Lack of $M_4$ muscarinic receptors in the striatum, thalamus and intergeniculate leaflet alters the biological rhythm of locomotor activity in mice

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Abstract

The deletion of $M_4$ muscarinic receptors (MRs) changes biological rhythm parameters in females. Here, we searched for the mechanisms responsible for these changes. We performed biological rhythm analysis in two experiments: in experiment 1, the mice [C57Bl/6NTac (WT) and $M_4$ MR $-/-$ mice (KO)] were first exposed to a standard LD regime (12/12-h light/dark cycle) for 8 days and then subsequently exposed to constant darkness (for 24 h/day, DD regime) for another 16 days. In experiment 2, the mice (after the standard LD regime) were exposed to the DD regime and to one light pulse (zeitgeber time 14) on day 9. We also detected $M_1$ MRs in brain areas implicated in locomotor biological rhythm regulation. In experiment 1, the biological rhythm activity curves differed: the period ($\tau$, duration of diurnal cycle) was shorter in the DD regime. Moreover, the day mean, mesor (midline value), night mean and their difference were higher in KO animals. The time in which the maximal slope occurred was lower in the DD regime than in the LD regime in both WT and KO but was lower in KO than in WT mice. In experiment 2, there were no differences in biological rhythm parameters between WT and KO mice. The densities of $M_1$ MRs in the majority of areas implicated in locomotor biological rhythm were low. A significant amount of $M_1$ MR was found in the striatum. These results suggest that although core clock output is changed by $M_4$ MR deletion, the structures involved in biological rhythm regulation in WT and KO animals are likely the same, and the most important areas are the striatum, thalamus and intergeniculate leaflet.

Keywords Locomotor activity · Biorhythm · $M_4$ muscarinic receptors · $M_1$ muscarinic receptors · Intergeniculate leaflet

Introduction

The generation of the rhythmic pattern controlling locomotion is formed by the activity of intrinsically oscillating interneurons in the spinal cord (Wyartt 2018). Researchers now agree that locomotion is generated centrally in the spinal cord by circuits referred to as central pattern generators (CPGs, see Table 1 for list of abbreviations). CPGs are triggered by descending commands from the brain (initializing or halting). In addition to on-demand triggering of these commands to meet the homeostatic needs of the organism, locomotion itself reveals a diurnal pattern directed by a series of pacemakers. The main circadian pacemaker is localized in the hypothalamic suprachiasmatic nuclei (SCN) (Ballesta et al. 2017). However, some other brain structures have recently been identified as important in locomotor biological rhythm regulation: the subparaventricular zone (SPVZ), intergeniculate leaflet (IGL) (Morin 2013), and posterior hypothalamic area (PHA) (Abrahamson and Moore 2006; Hughes and Piggins 2012). Locomotor activity can also be considered a nonphotic entraining signal of circadian rhythmicity (Hughes and Piggins 2012).

SCN has long been considered a structure rich in cholinergic neurons, i.e., nicotinic cholinergic receptors have been identified there by the end of the 70s (Silver and Bil liar 1976; Segal et al. 1978). Immunohistochemical study by (van der Zee et al. 1991) has identified colocalization of nicotinic and muscarinic receptors in the SCN. SCN has been shown to be innervated by cholinergic nerves (Hut and Van der Zee 2011) but does not have to be necessarily intrinsically cholinergic (van den Pol and Tsujimoto 1985). It receives cholinergic projections from basal forebrain and
brain stem tegmentum (Bina et al. 1993). Another approach, determining especially cholinergic neurons using choline-
acetyltransferase positivity, has been used by Ichikawa and
Hirata (1986). This study concluded that among other struc-
tures, SCN (ventromedial aspect) is rich in cholinergic ter-
minals. No cholinergic terminals have been found in other
hypothalamic areas.

The cholinergic agonist carbachol has been shown to have
similar effects on the circadian rhythm in rat pineal serotonin
N-acetyltransferase activity (Zatz 1979; Zatz and Brownstein
1979). It has also been demonstrated that carbachol pellet,
implanted near the SCN, shortened the free-running period
under constant darkness (Furukawa et al. 1987). However,
there are species differences in the presence of cholinergic
neurons in the SCN in rat, hamster and mouse (Hut and Van
der Zee 2011). For example, carbachol (i.c.v. injection) was
able to bring about phase shifts but was not able to induce
these effects when injected directly into the SCN (Buchanan
and Gillette 2005) in mice while in hamsters the phase shifts
were remarkable both after injection into the ventricle or
SCN (Bina and Rusak 1996).

Muscarinic receptor subtype expression in the SCN is
still a matter of debate. An initial paper used autoradiog-
raphy with the aim to determine the presence of MR in the
SCN (Bina et al. 1998). These authors revealed that MR
density in the SCN is very low, especially when compared
to the striatum. Further research has identified M₄ MR (Liu
and Gillette 1996) to be present in the SCN. Another report
indicated the presence of MR (generally) using immunohis-
tochemistry (Hut and Van der Zee 2011). It is not surprising
that PCR technique identified all five MR subtypes in the rat
SCN (Yang et al. 2010). This study also determined carba-
chol inhibitory effects (carbachol hyperpolarization) in the
SCN and found that both M₄ and M₁ receptors are involved
(Yang et al. 2010). Carbachol induced phase advance in the
circadian rhythm of spontaneous neuronal activity (Gillette
et al. 2001) was assigned to M₁ MR. Another recent data
suggest the role of M₄ MR in biological rhythm regulation:
the M₄ positive allosteric modulator LY2033298 enhanced
oxotremorine (muscarinic agonist) inhibitory effect on light-
duced phase delays but had no effect by itself (Gannon and
Millan 2012).

