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# Bradykinin B2 receptor is essential to running-induced cell proliferation in the adult mouse hippocampus

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## Abstract

Physical exercise is a strong external effector that induces precursor cell proliferation in the adult mouse hippocampus. Research into mechanisms has focused on central changes within the hippocampus and we have established that serotonin is the signaling factor that transduces physical activity into adult neurogenesis. Less focus has been given on potential peripheral signals that may cause pro-mitotic running effects. Vasoactive kinin peptides are important for blood pressure regulation and inflammatory processes to maintain cardiovascular homeostasis. Acting via the two receptors termed B1 (B1R) and B2R, the peptides also function in the brain. In particular, studies attribute B2R a role in cell proliferation and differentiation into neurons *in vitro*. Here, we determined B1R and B2R mRNA expression levels in the adult mouse hippocampus and prefrontal cortex *in vivo,* and in response to running exercise. Using mice depleted in either or both receptors, B1-knockout (KO), B2KO and B1/2KO we observed changes in running performance overnight and in running distances. However, voluntary exercise led to the known pro-mitotic effect in the dentate gyrus of B1KO mice while it was attenuated in B2KO accompanied by an increase in microglia cells. Our data identify B2R as important factor in running-induced precursor cell proliferation.



## Keywords

Dentate gyrus; bradykinin; BrdU; cell proliferation; physical activity


## Introduction

External stimuli and intrinsic factors modulate the generation of new neurons in the adult mouse dentate gyrus (Kempermann et al., 1997, van Praag et al., 1999, Klempin et al., 2013, Klempin et al., 2018). The niche's intrinsic components are neurotransmitters, growth and neurotrophic factors, cell autonomous molecules and transcription factors with distinct effects on cell proliferation and differentiation (Goncalves et al., 2016). Bradykinin, an important mediator of cardiovascular homeostasis, has been identified to also function in the brain (Chen et al., 2000, Shughrue et al., 2003). Kinins, with the active peptide bradykinin, exert their effects via two G-protein-coupled receptors primarily known to mediate inflammatory processes by causing endogenous vasodilation (Marceau and Regoli, 2004): Bradykinin B2 receptors (B2R) are abundant in the rat brain and exhibit high affinity to bradykinin. B1 receptors (B1R) are relatively low expressed, synthesized *de novo* at sites of injury and inflammation (Ongali et al., 2003), and interact with des-Arg$^9$-bradikinin.

Most of the physiological functions of kinins are mediated by B2R. In the central nervous system, bradykinin via B2R participates in signaling pathways for nitric oxide (NO) formation, glutamate release (Parpura et al., 1994, Kohno et al., 2008) and in the development of temporal lobe epilepsy (Perosa et al., 2007, Silva et al., 2008). In particular, B2R plays a role in cell proliferation and fate determination towards neurons as shown in mouse embryonic stem cell cultures, and B2R knockout rats (Trujillo et al., 2012, Nascimento et al., 2015, Pillat et al., 2016). Furthermore, B2R is involved in angiogenesis and gliogenesis at lesion sites following ischemic stroke, and promotes neurogenesis in cultured neuronal cells (Xia et al., 2006) – adding novel actions to kinins as neuropeptides (Negraes et al., 2015).

Physical exercise is also neuroprotective and promotes the proliferative phase in adult neurogenesis (van Praag et al., 1999). Given the potential role of kinin receptors in cell proliferation, we investigated B1R and B2R mRNA expression levels in the adult mouse brain *in vivo* at baseline, and after 6 days of voluntary running. We took advantage of mice depleted in either or both receptors, B1-knockout (KO), B2KO or B1/2KO, to study their effects in running-induced precursor cell proliferation. At baseline, our data show an overexpression of B1R mRNA in

absence of B2R, whereas running decreases B1R expression in the adult hippocampus of wild type [WT] mice. Absence of kinin receptors led to changes in running performance overnight in that mice are getting exhausted fast. Voluntary wheel running led to the known robust increase in cell proliferation in B1KO mice while it was attenuated in B2KO as well as B1/2KO mice. Furthermore, lack of B2R led to alterations in cell phenotype distribution and increased microglia cells upon exercise. Our data identify B2R as essential to running-induced precursor cell proliferation in the dentate gyrus.

