) injection of 50 μg BrdU (Sigma-Aldrich)/g body weight at a concentration of 10 mg/ml BrdU in sterile 0.9% NaCl solution for 3 consecutive days, and they were examined 4 weeks later if not stated otherwise. Please note that the BrdU injections for experiments depicted in Figure 1 were done in a different lab (Charité Berlin) than the BrdU injections in Figures 4A (Cleveland Clinic), 4C, and 4E (University of Magdeburg). Despite keeping protocols and substances similar across labs, individual injection techniques and volumes account for differences in baseline BrdU cell numbers. We always included a matching control group in each individual experiment and one should not compare baseline BrdU numbers across experimental groups.

Cell Counting and Unbiased Stereology

Cell counts were determined in an unbiased approach using the optical fractionator procedure of the StereoInvestigator software (MBF Bioscience) for every sixth brain section containing the hippocampus. The obtained cell number was multiplied by the number of slices analyzed to calculate the number of BrdU-positive cells in the whole hippocampus. Since both hippocampi were counted, we used as a unit the total number of BrdU-positive cells per brain. For further phenotypic analysis of BrdU-positive cells, 50 cells in the DG from each brain were counted on a confocal microscope for colocalization with Dcx or NeuN. Six different tissue sections were analyzed throughout the hippocampus and tissue from at least eight individual mice per group were used for phenotyping.

Novel Object Recognition Test

It was shown by Jessberger et al. (2009) that a DG-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. We adapted the protocol for mice. The novel object recognition task takes advantage of a rodent's spontaneous preference to explore novel objects relative to familiar objects. This test is the benchmark test of recognition memory in the rodent and is dependent on the integrity of the hippocampus (for review, see Squire et al., 2007). The test was conducted in a white plastic chamber (35 × 40 × 40 cm). A video camera connected to a computer was used to record the exploration and testing sessions. A single trial consists of a familiarization phase (encoding) followed by a delay interval and then a test phase (retrieval). During the familiarization phase, the mouse was allowed to explore and become familiar with two identical objects placed side by side in the chamber. A single 10-min familiarization phase was presented before the test phase. We tested the mice after delay intervals of 1 min and 3 hr. Before the test phase, one object was replaced by an unfamiliar object to the mouse (novel object). During the test phase, the mouse was allowed to explore two objects placed side by side (one novel object). The experimentor scored object exploration until the mouse accumulated 1 min of contact time with the objects in total (nose within 1 cm of the objects). The dependent measure was the percentage of time that a mouse spent exploring the novel object during the 1 min of object exploration. Which object was novel and the left/right position of the novel object was counterbalanced within each group. The experimenter was blind to the group membership of the mouse during offline data analysis.

Flow Cytometry

Single-cell suspensions were incubated with an anti-FcγIII/II receptor antibody (clone 93, eBioscience, 14-0161) to block unspecific binding. Thereafter, cells were stained with the following fluorochrome-conjugated antibodies against cell surface markers: CD45 (30-F11, eBioscience, 48-0451), CD11b (M1/70, eBioscience, 17-0112), Ly6G (1A8, BD Biosciences, 561104), Ly6C (HK1.4, eBioscience, 45-5932), CD3 (17A2, eBioscience, 11-0032) in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% fetal calf serum [FCS] and 0.1% NaN_3) for 30 min on ice and then washed and fixed in 4% paraformaldehyde for 10 min. Cell acquisition was performed on a BD FACS Canto II flow cytometer. Data were analyzed using FlowJo software (Tree Star).

Depletion of Monocytes

Depletion of monocytes with 75 μg/injection anti-CCR2 monoclonal antibody (MC-21, kindly provided by M. Mack, University of Regensburg) and neutrophil granulocytes with 440 μg/injection anti-Ly6G monoclonal antibody (1A8, BioXCell, BE0075-1) was started on day 0 by i.p. injection of the respective antibody in 300 μl PBS. BrdU was injected on days 1–3. Antibody injections were continued every third day until day 20 and animals were sacrificed on day 21. Control animals received 75 μg polyclonal rat IgG (BioXCell, BE0094).

Adoptive Transfer of Monocytes

$Ly6C^{pos}$ cells were isolated from the bone marrow of C57BL/6 mice using flow cytometry and sorting for adoptive transfer. $Ly6C^{pos}$ cells (10^6) were injected into the tail vein of adult female C57BL/6 Abx-treated mice. The procedure was repeated 48 hr later followed by BrdU injections.