The role of neurotransmitter systems in circadian rhythms
has been reviewed by Rusak and Bina (1990). In addition,
biological rhythms of many receptors, including muscarinic
receptors, have been identified in the whole forebrain and
the striatum that varied during the year and in various light
conditions (Kafka et al. 1983; Wirz-Justice 1987).

Not only SCN but also other structures are involved in
light-like effects. Thus, it has been shown that extra-SCN

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Table 1 List of abbreviations

| Abbreviation | Explanation |
|--------------|-------------|
| CPGs         | Central pattern generators |
| FFT          | Fast Fourier transformation |
| SCN          | Suprachiasmatic nuclei |
| SPVZ         | Subparaventricular zone |
| IGL          | Intergeniculate leaflet |
| PHA          | Posterior hypothalamic area |
| MR           | Muscarinic receptors |
| WT           | Wild types |
| KO           | Knockout animals |
| LD regime    | 12/12-h light/dark cycle, 22 ± 1 °C, light on at 7:00 AM |
| DD regime    | Constant darkness (for 24 h/day) |
| WT1-8        | WT animals, 1st to 8th day |
| WT9-16       | WT animals, 9th to 16th day |
| KO1-8        | KO animals, 1st to 8th day |
| KO9-16       | KO animals, 9th to 16th day |
| D<sub>mean</sub> | Day mean |
| N<sub>mean</sub> | Night mean |
| N–D<sub>mean</sub> | Difference between night and day mean |
| mesor        | A midline based on the distribution of values across the cycles of the circadian rhythm, computed using a cosine function |
| τ            | Period, the time after which a defined phase of the oscillation re-occurs |
| Periodogram  | Lomb-Scargle analysis of the period(s) power spectrum |
| Actogram     | The graphic representation of animal activity expressed by black sections (activity) and white sections (no activity) |
cholinergic synapse mediates the light-like cholinergic clock resetting reported previously (Buchanan and Gillette 2005). Another way how to induce phase shift is muscarinic input into the IGL (Cain et al. 2007). Moreover, there could be a fine-tuned balance between cholinergic and glutamatergic neurons in the IGL, as has been shown by Pekala et al. (2007). These authors have demonstrated that in the presence of a cholinergic agonist, glutamate-induced activity was either decreased or increased or not changed.

M4 muscarinic receptors (MRs) have been suggested to play a role in motor coordination. Previous studies have shown different results depending on genetic background and number of backcrosses (Fink-Jensen et al. 2011; Gomez et al. 1999; Koshimizu et al. 2012; Schmidt et al. 2011; Woolley et al. 2009). However, no attention has been given to biological rhythms. In our recent work (Valuskova et al. 2018b), we analyzed telemetrically obtained biological rhythms under a light/dark cycle (LD 12/12 h, lights on at 6:00) in intact M4KO mice (activity, body temperature) grown on the C57Bl6 background (i.e. the mice were backcrossed to C57Bl6 line for 12 generations) using ChronosFit software. We showed that M4KO female mice motor activity did not differ substantially from that of wild-type mice during the light period, while in the dark phase (the active part of the day for mice), the M4KO mice revealed an increase in the mesor, in the night values, in the night-day difference, in the area under curve, in the highest value, in the night area under curve, in the highest value measured during the night period, and in the amplitudes of the 24-h, 12-h, 6-h, and 4.8-h rhythm. Similar differences have also been found between female and male KO mice.

As the brain areas implicated in locomotor activity biological rhythm changes comprise more structures (see also above), we employed in vitro autoradiography and identified potential brain areas likely involved in locomotor activity biological rhythm regulation. We investigated the following areas: the motor cortex, the striatum, the thalamus, and the intergeniculate leaflet. M4 MR expression was negligible in the subparaventricular zone, the posterior hypothalamic area, and the suprachiasmatic nuclei (Valuskova et al. 2018b).

In subsequent work (Valuskova et al. 2019), we also identified important differences in the morning vs. evening muscarinic drug (scopolamine, oxotremorine) effects, both in WT and M4 KO animals. Acutely, scopolamine induced an increase in motor activity in WT and M4KO at 9:00, yet no significant increase was observed at 21:00. Oxotremorine induced hypothermic effects in both WT and M4KO. Hypothermic effects were more evident in WT than in M4KO. Hypothermia in both cases was more pronounced at 9:00 than at 21:00. We have also tested cocaine as a drug that can disrupt the balance between dopamine and acetylcholine levels. Cocaine increased motor activity when compared to saline, but no differences were found in morning vs. evening effects. Another part of our study focused on behavior in a novel environment. There was no difference in behavior in the open field between WT and M4KO when tested at 9:00; however, at 21:00, the activity of M4KO mice was doubled in comparison to that of WT mice. Both WT and KO animals spent less time climbing in their active phase. Autoradiography of muscarinic receptors, GABA_A receptors, dopamine D_1-like receptors, D_2-like receptors, kainate receptors and NMDA receptors revealed no significant morning vs. evening differences.

Here, we searched for a potential mechanism of locomotor biological rhythm changes using constant darkness to distinguish light responsiveness from real circadian effects. We tested the hypothesis that the increased locomotion in females is caused by circadian rhythmicity changes. In addition, we also identified specific structures involved in locomotor biological rhythm changes. We used a light pulse to cause a phase shift. We tested the hypotheses that the phase shift would reveal similar parameters in WT and KO animals, thus suggesting that similar CNS structures are involved in M4 MR-affected changes in biological rhythm. As there were evident differences between brain areas in M4 MRs density, we also investigated another MRs subtype that is believed to be involved in motor coordination—the M1 MRs.