## Materials and methods

Adult female B1R-, B2R-deficient (B1KO, B2KO), double knockout (B1/2KO), and C57BL/6N WT matched mice were used in this study ($n$ = 40, 8 to 12 weeks old). The generation of mice has been described in detail earlier (Borkowski et al., 1995, Pesquero et al., 2000, Cayla et al., 2007). Mice were randomly divided into two groups for "baseline" (2 to 3 animals per standard cage) and "running" conditions (single housed in a standard cage plus running wheel), and held with a 12 hours light/dark cycle and *ad libitum* access to food and water. Mice in running conditions had unlimited access to the running wheel for 6 days and running distance, time and performance was monitored daily (by both commercial bicycle computers and Tinytag Data Logger Chambers that allows monitoring the running performance per every 5 minutes). We have used single housing that allows examining the running performance per individual mouse. Others and we have previously reported that no isolation effects occur for different mouse strains (Marlatt et al., 2010, Klempin et al., 2013). To analyze proliferation, animals received three intraperitoneal injections (i.p.) of bromodeoxyuridine (BrdU, SIGMA-Aldrich, Germany; 50 mg/kg body weight dissolved in 0.9% NaCl) on day 6, 6 hours apart and were killed 24 hours after the first injection (Fig. 1a). All experiments were performed according to national and institutional guidelines and were approved by the appropriate authority.

*Immunohistochemistry and quantification*

Mice were deeply anesthetized and transcardially perfused with 0.9% sodium chloride followed by 4% paraformaldehyde (PFA, Otto Fischar GmbH & Co. KG). Brains were removed from the skulls, postfixed in 4% PFA at 4°C overnight, and transferred to 30% sucrose. Sequential 40 µm coronal sections were cut on a microtome and cryoprotected for long-term storage. For BrdU staining, DNA was denatured in 2N HCl for 20 min at 37°C. Sections were rinsed in 0.1 M borate buffer and washed in Tris-buffered saline (TBS). Sections were stained free-floating with all antibodies diluted in TBS containing 3% donkey serum and 0.1% Triton X-100. Primary antibodies were applied in the following concentrations: anti-BrdU (rat, 1:500; Biozol/AbD serotec), anti-doublecortin (DCX; goat, 1:250; Santa Cruz Biotechnology), anti-Sox2 (goat, 1:1000; Santa Cruz Biotechnology), anti-iba1 (rabbit, 1:500; Wako), anti-NeuN (rabbit, 1:500; Millipore). For immunofluorescence, Alexa488-conjugated, Cy3-conjugated, or Cy5-conjugated secondary antibodies (Invitrogen) were used. Immunohistochemistry for BrdU-only followed the peroxidase method with biotinylated secondary antibody (Donkey anti-rat Biotin, 1:500, Jackson laboratories), ABC Elite reagent (Vector Laboratories), and di-aminobenzidine (DAB; Vector Laboratories) used as chromogen.

For quantification, one-in-six series of sections of each brain were BrdU stained for light microscopy (peroxidase method), and immunoreactive cells were counted throughout the rostro-caudal extent of the dentate gyrus. Results were multiplied by six to obtain the total number of BrdU-positive cells per dentate gyrus. For phenotypic analysis, one-in-twelve series of sections were labeled for multiple-immunofluorescence staining. Fifty to 100 randomly selected cells per animal were evaluated for three-dimensional colocalization using a Leica TCS SP5 (Leica, Germany) confocal microscope.

*RT-PCR*

Another set of animals ($n$ = 12 for WT; $n$ = 3 B1KO, $n$ = 3 B2KO) was used to analyze the expression of B1R and B2R in the hippocampus and prefrontal cortex at baseline, and following running using RT-PCR. The designated regions of both hemispheres were dissected and pooled, RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA was generated using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). The following primers were used: B1R-Forward-5-TGGAGTTGAACGTTTTGGGTTT-3, B1R-Reverse-5-GTGAGGATCAGCCCCATTGT-3 (GenBank: EY274959), B2R-Forward-5-GGTGCTGAGGAACAACGAGA-3, B2R-Reverse-5-CCCAACACAGCACAAAGAGC-3 (GenBank: EY275228), gapDH-Forward-5-GCTGTGGGCAAGGTCATCC-3 and gapDH-Reverse-5-CTTCACCACCTTCTTGATGTC-3 (GenBank: XM_017321385). Quantitative PCR was performed with the TaqMan system (Applied Biosystems), and the protocol was as follows: 95°C for 10 min, 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Target mRNA expression was normalized to levels of Gapdh using specific primer set obtained from BIOTEZ-Berlin. Data are shown as value ΔΔCt and normalized to WT baseline expression.