Sphere Formation Assay

The number of newly formed spheres was assessed by plating 10^5 NPCs/ ml (96-well plate) and incubated in 200 μl cultivation medium. $Ly6C^{hi}$ cells were isolated from bone marrow and sorted with FACS. Either $Ly6C^{pos}$ or $Ly6C^{neg}$ cells were co-cultured on top of the trans-well in different concentrations, as indicated in Figure 5. Twelve wells were seeded per cell concentration. After 72 hr, the trans-well was removed and the number of spheres per well was counted using an inverted light microscope. An Alamar-blue assay was performed to control for cell viability.

Statistical Analysis

Datasets were analyzed statistically using GraphPad Prism 3. Before we started statistical analysis, we tested for normality with a normality probability plot. We performed Dixons test for single outlier, but we did not detect outliers in our dataset. We used either two-way or one-way ANOVA, with Bonferroni

post hoc or unpaired two-tailed Student's t test where applicable. We mention the respective statistical test in the figure legends. Significances are depicted in the respective figure legends as mean ± SEM. When we sacrificed the animals (D.M. and S.A.W.), we prepared a list with animal IDs. We prepared the specimens (brain, blood, feces, and bone marrow) for further analysis and coded the samples in order to do a blind analysis afterward. The investigator who analyzed the samples (L.M., D.M., I.R.D., S.A.W., and A.F.) was blind to the animal's treatment group.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.074.

AUTHOR CONTRIBUTIONS

S.A.W. and I.R.D. designed the experiments and supervised the project. L.M., D.M., M.M.H., S.A.W., I.R.D., A.F., M.A., T.F., and D.H. performed the experiments. L.M., D.M., M.M.H., S.A.W., I.R.D., S.B., and A.F. interpreted the data. L.M., S.A.W., I.R.D., and P.M. contributed to manuscript preparation. M.M.H., I.R.D., and S.B. funded the project. P.M. inspired the experiments and discussed results.

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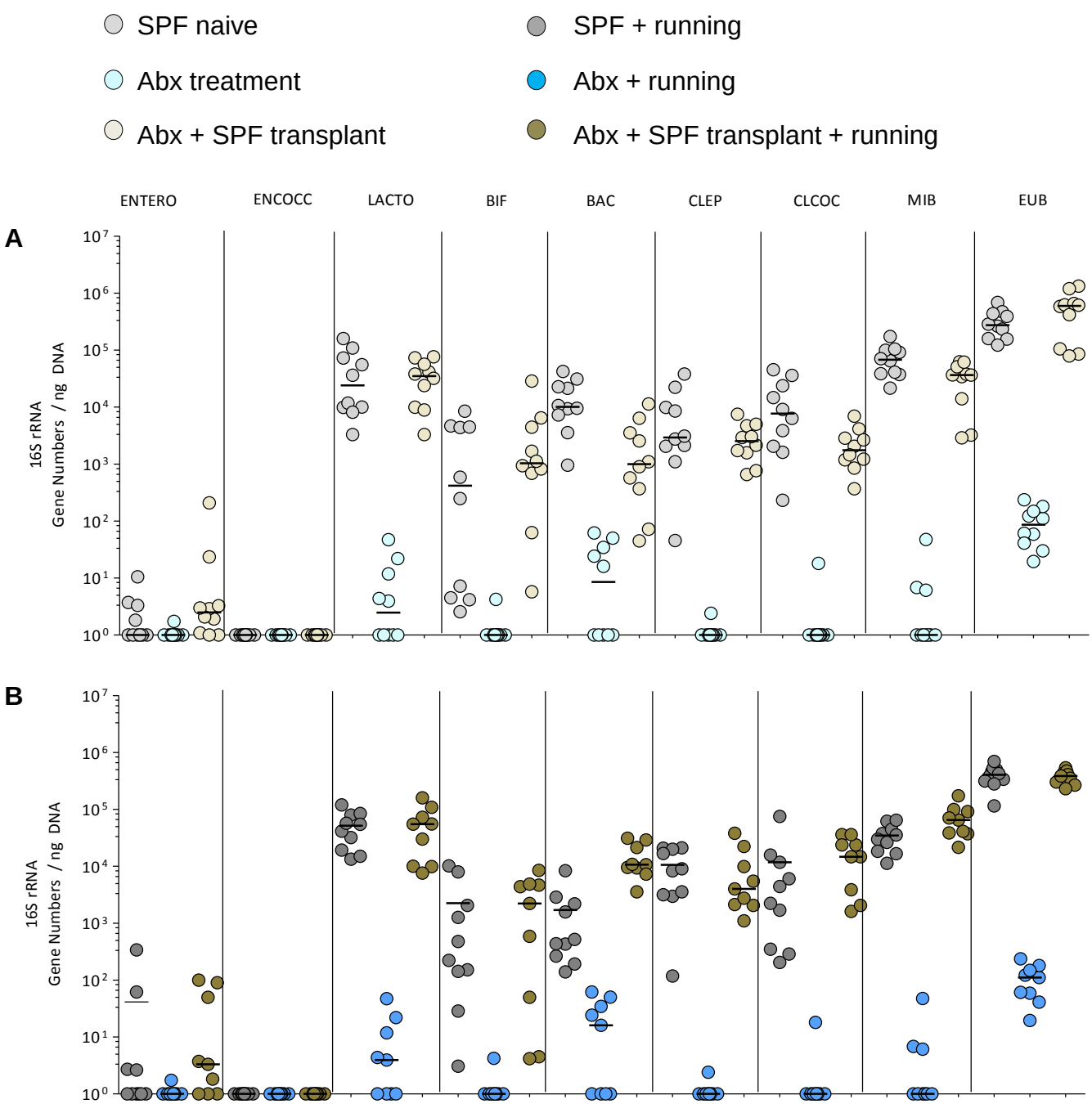
Cell Reports, Volume *15*