Methods

Animals

Mice lacking the M4 muscarinic receptor were generated in Wess’ laboratory (Gomeza et al. 1999) and then bred in our animal facility (Prague, Czech Republic). Their genetic background was C57Bl/6NTac. Animals were treated in accordance with the legislation of the Czech Republic and the EU legislature (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [Council of Europe No 123, Strasbourg 1985]), and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the 1st Medical Faculty, Charles University, Prague and by the Ministry of Education of the Czech Republic under No MSMT-2409/2017-3. The wild-type line was the C57Bl/6NTac line. We studied fully backcrossed (14 generations) muscarinic M4−/− and M4+/+ littermates. The animals were maintained under controlled environmental conditions (12/12-h light/dark cycle, 22 ± 1 °C, light on at 7:00). Food and water were available ad libitum. A total of 36 females (weighing 20–26 g, age 3–6 months) were used in the study, of which there were 18 M4 KO animals and 18 WT animals. Prior to the experiments, the mice were genotyped, and only homozygous
mice were used. The females were housed separately from males, thus revealing the Lee-Boot effect (i.e., suppression of the estrus cycle—anestrous), which made the female group homogenous in hormone levels. Moreover, no differences were observed in light microscopy of vaginal lavage or actograms in females for 15 consecutive days (control animals, not included in the experiment because of the stressful procedure associated with lavage acquisition).

Telemetry

To judge the biological rhythm changes, we employed a telemetric apparatus able to measure body temperature and overall motor activity. The telemetry system used was commercially available from Mini Mitter (Starr Life Sciences Corp., Oakmont, PA, USA, originally from Respironics, Andover, MA, USA). The transponders (E-Mitter, G2, length 15.5 mm, 1.1 g) were implanted in the peritoneal cavity under anesthesia (Zoletil® 100, Rometar® 2% 5:1, diluted 10 times, 3.2 ml kg⁻¹). During the implantation, the mice were kept on a thermostable pad. Mice were allowed 2 weeks for recovery from the surgery and then used in the experiment. The activity data was acquired directly from the transponders in the sample period for three consecutive days, during which the animals were not disturbed. Similar rhythms were recorded before and after this sample period. The activity was recorded in home cages of typical size (38 × 22 × 15 cm). Receivers were connected in series and overall motor activity. The telemetry system used was a telemetric apparatus able to measure body temperature and overall motor activity. The telemetry system used was commercially available from Mini Mitter (Starr Life Sciences Corp., Oakmont, PA, USA, originally from Respironics, Andover, MA, USA). The transponders (E-Mitter, G2, length 15.5 mm, 1.1 g) were implanted in the peritoneal cavity under anesthesia (Zoletil® 100, Rometar® 2% 5:1, diluted 10 times, 3.2 ml kg⁻¹). During the implantation, the mice were kept on a thermostable pad. Mice were allowed 2 weeks for recovery from the surgery and then used in the experiment. The activity data was acquired directly from the transponders in the sample period for three consecutive days, during which the animals were not disturbed. Similar rhythms were recorded before and after this sample period. The activity was recorded in home cages of typical size (38 × 22 × 15 cm). Receivers were connected in series and connected directly to the PC into a single computer port, allowing for the determination of all parameters. The data were collected every 60 s. VitalView software was used for the acquisition and first processing of data.

Biological rhythm analysis

The data collected by telemetry were grouped into 10-min sequences, and the calculated means were used for further analysis. The analysis was performed by Lomb-Scargle spectral analysis (period length and power spectra determination), by fast Fourier transformation (FFT) and the stepwise regression technique as described earlier (Valuskova et al. 2018a, b) with the determination of biological rhythm parameters. Phase shifts of activity were calculated by determining the horizontal distance between regression lines fitted through activity onsets and offsets in the LD and DD regimes. The times of the minimal and maximal slopes were compared between control (WT) and experimental (M₄ KO) groups in the LD and DD regimes. The data were also presented as periodograms and actograms. For further statistical analysis GraphPad Prism 8. 4.0.0 program (San Diego, USA) was used.

Autoradiography detection of M₁ muscarinic receptors

For receptor determination, autoradiography was performed in several brain areas previously shown to be connected with locomotor biological rhythm changes (striatum [Str], thalamus [TH], SCN, SPVZ, PHA, and IGL). Brains were rapidly removed (4–6 brains per group), frozen in dry ice, and then stored at − 80 °C until cryostat sectioning. Sixteen-micrometer-thick sagittal or frontal sections were cut on a cryostat at − 20 °C, thaw-mounted on Superfrost® Plus glass slides (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and stored in storage boxes at − 80 °C until use. To assess M₁ muscarinic receptor binding, the sections were allowed to thaw and dry for 30 min at 22 °C, and the density of receptors was determined using the M₁ MR selective protocol as previously described (Valuskova et al. 2018a). Briefly, sections were incubated for 1 h with 5 nM ³H-pirenzepine at room temperature. Nonspecific binding was assessed on adjacent sections in the presence of 10 μM atropine sulfate. After incubation, the sections were washed two times for 5 min and gently dried. Dry sections were apposed to the tritium-sensitive Fuji BAS imaging plates (GE Healthcare Europe GmbH, Freiburg, Germany) in Kodak Biomax autoradiographic cassettes (Carestream Health, Inc., Rochester, NY, USA) for 5 days. The linearity of the signal and conversion of photostimulated luminescence to radioactivity was assessed using tritium autoradiographic standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA). The film autoradiograms were scanned, and densitometry was performed with the PC-based analytical software, MCID analysis software. Measurements were taken and averaged from at least three sections for each animal and brain region. We compared the densities in the left and right hemisphere. Since there were no differences in laterality, both sides were taken together.