*Statistical analysis*

Statistical significance was evaluated by Two-way analysis of variance (ANOVA) for BrdU numbers followed by Tukey's *post hoc* tests in cases where a significant F statistics was obtained. For individual comparisons an unpaired two-tailed Student's *t*-test was used. All values are expressed as mean ± sem. *P*-values of ≤ 0.05 were considered statistically significant.

# Results

***B1R is over-expressed in the adult hippocampus in the absence of B2R***

We first quantified B1R and B2R mRNA expression levels in the hippocampus and prefrontal cortex of WT and knockout mice (n = 4 WT, n = 3 B1KO, n = 3 B2KO). At baseline, B1R expression is significantly increased in B2KO mice in the adult hippocampus (ΔΔCT B1R: WT 1.00 ± 0.145 vs. B2KO 2.400 ± 0.507, $p$ = 0.0478; Fig. 1b); a trend was seen in the prefrontal cortex (ΔΔCT B1R: WT 1.00 ± 0.350 vs. B2KO 1.850 ± 0.235, $p$ = 0.1027; Fig. 1b). No change in mRNA level was observed vice versa for B2R expression in B1KO mice in both hippocampus (ΔΔCT B2R: WT 1.00 ± 0.233 vs. B1KO 1.140 ± 0.289, $p$ = 0.6485) and prefrontal cortex (ΔΔCT B2R: WT 1.00 ± 0.199 vs. B1KO 0.800 ± 0.127, $p$ = 0.3254; Fig. 1b).

***B1R expression is downregulated following running***

Following physical exercise for 6 days, B1R expression is significantly downregulated in the hippocampus of WT animals (n = 8; ΔΔCT B1R: WT RUN 0.611 ± 0.131, $p$ = 0.0289; Fig. 1b) with no changes observed in the B2R expression level (ΔΔCT B2R: WT RUN 0.766 ± 0.378, $p$ = 0.396; Fig. 1b). Receptor expression in the prefrontal cortex is not affected by running. WT animals ran the total amount of 47.1 ± 6.3 km in 6 days with a temporal average per night of 11.36 ± 0.15 h (Fig. 1c). Knockout mice were running significantly less distances and time compared with WT: B1KO (27.1 ± 3.9 Km, $p$ = 0.026; 8.01 ± 0.86 h, $p$ = 0.0065), B2KO (24.5 ± 7.3 km, $p$ = 0.05; 9.40 ± 0.35 h, $p$ = 0.0005), and B1/2KO (18.3 ± 5.5 km, $p$ = 0.005; 7.76 ± 0.62 h, $p$ = 0.0002, Fig. 1c). Notably, running less was reflected by changes in the running performance at night: WT mice ran enduringly at high level all night long, B2KO

animals run the first half of the night at highest speed and were satiated during the second half, B1KO mice started at high level but run overall at less speed with pauses in between (Fig. 1c).

***B2R expression is important for running-induced cell proliferation***

We next determined precursor cell proliferation at baseline and following exercise in knockout (n = 5 for baseline, n = 5 for running per receptor knockout) and WT mice (n = 5 for baseline, n = 5 for running). At baseline, absence of B1R, or B2R, or both did not alter the number of BrdU-positive cells in the dentate gyrus (Fig. 1d). However, when animals were subjected to voluntary wheel running, a condition as well as genotype effect was observed (Two-way ANOVA $F(1,29)=75.57$, $p_{\text{condition}} < 0.0001$; $F(3,29)=3.669$, $p_{\text{genotype}} = 0.0235$; Fig. 1d). The running stimulus robustly increased the number of proliferating cells in WT (baseline 745 ± 104 cells vs. running 2408 ± 209 cells, Tukey's *post hoc* $p < 0.0001$), and B1KO animals (baseline 854 ± 112 cells vs. running 2430 ± 302 cells, Tukey's *post hoc* $p < 0.0001$; Fig. 1d) although B1KO ran significantly less. In B2KO (and also B1/2KO) running groups, the increase in BrdU-positive cells was markedly attenuated and significantly less compared with WT and B1KO (WT vs. B2KO, and B1KO vs. B2KO Turkeys *post hoc* $p < 0.05$; Fig. 1d). Nevertheless, a statistical significant increase after running was reached using Student's *t*-test for B2KO (baseline 681 ± 115 cells vs. running 1509 ± 224 cells, $p = 0.019$) and B1/2KO groups (baseline 766 ± 52 cells vs. running 1512 ± 153 cells, $p = 0.0006$; Fig. 1d).