Supplemental Information

Ly6C^{hi} Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis

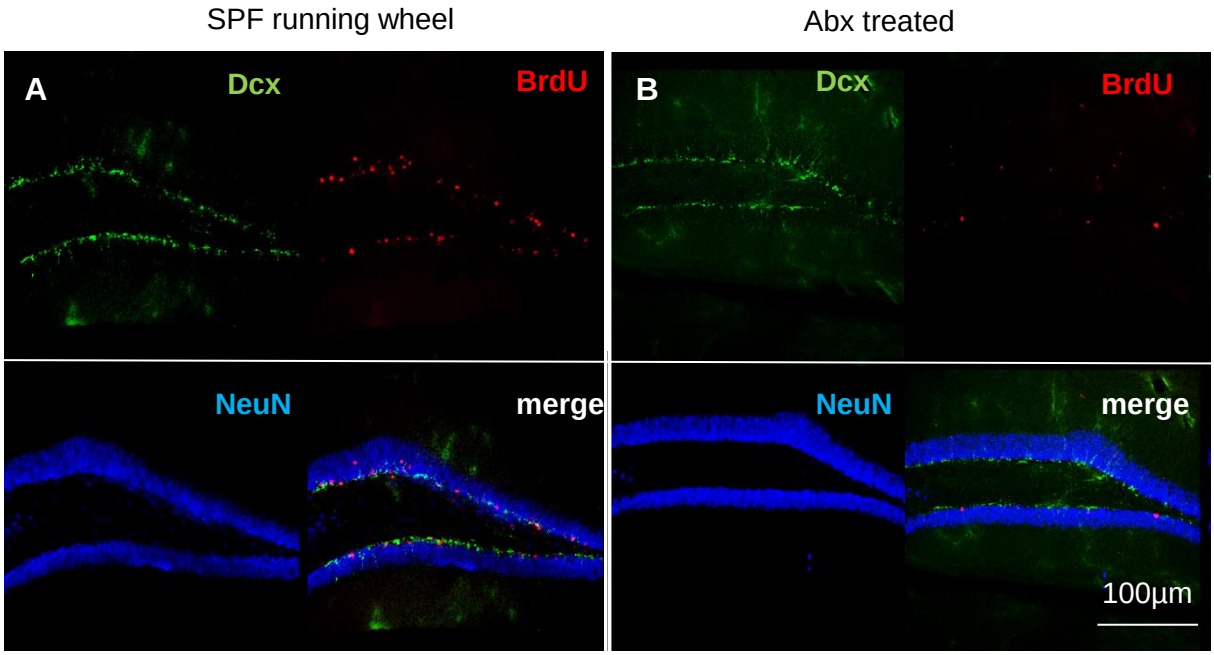
Luisa Möhle, Daniele Mattei, Markus M. Heimesaat, Stefan Bereswill, André Fischer, Marie Alutis, Timothy French, Dolores Hambardzumyan, Polly Matzinger, Ildiko R. Dunay, and Susanne A. Wolf