Histology

Nissl staining was used for SCN, SPVZ, IGL and PHA identification in MR autoradiography determination. Briefly, parallel sections were obtained using a cryostat [the appropriateness of the section was assessed using a mouse atlas (Paxinos and Franklin 2008)], and the sections were collected and divided into four sets. The first section from the set was placed on the first glass slide and used for Nissl staining, while the remaining four sections from the set were placed on other glass slides (three sections from different sets on one glass slide) and used for autoradiography. The sections used for Nissl staining were immersed in a solution of alcohol (70%, 80%, 96%) for two minutes each, stained with Nissl solution (1% cresyl violet and 0.2 mol/l acetic acid + 0.2 mol/l sodium acetate, 4:1, pH = 3) for 20 min, then
twice washed in distilled water and immersed in a solution of alcohol (96%, 80%, 70%) for two minutes each. Then, the samples were immersed in xylene (xylene, mixture of isomers, p.a., Penta, Czech Republic) for 5 min. Then, the sections were incubated for another 45 min in xylene (p.a., Penta, Czech Republic) and mounted using DPX (SigmaAldrich, Czech Republic) with a coverslip.

The area, clearly visible with Nissl staining, was then marked (using border transposition) on a scanned autoradiogram and used for densitometry with PC-based analytical software (MCID software).

**Experiment 1**

The mice were first exposed to a standard LD regime (the same as described above, i.e., 12/12-h light/dark cycle, light on at 7:00) for eight consecutive days and then exposed to constant darkness (for 24 h/day, DD regime) for the other sixteen days. The biological rhythm was analyzed using the FFT and Lomb-Scargle spectral analysis. Lomb-Scargle periodograms were computed and power spectrum in WT and M1 KO animals was compared using repeated measures two way-ANOVA with Sidak post hoc correction.

**Experiment 2**

The mice were first exposed to a standard LD regime (the same as described above, i.e., 12/12-h light/dark cycle, light on at 7:00) for eight consecutive days and then exposed to constant darkness (for 24 h/day, DD regime) for the other sixteen days. On day 9 from the beginning of the experiment, the animals were exposed to one light pulse (300 lx, 1 h, administered at zeitgeber time 14, the onset of subjective night). The biological rhythm was analyzed using the FFTand Lomb-Scargle spectral analysis.. Lomb-Scargle periodograms were computed and power spectrum in WT and M1 KO animals was compared using repeated measures two way-ANOVA with Sidak post hoc correction.

**Statistical analysis**

Repeated-measures three-way ANOVA was used for comparison of data in biological rhythm curves. Repeated-measures two-way ANOVA and its alternative mixed-effects model (Restricted Maximum Likelihood, REML) with Sidak post hoc correction were used for specific biological rhythm parameter changes between days 1–8 (LD regime), 9–16 and 17–24 (DD regime). The data reported for REML analysis are similar to ANOVA, except \( \eta^2 \), as the mixed-effects model compares the fit of a model where subjects are a random factor vs. a model that ignores the difference between subjects. This results in \( \chi^2 \) ratio and \( p \) value. For comparison of time shifts, an unpaired \( t \) test was used. For M1 MR autoradiography analysis, two-way ANOVA with Sidak post hoc correction was used. Generally, values of \( p < 0.05 \) were considered significant. The specific test that was used and the factor significance and/or interaction significance are presented in the Results section. The statistics were calculated using GraphPad Prism 8.0.4.0.

**Results**

**Experiment 1**

**Biological rhythm of locomotor activity**

Lomb-Scargle spectral analysis showed differences between days 1–8 of the experiment (LD regime) and 9–16 (DD regime) both in WT and KO animals in the period length. REML analysis (\( \chi^2 \) ratio = 5.301, \( df = 1, p = 0.0213 \), main effect of time: \( F_{1,171,32.79} = 12.23, \eta^2 = 0.008 \) (\( \eta^2 \) where \( \eta^2 \) is the degrees of freedom numerator, and \( \eta^2 \) is the degrees of freedom denominator; no effect of genotype: \( F_{1,34} = 0.027, p = 0.87 \) and significant genotype-time interaction: \( F_{2,56} = 12.72, p < 0.0001 \) showed a shortening of the period length in WT and KO animals between days 1–8 (LD regime) and days 9–16 (DD regime). The values were: day 1–8: 24.21 ± 0.023 vs. 23.91 ± 0.016, day 9–16: 23.78 ± 0.06 vs. 23.72 ± 0.02, day 17–24: 23.7 ± 0.11 vs. 24.03 ± 0.12, in WT and KO, respectively.