***Assessment of phenotype of newly generated cells***

We next assessed phenotypes of BrdU-positive cells at baseline and following running. Quantification at baseline revealed no differences between groups in cell number and ratio for the neural precursor marker Sox2 and the transient immature neuronal marker DCX, but decreased co-labeling of BrdU and the nuclear marker NeuN was observed in B1/2KO mice compared with WT (ratio 6.9 ± 0.2% v 13.3 ± 2.1% BrdU/NeuN-positive cells, $p = 0.049$; Table 1). Following running, the boost in absolute numbers in WT and B1KO animals was reflected by a significant increase in BrdU/Sox2-positive type-2a precursors (3.7-times, WT $p = 0.0020$, B1KO $p < 0.0001$) and BrdU/DCX-positive type-2b/3 immature neurons (2.8-times, WT $p = 0.0038$, B1KO $p < 0.00010$). Table 1 –nomenclature by (Kempermann et al., 2004); note that up to 3% of BrdU/Sox2-positive cells may overlap with DCX (Steiner et al., 2006). The number of BrdU/NeuN-positive cells after running was increased by 3.7- (WT; $p = 0.0009$) and 2.7-times (B1KO, $p = 0.0111$; Table 1), respectively. In B2KO, almost half of BrdU-positive cells after running expressed Sox2; which is the largest increase in percentage of all groups (B2KO baseline 35.8 ± 2.0% vs. running 48.8 ± 1.9%, $p = 0.010$). In turn, the number of immature and mature neurons only doubled in B2KO and B1/2KO after running and only reached statistical difference for BrdU/DCX in B1/2KO compared with baseline ($p = 0.0190$; Table 1). We have also looked at co-expression of BrdU and the microglia marker Iba-1. Interestingly, after 6 days of running significantly more BrdU/Iba1-positive microglia cells were found in B2KO (8-times) and B1/2KO mice (3.9-times; Table 1).

**Table 1 Number and phenotypes of BrdU-positive cells in the SGZ/GCL**

BrdU-positive cells co-expressing Sox2 (precursor cells), DCX (transient immature neurons), NeuN (immature, and mature granule cells), iba-1 (microglia), or neither marker are presented. All data are shown as mean ± sem

| Baseline | Cell number | Sox2 | DCX | NeuN | Iba-1 | Other |
|---|---|---|---|---|---|---|
| **WT** | 745 (104) | 283 (51) | 240 (31) | 99 (16) | 19 (7) | 134 |
| **B1KO** | 854 (112) | 286 (11) | 231 (29) | 136 (33) | 14 (8) | 187 |
| **B2KO** | 681 (115) | 214 (37) | 156 (9) | 89 (38) | 7 (4) | 215 |
| **B1/2KO** | 766 (52) | 219 (30) | 211 (37) | 52 (5)$^{\#}$ | 14 (4) | 276 |
| **Running** | | | | | | |
| **WT** | 2408 (209)$^{****}$ | 1052 (106)$^{**}$ | 679 (115)$^{**}$ | 366 (51)$^{**}$ | 38 (11) | 273 |
| **B1KO** | 2430 (302)$^{****}$ | 1061 (55)$^{****}$ | 722 (18)$^{****}$ | 370 (76)$^{*}$ | 24 (15) | 253 |
| **B2KO** | 1509 (224) | 736 (137)$^{*\#}$ | 352 (117)$f$ | 164 (46)$^{\#}f$ | 57 (9)$^{*}$ | 169 |
| **B1/2KO** | 1512 (153) | 581 (102)$^{*\#}$ | 488 (57)$^{*}f$ | 111 (28)$^{\#\#}f$ | 55 (11)$^{*}$ | 301 |

Tukey's *post hoc* $*p < 0.05$, $**p < 0.01$, $****p < 0.001$ to baseline of same genotype, $\#p < 0.05$, $\#\#p < 0.01$ to WT of same condition, $f$p < 0.05 to B1KO of same condition

# Discussion

Our data demonstrate that B1R and B2R are expressed in the adult mouse hippocampus and prefrontal cortex. Furthermore, B1R mRNA expression levels are affected by the running stimulus, and they compensate for the lack of B2R in the hippocampus at baseline. Our most prominent result is an attenuation of running-induced precursor cell proliferation in the dentate gyrus in the absence of B2R that is accompanied by a shift in cell phenotypes and increased microglia numbers.