Supplementary figure 1
Example of analysis of fecal samples of animals used in main figures 1 - 4



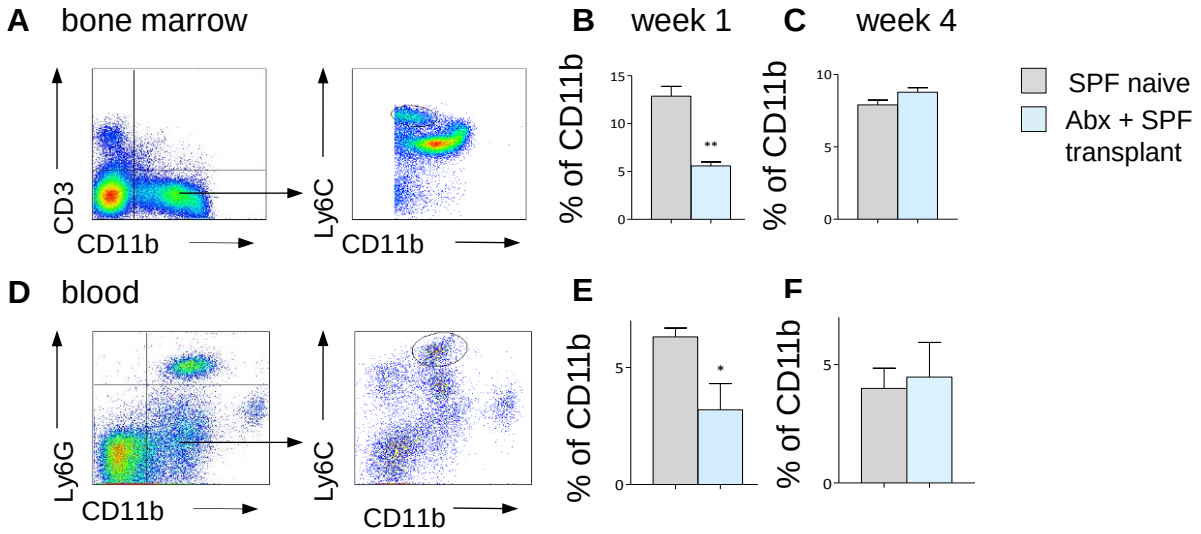
(A) Analysis of feces from specific pathogen free (SPF naive) control animals and animals treated with the antibiotic cocktail (Abx) for 7 weeks and antibiotic treated mice that received a fecal transplant from SPF mice 2 days after the antibiotic treatment stopped. (Abx + SPF transplant). The analysis was done 21 days after the fecal transplant to show successful repopulation with the major gut bacteria strains: ENTERO – Enterobacteria, ENCOCC - Enterococci, LACTO – Lactobacilli, BAC – *Bacterioides*, CLEP – *Clostridium leptum* subgroup, COER – *Clostridium coccoides/Eubacterium rectale* subgroup, MIB – Mouse Intestinal *Bacterioides*, BIF – Bifidobacteria, EUB – 338 positive control. The plot shows a representative analysis from one individual experimental set including 10 animals per group. Each dot represents the data from one animal. **(B)** Here we show the same groups like in A ten days after running.

Supplementary figure 2
Examples for BrdU cell staining in the hippocampus depicted in representative micrographs used for analysis of total BrdU number in main figures 1 and 4