The biological rhythm of locomotor activity, as revealed by FFT, differed between WT and KO animals and between days 1–8 of the experiment (LD regime) and days 9–16 (DD regime, see Fig. 1; repeated-measures three-way ANOVA, genotype: wild-type/knockout; day: days 1–8 (LD regime)/days 9–16 (DD regime); time: time in 24-h cycle from FFT calculation), with a significant main effect of the geno-type, time, and day and an interaction of genotype and time (genotype: \( F_{1,1400} = 319.7, p < 0.0001 \); \( \eta^2 = 0.07 \); day: \( F_{1,1400} = 1144, p < 0.0001 \); \( \eta^2 = 0.07 \); time: \( F_{99,1400} = 93.43, p < 0.0001 \); \( \eta^2 = 0.07 \) ; genotype–time interaction: \( F_{99,1400} = 19.54, p < 0.0001 \); \( \eta^2 = 0.06 \); interaction time–day: \( F_{99,1400} = 13.33, p < 0.0001 \); \( \eta^2 = 0.08 \); genotype–day interaction: \( F_{1,1400} = 10.18, p = 0.0014 \); \( \eta^2 = 0.0017 \); interaction time–genotype–day: \( F_{99,1400} = 3.397, p < 0.0001 \); \( \eta^2 = 0.017 \) ). To further analyze and identify the significance of differences between WT/KO and days 1–8/9–16, we simplified the analysis design (as the main effect of genotype and day and its interaction was highly significant in the three-way ANOVA, we used WT1-8, KO1-8, WT9-16, and KO9-16 as separate groups without distinguishing genotype and day) and used repeated-measures two-way ANOVA with post hoc Sidak corrections. This analysis (group: wild-type days 1–8/knockout days 1–8/wild-type days 9–16/knockout days 9–16; time: time in a 24-h cycle from the FFT
of values across the cycles of the circadian rhythm, computed using a cosine function by fitting the partial Fourier series) and period (τ) computed by the FFT (see Fig. 2 top). The switch to the DD regime caused (REML analysis, χ² ratio = 5.399, df = 1, p = 0.0201, main effect of time: F₁,₈₅₇,₂₂₃.₇ = 13.46, p < 0.001; main effect of genotype: F₁,₁₃₇ = 0.97, p = 0.33 and significant genotype-time interaction: F₂,₂₄₁ = 4.375, p = 0.014) a shortening of the period from τ = 23 ± 0.44 on days 1–8 to τ = 21 ± 0.87 on days 9–16 (p = 0.02) in WT animals. In KO animals, the period shortened from τ = 23 ± 0.38 on days 1–8 to τ = 21 ± 0.74 on days 9–16 (p = 0.029) and to τ = 18 ± 1.0 (p < 0.001) on days 17–24.

N−D_mean REML analysis (χ² ratio = 55.32, df = 1, p < 0.0001) showed the following results: a significant main effect of time (F₁,₈₀₆,₂₄₆.₅ = 130.5, p < 0.001) and genotype (F₁,₁₄₀ = 11.05, p < 0.01) but no significant interaction between genotype and time (F₂,₂₇₃ = 2.887). Post hoc Sidak analysis was, therefore, performed to assess the time effects in the WT and KO groups separately. This analysis showed differences between time slots in WT animals (p < 0.001), i.e., between days 1–8 and 9–16 and between days 1–8 and days 17–24. The same was true for KO animals. D_mean REML analysis (χ² ratio = 16.47, df = 1, p < 0.0001) showed the following results: a significant main effect of time (F₁,₇₀₁,₂₃₂.₂ = 116.2, p < 0.001) and genotype (F₁,₁₄₀ = 35.12, p < 0.001) and a significant interaction between genotype and time (F₂,₂₇₃ = 8.88, p < 0.001). Post hoc Sidak analysis showed differences between time slots in WT and KO animals (p < 0.001) and differences between the WT and KO groups on days 9–16 and 17–24 (p < 0.001). REML analysis showed differences in the N_mean (χ² ratio = 126.9, df = 1, p < 0.0001) including a significant main effect of time (F₁,₉₀₅,₂₆₀ = 76.3, p < 0.001) and genotype (F₁,₁₄₀ = 27.81, p < 0.001) and a significant interaction between genotype and time (F₂,₂₇₃ = 4.211, p = 0.016). Post hoc Sidak analysis showed differences between time slots in WT animals (p < 0.001) and between days 1–8 and 17–24 in KO animals, as well as a significant difference between WT and KO on days 1–8, 9–16 and 17–24 (p < 0.001). REML analysis also showed differences in the mesor (χ² ratio = 167.1, df = 1, p < 0.0001) including a significant main effect of time (F₁,₉₈₇,₂₇₁.₃ = 14.25, p < 0.001) and genotype (F₁,₁₄₀ = 41.56, p < 0.001) and a significant interaction between genotype and time (F₂,₂₇₃ = 9.26, p < 0.001). Post hoc Sidak analysis showed differences between time slots in WT animals (p < 0.001) and between days 1–8 in KO animals, as well as a significant difference between the WT and KO groups on days 1–8, 9–16 and 17–24 (p < 0.001).

Another aspect of the activity shift is depicted in Fig. 3 left, where the times at which the slope was maximal or minimal are shown. REML analysis of f_MinSlope (χ² ratio = 1.096, df = 1, p = 0.29) showed that the p value is high

**Biological rhythm parameters**

Thus, we compared the main biological rhythm parameters using the mixed-effects model (REML) with Sidak correction. We compared the following parameters on days 1–8 (LD regime), 9–16 (DD regime), and 17–24 (DD regime): day mean (D_mean), night mean (N_mean), difference between night and day mean (N−D_mean), mesor (Midline Estimating Statistic of Rhythm, a midline based on the distribution calculation) showed the following: significant main effect of time (F₂,₃₇₈,₁₆₆₅ = 582.4, p < 0.0001, η² = 0.086) and group (F₁₀₀₂,₂₁₀₀ = 1.479, p < 0.0001, η² = 0.051) and an interaction between group and time (F₁₉₇,₂₁₀₀ = 13.16, p < 0.0001, η² = 0.19). Post hoc Sidak analysis showed differences between every group (p < 0.0001).