Vasoactive kinin peptides are important for blood pressure regulation and inflammation, and also function in the brain. On cellular level, B2R immunoreactivity has been described in neurons of the rat hippocampus and olfactory bulb; B1R mRNA is expressed throughout the primate brain and spinal cord (Chen et al., 2000, Shughrue et al., 2003). Both receptors are upregulated under pathological conditions mediating pro-inflammatory effects (Austinat et al., 2009, Su et al., 2009), e.g. in temporal lobe epilepsy (Perosa et al., 2007, Silva et al., 2008) or in ischemic stroke (Howl and Payne, 2003). However, the receptors differ in their cellular distribution in that B1R is expressed in astrocytes, while B2R remains present in neurons in diabetic rats after ischemic stroke (Howl and Payne, 2003). Studies pharmacologically targeting the two receptors also revealed differential effects with B1R expression strongly responsive to inflammation (Sriramula and Lazartigues, 2017) and to changes in the environment (Ongali et al., 2003, Arganaraz et al., 2004). Furthermore, blockade of B1R in rats led to the improvement of cognitive function and age-related cognitive decline (Prediger et al., 2008, Bitencourt et al., 2017). B2R is crucial in long-term facilitation of angiogenesis and neurogenesis (Xia et al., 2006, Trujillo et al., 2012) playing a protective role against cell-death (Arganaraz et al., 2004).

In our study, B1R mRNA is overexpressed in the hippocampus of B2KO mice, probably as compensation for the absence of B2R. In mammals, genes encoding both receptors are located in 'tandem orientation' on the same chromosome (Cayla et al., 2002); compensation of one receptor in lack of the other has been described (Fonseca et al., 2013). B1R mRNA expression levels were downregulated in WT animals upon the external running stimulus with unaffected cell proliferation in the absence of B1R. In both, WT and B1KO mice the typical robust pro-mitotic effect of physical exercise was observed (van Praag et al., 1999). In contrast, B2R mRNA levels were neither affected by running nor by the lack of B1R. However, B2R is important to induce precursor cell proliferation in the dentate gyrus upon running. Given that no specific antibodies exist to date, we cannot say whether B2R plays a direct or indirect role on cell proliferation. Our findings further demonstrate alterations in the number of cells towards a

neuronal lineage in B2KO as shown by an elevation in BrdU/Sox2 co-labeling and microglia, while a decrease in DCX or NeuN co-expression was observed in comparison to WT. This data substantiates a neuroprotective role for B2R.

We observed changes in running performance for both receptor knockout models in that mice are getting exhausted fast~~er~~. B1KO mice paused often, B2KO almost stopped running for the second half of the night resulting in less distances and running time over 6 days. Voluntary running activity was also markedly decreased in a recent study on dopamine 2 receptor (D2R) know-down mice due to altered energy expenditure (Beeler et al., 2016). Since B2R-D2R can function through the formation of a dimer (Niewiarowska-Sendo et al., 2017), D2R activity may participate in the observed effect of physically running in B2KO mice. Nevertheless, only in the absence of B2R running-induced cell proliferation was affected. Shorter running distances could influence the results on cell proliferation. However, it has been shown that running 3 hours during the middle of the night is sufficient to induce the pro-mitotic effect (Holmes et al., 2004), as was also seen for B1KO mice. We conclude the neurogenic response to physical exercise is at least partially mediated by B2R. Furthermore, the population of BrdU/Iba1-positive microglia is significantly increased after running in B2KO mice. Increased Iba1 expression might be a response to increased inflammation mediated by amplified 'inducible' B1R in B2KO mice; as has been described earlier, when bradykinin-induced migration of microglia to the injury site was observed, mediated by upregulation of B1R (Ifuku et al., 2007); yet, our results on B1/2KO do not support this.