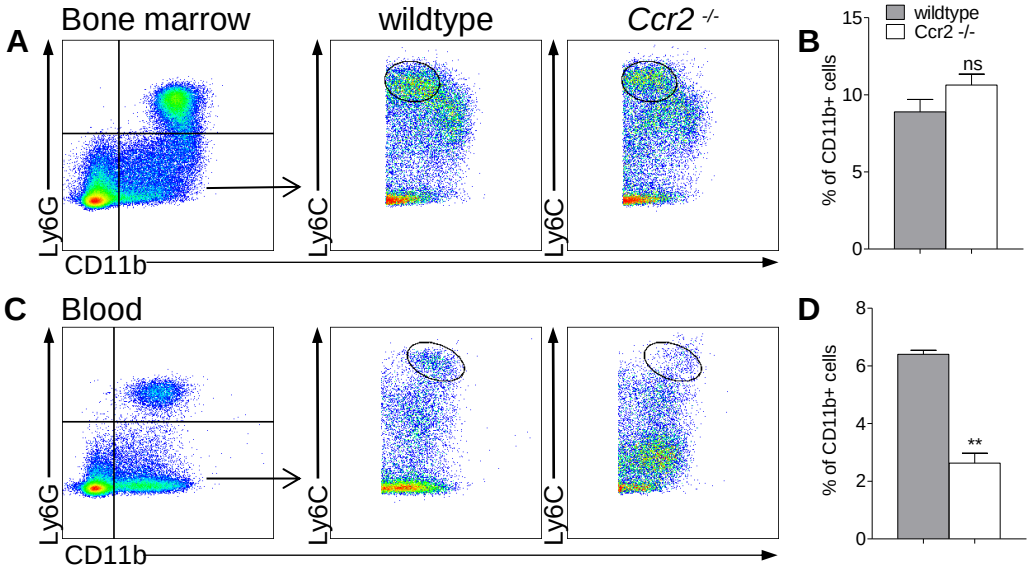
(A) The first panel shows representative micrographs of the dentate gyrus in the hippocampus from SPF naïve mice after ten days of running. This was the highest number of BrdU cells in a hippocampal section within our experimental groups.
(B) The lowest number of proliferating BrdU cells we detected in the hippocampal sections from mice treated with antibiotics, which is illustrated in the right panel. Proliferating cells labeled with BrdU (red), Doublecortin (Dcx, green) as a marker for mitotic neuronal progenitor cells and NeuN (blue) as a marker for mature neurons. Images were taken with an 10x objective.

Supplementary figure 3
Ly6C^{hi} cell number in bone marrow and blood in Abx+SPF transplant mice vs. SPF naïve mice complementary to the Ly6C^{hi} numbers in the brain shown in main figure 3



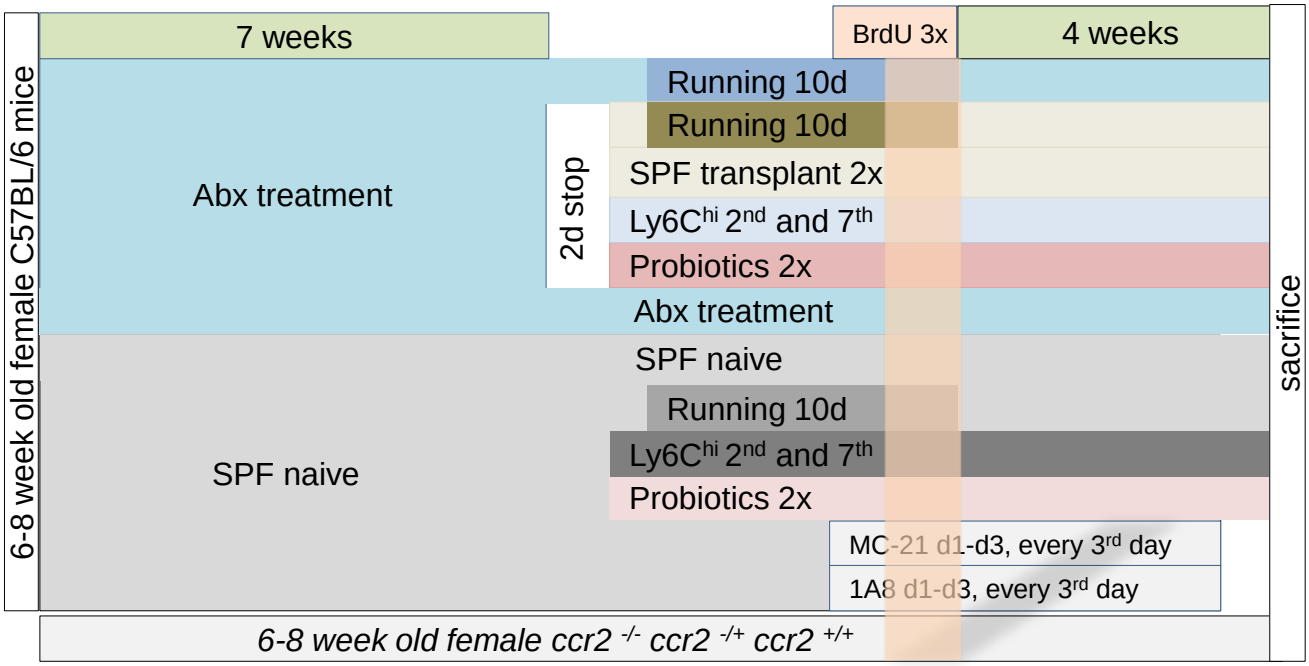
(**A,D**) Cells isolated from peripheral organs were stained against specific surface markers for flow cytometric analysis. Representative pseudocolor plots demonstrate the gating strategies used to identify Ly6C^{hi} monocytes from (**A**) bone marrow and (**D**) blood. In (A) we gated for the CD3^{neg}CD11^{pos} population. In (D) we gated for the Ly6G^{neg}CD11b^{pos} population. The Ly6C^{hi} population is encircled in the secondary dot plots as a percentage of CD11b. (**B**) In the bone marrow, we found a lower proportion of Ly6C^{hi} monocytes in Abx treated animals reconstituted with SPF flora compared to SPF naïve ($p = 0.0024$, $t_{(30)} = 4.352$) one week after discontinuation of Abx treatment. (**C**) Four weeks after discontinuation of the Abx treatment and start of SPF fecal transplant, no differences in the proportion of Ly6C^{hi} monocytes in the bone marrow were measured between the groups. (**E**) In the blood, there were fewer Ly6C^{hi} monocytes in Abx treated than in SPF naïve mice one week after discontinuation ($p = 0.0303$, $t_{(30)} = 2.627$). (**F**) Four weeks after discontinuation of the Abx treatment and start of SPF fecal transplant, the differences were no longer present and the same proportion of Ly6C^{hi} monocytes was found in all experimental groups. (n = 8, two independent experiments, unpaired two tailed t test).