**Fig. 1** The locomotor activity biological rhythm in the LD regime (days 1–8), top, and in the DD regime (days 9–24), bottom. Gray parts represent the dark phase. Abscissa: time (h), ordinate: activity (cnts.min⁻¹). For significance, see “Results”. See legend for symbol explanation.
thus we have to conclude that the matching was not effective. REML analysis returned a significant main effect of time \(F_{1,941,233.9} = 5.746, p = 0.004\), whereas the main effect of genotype \(F_{1,137} = 0.04, p = 0.84\) and interaction between genotype and time \(F_{2,241} = 0.1, p = 0.9\) were not significant. It is, therefore, possible to conclude that there was no difference in \(t_{\text{MaxSlope}}\) between WT and KO in LD and DD regime. REML analysis also showed a high \(p\) value in \(t_{\text{MaxSlope}}\) \(\chi^2\) ratio \(= 0.017, df = 1, p = 0.896\) but returned a significant main effect of time \(F_{1,911,230.3} = 21.5, p < 0.001\) and genotype \(F_{1,137} = 14.28, p < 0.001\) and a significant interaction between genotype and time \(F_{2,241} = 3.3, p = 0.029\).

Post hoc Sidak analysis showed differences between time slots in WT \(p < 0.001\) and \(p < 0.01\), between days 1–8 and 9–16 and between days 1–8 and 17–24, respectively, and KO \(p = 0.038\) between days 1–8 and 9–16, and \(p < 0.001\) between days 1–8 and 17–24 animals and a difference between the WT and KO groups on days 1–8 \(p < 0.001\) and days 17–24. Taken together, the differences in \(t_{\text{MaxSlope}}\) between WT and KO in LD and DD regime are questionable.

Lomb-Scargle periodograms (see Fig. 4) showed an approximately 24-h period both in WT and KO animals. Moreover, it was possible to detect an additional, approximately 24-h period both in WT and KO animals. In KO animals, the period was shortened from \(\tau = 18.21 \pm 0.94\) days on days 9–16 \(p = 0.04\) and to \(\tau = 17.26 \pm 0.96 (p = 0.008)\) days 17–24.

Other parameters \([N – D_{\text{mean}}\) \(\chi^2\) ratio \(= 0.045, df = 1, p = 0.83\), \(D_{\text{mean}}\) \(\chi^2\) ratio \(= 0.99, df = 1, p = 0.75\), \(N_{\text{mean}}\) \(\chi^2\) ratio \(= 1.495, df = 1, p = 0.221\), mesor \(\chi^2\) ratio \(= 1.624, df = 1, p = 0.20\), \(t_{\text{MinSlope}}\) \(\chi^2\) ratio \(= 0.8, df = 1, p = 0.37\), \(t_{\text{MaxSlope}}\) \(\chi^2\) ratio not estimated\)] had very high \(p\) values. Thus, we have to conclude that the matching was not effective. In spite of that, the analysis returned some significant differences: \(N – D_{\text{mean}}\), a significant main effect of time \(F_{1,742,221.3} = 73.84, p < 0.001\) and genotype \(F_{1,128} = 7.25, p = 0.008\) and no significant interaction between genotype and time \(F_{2,254} = 2.988, p = 0.052\). Post hoc Sidak analysis was, therefore, performed to assess the time effects in the WT and KO groups separately. This analysis showed differences between time slots in WT animals \(p < 0.001\), i.e., between days 1–8 and 9–16 and between days 1–8 and 17–24. The same was true also for KO animals.

\(D_{\text{mean}}\) REML analysis showed the following results: a significant main effect of time \(F_{1,961,276.5} = 184.2, p < 0.001\) and genotype \(F_{1,142} = 40.34, p < 0.001\), but the genotype-time interaction was not significant \(F_{2,282} = 2.05, p = 0.13\). Post hoc Sidak analysis showed differences between time slots (days 1–8 vs. 9–16, and 1–8 vs. 17–24) in WT and KO animals \(p < 0.001\). REML analysis showed differences in \(N_{\text{mean}}\) a significant main effect of time \(F_{1,842,259.8} = 19.56, p < 0.001\) and genotype \(F_{1,142} = 43.08, p < 0.001\), but the interaction between genotype and time was not significant \(F_{2,282} = 1.4, p = 0.25\). Post hoc Sidak analysis showed differences between days 1–8 and 17–24 in WT animals \(p = 0.028\) and between days 1–8 and 9–16 \(p < 0.001\) and days 1–8 and 17–24 \(p < 0.001\) in KO animals. REML analysis also showed a significant main effect of genotype in the mesor \(F_{1,142} = 67.82, p < 0.001\). Other parameters were not significant: main effect of time \(F_{1,936,273} = 2.142, p = 0.12\) and genotype-time interaction \(F_{2,282} = 0.66, p = 0.52\). However, in the view of non-effective matching these differences are questionable.

The comparison of the main biological rhythm parameters was performed using FFT analysis with stepwise regression technique and statistically analysed by the mixed-effects model (REML) with Sidak correction. We compared the same parameters in the same time slots as in Experiment 1 (see Fig. 2 bottom). Using this analysis, the light pulse caused (REML analysis, \(\chi^2\) ratio \(= 4.706, df = 1, p = 0.003\), main effect of time: \(F_{1,952,239.1} = 15.94, p < 0.001\); main effect of genotype was not significant: \(F_{1,141} = 0.26, p = 0.61\); genotype-time interaction was not significant: \(F_{2,245} = 0.53, p = 0.59\) a shortening of the period from \(\tau = 21.64 \pm 0.71\) on days 1–8 to \(\tau = 17.01 \pm 1.04\) on days 9–16 \(p = 0.002\) and to \(\tau = 16.64 \pm 1.00\) on days 17–24 \(p = 0.002\) in WT animals. In KO animals, the period was shortened from \(\tau = 21.17 \pm 0.79\) on days 1–8 to \(\tau = 18.21 \pm 0.94\) on days 9–16 \(p = 0.04\) and to \(\tau = 17.26 \pm 0.96 (p = 0.008)\) on days 17–24.