Together, our data identify B1R as strongly responsive to changes in the environment, while B2R is necessary on the cellular level to induce cell proliferation and fate choice toward a neuronal lineage after physical exercise.

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**Ethical Responsibility of Authors**

The manuscript has been read and approved for submission by all authors. All persons listed have contributed to preparing the manuscript. All of the forms and manuscript components have been formatted according to the journal's instructions and are included in the upload. All figures and tables are original and have not been published elsewhere.

**Compliance with Ethical Standards, conflict of interests**

The authors declare no competing financial interests.

All experiments were performed according to national and institutional guidelines and were approved by the appropriate authority.

# Figure legend

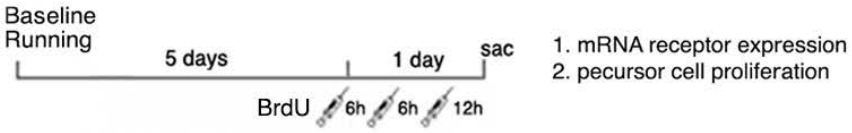
**a** Experimental design. Wild type [WT] and B1KO, B2KO, B1/2KO mice were randomly assigned to either baseline or running groups. Readout: 1) mRNA receptor expression levels, and 2) number of proliferating cells at day 7, 24 hours after the first of three intraperitoneal injections of BrdU

**b** Receptor mRNA expression revealed increased B1R levels in B2KO, and downregulation after running in the hippocampus of WT mice; B2R expression was not affected (n = 4 WT, n = 3 B1KO, n = 3 B2KO at baseline; n = 8 for WT baseline vs. running)

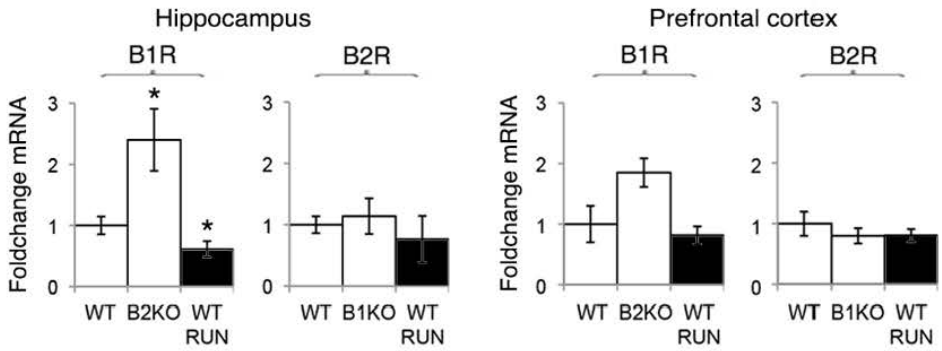
**c** Running distances and time was significantly shorter in knockout mice; running performance overnight reveals early satiation in B1KO, and exhaustion of B2KO mice during the second half of the night (Representative graphs per genotype)

**d** Absolute BrdU numbers are unchanged at baseline between genotypes; however, physical exercise significantly increased cell proliferation in WT and B1KO and attenuated levels in B2KO, B1/2KO mice (n = 5 per group, condition, and genotype); representative images of BrdU-labeled cells after running, SGZ, subgranular zone; Scale bar 100 μm

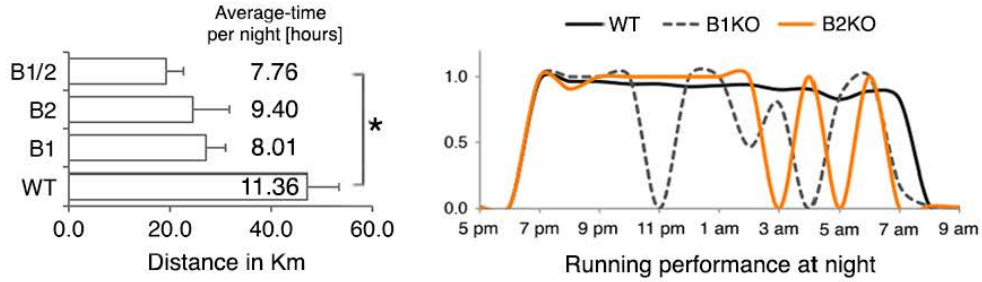
A Experimental design




B B1R and B2R expression pattern




C Running performance




D Precursor cell proliferation