Supplementary figure 4
Ly6C^{hi} cell number in bone marrow and blood in *Ccr2*$^{-/-}$ mice complementary to the Ly6C^{hi} numbers in the brain shown in main figure 4B



(**A,C**) Cells isolated from peripheral organs were stained against specific surface markers for flow cytometric analysis. Representative pseudo color plots demonstrate the gating strategies used to identify Ly6C^{hi} monocytes from **(A)** bone marrow and (**C**) blood of *Ccr2*$^{-/-}$ (right plots) compared to *Ccr2*$^{+/+}$ littermates (middle plots). In the first (left) plots we gated for CD11b^{pos} Ly6G^{neg} cells. The round gates in the middle and right plots circle the Ly6C^{hi}CCR2^{pos} population that was analyzed. **(B)** In bone marrow there is no difference between the genotypes. (**D**) $p = 0.0004$, $t_{(3)} = 17.45$ the Ly6C^{hi}CCR2^{pos} population was significantly reduced. ($n = 3$, unpaired two tailed t test).

Supplementary figure 5
Experimental design showing the experimental groups and setup used in main figures 1-4.



We here show the experimental design. Mice were treated with the antibiotic cocktail for 7 weeks.

Treatments: The antibiotic treatment was stopped for 2 days before treating with either SPF transplant (2 gavages on 2 consecutive days), Ly6C^{hi} cells (2 days and 7 days after antibiotic discontinuation) or Probiotics (2 gavages on 2 consecutive days). Ly6C^{hi} transfers and probiotic treatment have been done in mice from the SPF naïve group in parallel to the Abx treated mice.

Running: Another group of Abx treated mice , SPF naïve mice and Abx treated mice with SPF transplant had access to a running wheel for 10 consecutive days.

Antibodies: Additional SPF naïve mice were treated either with the monocyte depletion antibody MC-21 or with the antibody 1A8 that depletes all neutrophil cells. First antibody injections were done one day before the first BrdU injection and co-injected with the BrdU for 3 days. Until day 21 after the first antibody injection, the injections were repeated every 3rd day.

Knock out animals: In addition a cohort of *ccr2* $^{-/-}$ and *ccr2* $^{-/+}$and *ccr2* $^{+/+}$ littermates were investigated.

BrdU injections: Apart from the antibody injected mice, who were sacrificed 3 weeks after the first of three BrDU injections, all other mice despite of treatment were sacrifices 4 weeks after the last of three BrdU injections. The BrdU injections for mice subjected to a running wheel were done on the last 3 consecutive days of running.