\(\chi^2\) values.
Post hoc Sidak analysis showed differences between time periods 1–8 and 9–16, and a significant difference between days 1–8 and 17–24 (Δ
\[\text{p} < 0.01\), difference from the same time slot in WT animals. See legend for symbol explanation. Bottom: Changes in biological rhythm parameters, N–Dmean, Dmean, Nmax, mesor and period (\(\tau\)), before and after the shift from the LD to DD regime with a light pulse (300 lx) applied on day 9. *\(\text{p} < 0.05\), difference from the LD regime (days 1–8); **\(\text{p} < 0.01\), difference from the LD regime (days 1–8); ***\(\text{p} < 0.001\), difference from the LD regime (days 1–8); no difference between WT and KO animals was found.

Another aspect of the activity shift is depicted in Fig. 3 right, where the times in which the slope was maximal or minimal are shown. REML analysis showed differences in \(t_{\text{MaxSlope}}\), a significant main effect of time \(F(1,921,235.3) = 31.33, p < 0.001\) and genotype \(F(1,141) = 6.673, p = 0.01\) and a significant genotype–time interaction \(F(2,245) = 4.008, p = 0.019\). Post hoc Sidak analysis showed differences between time slots in WT \(p < 0.001\) and KO animals \(p < 0.001\) between days 1–8 and 9–16, and \(p = 0.013\) between days 1–8 and 17–24, respectively. The difference between WT and KO animals was found on days 17–24 \(p = 0.01\). REML analysis also showed differences in \(t_{\text{MaxSlope}}\), a significant genotype–time interaction \(F(2,386) = 3.044, p = 0.048\). However, the main effects were not significant: time \(F(1,86,359) = 2.286, p = 0.11\) and genotype \(F(1,386) = 2.833, p = 0.11\). However, in the view of non-effective matching these differences are questionable.

Time shifts between WT and KO animals were not significantly different between days 1–8 and days 9–16 \(\Delta\phi_{\text{WT}} = 1.08 \pm 0.12, (\Delta\phi_{\text{KO}} = -0.83 \pm 1.58, \text{t test}, p = 0.35)\) and between days 1–8 and 17–24 \(\Delta\phi_{\text{WT}} = 0.55 \pm 0.95, (\Delta\phi_{\text{KO}} = -0.73 \pm 1.34, \text{t test}, p = 0.52)\).

Lomb-Scargle periodograms showed (see Fig. 5) an approximately 24-h period both in WT and KO animals. Moreover, it was possible to detect an additional, approximately 12-h period in WT and KO animals but the differences between WT and KO were non-significant (day 9–16: WT: 12.13 \pm 0.04, KO: 12.14 \pm 0.06, day 17–24: WT: 12.03 \pm 0.02, KO: 12.04 \pm 0.04). The calculation of power spectrum in the 24-h period showed [mixed-effects model (REML) with Sidak correction, genotype: wild-type and knockout, time: days 1–8, 9–16, 17–24] a significant main effect of time \(F(1,526,19,84) = 58, p < 0.0001\), no effect of genotype \(F(1,15) = 1.015, p = 0.3297\) and no interaction genotype/time \(F(2,26) = 0.77, p = 0.475, \chi^2\text{ ratio} = 26.75, df = 1, p < 0.0001\). Post hoc Sidak analysis showed differences between days 1–8 and 9–16, and 1–8 and 17–24 in WT and in KO. The REML analysis showed no differences in the 12-h period (time: \(F_{1,10} = 0.4520, p = 0.5166\), genotype: \(F_{1,12} = 0.2203, p = 0.6472\), interaction between time and genotype: \(F_{1,10} = 1.030, p = 0.344; \chi^2\text{ ratio} = 4.274, df = 1, p = 0.0393\).

The sample periodograms showing the time shift are shown in Fig. 6 (bottom).

**M1 MR autoradiography**

Two-way ANOVA showed a significant main effect of genotype \(F(5,79) = 6.378, p = 0.0135, \eta^2 = 0.069\) and area analyzed \(F(5,79) = 166.7, p < 0.001, \eta^2 = 0.90\) and a significant genotype–area interaction \(F(5,73) = 3.557, p = 0.0059, \eta^2 = 0.019\). Post hoc Sidak analysis showed differences between WT and KO M1 MR density in the striatum (see Fig. 7).

**Discussion**

Here, we have shown that the biological rhythm activity curves differed after switching to the DD regime. These results suggest that the core clock output is changed. In the freely running clock condition (DD regime), the period \(\tau\), duration of diurnal cycle was shorter. This is in agreement with the Aschoff’s rule: Aschoff (Aschoff 1960) noted that under constant light conditions, the activity phase shortens in nocturnal organisms and lengthens in diurnal organisms.

Moreover, the day mean, night mean and their difference were higher in KO animals. Additionally, the mesor (midline value) was higher in KO animals than in WT. Therefore, KO animals retain increased locomotor activity in the active period in the freely running clock condition. There was no difference in the time in which the minimal slope occurred between WT and KO animals. The time difference, in which the maximal slope occurred, is questionable. However, our conclusion, that the core clock output is changed, is not affected by this finding. The periodograms showed differences between the LD and the DD regime both in WT and KO animals.