Additional information regarding animals with respect to main experimental procedures section

In preliminary experiments we obtained a standard deviation in the treatment group (antibiotics) σ = 25. For the sample size calculation, we assumed a common standard deviation of σ = 30, as we could not rule out an increase in variability in some of the groups with double treatment (e. g. antibiotic-treated and running wheel). nQuerys 'Two-way analysis of variance' was calculated for a 80% power factor during treatment on the significance level of 5 % and revealed a minimum sample size of n=8 per group. For each individual experiment, we randomly assigned mice from different litters to the initial two treatment groups: 30 SPF naïve, 50 antibiotic (Abx) treated. After seven weeks, we maintained 10 animals in each of the initial groups for baseline measurements and divided the remaining animals from the Abx group further. These animals were randomly assigned to: Abx treated plus fecal transplant, Abx treated plus probiotics, SPF naïve with access to running wheel, SPF naïve plus probiotics, Abx treated with access to running wheel, Abx plus fecal transplant plus access to running wheel, Abx plus Ly6C^{hi} cell transplantation with a minimum of 8 animals per group. For the behavior experiments depicted in Fig. 2, we used additional 12 animals for the following groups: SPF naïve, SPF running, SPF probiotics, Abx treatment, Abx running, Abx probiotics, that were obtained like described above. As donors for bone marrow and subsequent Ly6C^{hi} cells for *in vitro* culture or *in vivo* transfer, we used a total of 12 adult female C57BL/6 mice. In figure 4 we show a representative gating strategy and depict Ly6C^{hi} numbers in bone marrow and blood from 3 individual *ccr2*$^{-/-}$ and *ccr2*$^{+/+}$ mice. For figure 5 we used 10 *ccr2*$^{-/-}$ mice and their heterozygote and wild type littermates that were housed and treated under SPF conditions in the animal facilities of the Cleveland Clinic, Cleveland Ohio, USA according to the governmental and federal law and recommendation. To produce the representative images showing CCR2RFP and CX1CR3EGFP cells, we transcardially perfused 3 *ccr2*$^{RFP/wt}$ x *cx1cr3*$^{EGFP/wt}$ female mice. For depletion and adoptive transfer we started with a total of 60 female C57BL/6 mice for each individual experimental set up and randomly assigned them to the groups SPF naïve, SPF naïve + Ly6C^{hi} monocytes, IgG control, 18A, MC-21, Abx treated and Abx + Ly6C^{hi} monocytes with 6-8 animals in each group. We repeated the experiments twice. For figure 6 we used 2 adult female C57BL/6 mice as donors for Ly6C^{+} cells and 6 P16 female C57Bl/6 mice as donors for neuronal precursor cells. We repeated the experiment three times. The bars represent the average of 12 individual wells from all three experiments. If there is a discrepancy between initial animal numbers and numbers used for the statistical analysis this is due to mortality.

Gut flora analysis from fecal samples with respect to main experimental procedures section

Fecal samples were taken weekly to monitor the antibiotic-treated mice under antibiosis. After fecal transplants from SPF mice, the samples were taken 7, 21 and 33 days after the gavage of the mice. We show the representative results from 10 naïve SPF mice compared to 10 mice treated with the antibiotic cocktail and 10 mice transplanted with SPF flora 21 days after the gavage. DNA from fecal samples was extracted as described previously (Heimesaat et al., 2010). Briefly, DNA extracts and plasmids were quantified using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng/µl. Main bacterial groups abundant in the murine conventional intestinal microbiota were assessed by quantitative RT-PCR with group-specific 16S rRNA gene primers (Tib MolBiol, Germany). The number of 16S rRNA gene copies per ng DNA of each sample was

determined and frequencies of respective bacterial groups calculated proportionally to the eubacteria amplicon. For total CFU measures we counted the colonies formed by fecal samples from SPF probiotics, Abx treated and Abx probiotics mice.

Immunolabeling with respect to main experimental procedures section

The mice were anaesthetized with pentobarbital (Narcoren, Merial, Germany) at a dose of 500 µg/g body weight intraperitoneally and perfused with an intracardiac injection of a 0.9 % NaCl solution, followed by freshly prepared 4 % paraformaldehyde (PFA, Sigma-Aldrich) solution (30 ml per animal). Afterwards, the skull was opened and the brain was carefully removed and postfixed overnight in 4 % PFA. Then, the brains were cryopreserved in 30 % sucrose. The PFA perfused cryoprotected brains were rapidly frozen in dry ice and mounted onto a sliding microtome. 40 µm thick sections were collected into cryoprotecting buffer (25 % glycerol, 25 % ethylenglycol in 0.05 M phosphate buffer). Before immunolabeling, the sections were washed with buffer and for 3,3'-Diaminobenzidine (DAB) reaction blocked by incubating them in 0.6 % H_2O_2 (in PBS) for 30 min at room temperature on a shaker. For BrdU labeling, the sections were treated with 2N HCl for 30 min at 37 °C and 0.1 M borate buffer 10 min at room temperature. The sections were again washed and incubated in blocking buffer (3 % donkey serum / 0.1 % TritonX100) for 1 h for permeabilization. The sections were then incubated over night at 4 °C with the primary antibody (rat BrdU, 1:500, Biozol, catalogue number: BZL20633, Germany; goat Dcx, 1:200, Santa Cruz Biotechnology, catalogue number: sc-8066; mouse NeuN, 1:100, Millipore, catalogue number: MAB377). Sections were washed and blocked for 30 min. For DAB-staining, the slices were incubated for 2h in the secondary antibody (anti rat Biotin-SP-conjugated, Dianova, catalogue number: 712-065-153, at room temperature). After washes the third antibody was incubated for 1 h (HRP-conjugated streptavidin, Dianova, catalogue number: 016-030-084, at room temperature), following detection via DAB. The reaction was stopped with water. For immunofluorescence labeling, the slices were labeled by incubation with a fluorophore-labeled antibody (anti goat Alexa488, anti-mouse Cy5, and anti-rat Cy3, all 1:250, Dianova, catalogue numbers: 705-545-147, 715-175-151, 712-166-153 respectively).

Confocal microscopy with respect to main experimental procedures section

For confocal microscopy we used a Leica SPE microscope (Leica microsystems, Germany) with appropriate gain and black level settings (determined on control tissues stained with secondary antibodies alone). Images are recorded as z-stacks and analyzed using Volocity LE software (PerkinElmer).

Cell Isolation for flow cytometry analysis with respect to main experimental procedures section

Mice were perfused with PBS to remove circulating blood cells. For mononuclear cell isolation, one cortical hemisphere of each brain was homogenized in a buffer containing 1M Hepes pH 7.3 and 45% Glucose and then sieved through a 70µm strainer. The cell suspension was washed and re-suspended in 10ml 75% Percoll (GE Healthcare) in PBS and over layered with 10ml 25% Percoll in PBS and 5ml PBS. The gradient was centrifuged for 45min at 800g without brake. Cells were recovered from the 25%/75% interphase, washed and used immediately for further experiments.

Cell culture of mouse neural precursor cells (NPCs) experimental procedures

Mice were decapitated and the brain was dissected from the brain stem and transferred into cold PBS (Phosphate buffered saline; Life Technologies, Germany)/Glucose (4.5 g/l; Roth, Germany). The hippocampi were microdissected, collected in a 15 ml tube and centrifuged (500 g, 5 min, 4 °C). The collected tissue was incubated with occasional mixing in PDD solution (2.5 U/ml papain (Cell systems, Germany), 1 U/ml dispase II (neutral protease; Roche, Germany) and 250 U/ml DNAse (Worthington, USA) in DMEM 4.5 g/l glucose (Life Technologies)(5 ml/animal) for 40 min at 37 °C. The tissue was washed three times with PBS to remove the PPD solution. Cells were plated in 10 cm dishes in cultivation medium. Cultures were incubated at 37 °C, 5 % CO_2 and medium was changed on the next day. The cells were cultured until they formed neurospheres. For splitting, NPCs were collected by centrifugation (500 g, 5 min, 4 °C) and dissociated enzymatically by Accutase (Life Technologies) according to the manufacturer's instructions. The cells were counted and seeded at a clonal density of 0.5 x 10^6 per 10 cm dish in cultivation medium (20 ng/ml FGF-2 (Fibroblast growth factor-2), 20 ng/ml EGF (Epidermal growth factor; both from Cell systems), 100 U/ml penicillin, 100 µg/ml streptomycin (both from Biochrom, Germany), 3 mg/ml glucose (Roth), 20 % B-27 supplement, 0.2 mM glutamine in Neurobasal-A (all from Life Technologies).