The light pulse applied on day 9 caused no differences in biological rhythm parameters between WT and KO animals. The periodograms showed differences between the LD and the DD regime both in WT and KO animals. This conclusion supports the hypotheses that the structures involved in biological rhythm regulation in WT and KO animals are most likely the same. Different conclusions were found in a paper in which the temperature rhythm was entrained faster than the locomotor rhythm after a light-phase shift (Satoh et al. 2006). These results suggested different intrinsic pacemaker regulation. However, in our previous work (Valuskova et al. 2018b), we did not find differences in temperature biological rhythm in M1 KO animals, which could be the reason for this difference.

In our previous work, we detected a significant density of M1 MRs in the motor cortex, striatum and intergeniculate leaflet. These densities, using recalculation (not used in the original paper) were: MoCx: 16.49 \pm 2.12 nCi/mg,
Thus, here we examined the amount of $M_1$ MRs, another muscarinic receptor subtype implicated in locomotor coordination. The density of $M_1$ MRs, as detected using the $M_1$ MR-specific autoradiography protocol (Valuskova et al. 2018a), was not high in most brain areas implicated in locomotor activity biological rhythm changes. Very high amounts of $M_1$ MRs were found in the striatum (relative density approximately 57 nCi/mg). The other areas revealed $M_1$ MR densities between 2.1–4.2 nCi/mg. These densities were comparable to the low densities of $M_4$ MRs in these areas. This is, according to our best knowledge, the first report about $M_1$ and $M_4$ MRs detection in the SCN, SPVZ, IGL, and PHA.

In Syrian hamsters, the application of the $M_1/M_4$ agonist (McN-A-343) directly to the SCN caused phase advances during the day (Basu et al. 2016). At first glance, this observation may be in disagreement with our results. However, $M_4$ MRs are not present in the SCN (Valuskova et al. 2018b), and thus, these effects can be attributed to $M_1$ MRs. $M_1$ MRs are present in the SCN, although at a very low density. On the other hand, the SCN has a high density of cholinergic neurons (Abbott et al. 2013). In addition to that, (Yang et al. 2010) have demonstrated that carbachol (muscarinic non-specific agonist) induces hyperpolarization via background $K^+$ currents. These effects were blocked by $M_4$ antagonists and to a lesser degree by $M_1$ antagonists.

In conclusion, there are three possibilities explaining this SCN-directed locomotor biological rhythm regulation: first, the biological rhythm is directed by $M_1$ MRs, and the very low density is enough to regulate these events. Second, there is an interconnection between $M_1$ MRs and $M_4$ MRs that regulates biological rhythm. Third, structures other than the SCN are responsible for locomotor biological rhythm regulation. Our data on $M_4$ and $M_1$ density, however, very probably exclude the role of this muscarinic receptor subtype in SCN directed locomotor biological rhythm regulation what does not exclude its role in other SCN-mediated processes. Therefore, we suggest the third possibility as the most plausible.

Interestingly, WT female mice were shown to have a significantly larger phase response than estrogen receptor subtype 1 knock-out female mice during the early subjective...
night (Blattner and Mahoney 2013). This observation further supports the hypothesis regarding the connection between hormone levels and different types of locomotor activity as defined previously (Kuljis et al. 2013; Blizard et al. 1975; Morgan and Pfaff 2001; Ogawa et al. 2003).

The structures involved in locomotor biological rhythm regulation are, in many cases, the target or the source of acetylcholine action. The SCN is densely innervated by cholinergic neurons (Abbott et al. 2013). Muscarinic receptors have been shown to be involved in carbachol (muscarinic agonist)-induced phase shifts in circadian rhythms (Bina and Rusak 1996). However, our results suggest that structures other than the SCN are responsible for locomotor biological rhythm regulation. This suggestion is also in agreement with findings of the role of M4 MRs in SCN circadian rhythm (Liu and Gillette 1996; Gillette et al. 2001).

Cholinergic projections were also identified in the subparaventricular zone (Castillo-Ruiz and Nunez 2007). The density of M1 MRs in this area, as detected in our present study, was low, and there are no M4 MRs present. We also compared our results with in situ hybridization data in Allen Mouse Brain Atlas (Lein et al. 2007). However, it is necessary to stress that the amount of RNA does not necessarily correlate with the number of binding sites representing the real picture of receptors able to bind a specific ligand. For example, M4 MR showed an almost undetectable

**Fig. 4** Lomb-Scargle periodograms in WT (top) and M4 KO (bottom) animals before (i.e. in LD regime) and after switch to the DD regime. The horizontal dotted line shows the power with \( p < 0.05 \), the vertical dotted line shows the 24-h period. See legend for symbol explanation. The inset in the M4 KO periodogram shows the differences in power in WT and KO animals between specific time slots and the 1–8th day (LD regime). \( * p < 0.05, \) difference from the LD regime; \( ** p < 0.01, \) difference from the LD regime.

**Fig. 5** Lomb-Scargle periodograms in WT (top) and M4 KO (bottom) animals before (i.e. in LD regime) and after the switch to the DD regime when the light pulse was applied. The horizontal dotted line shows the power with \( p < 0.05 \), the vertical dotted line shows the 24-h period. See legend for symbol explanation. The inset in the M4 KO periodogram shows the differences in power in WT and KO animals between specific time slots and the 1–8th day (LD regime). \( * p < 0.05, \) difference from the LD regime; \( ** p < 0.01, \) difference from the LD regime.
hybridization in all brain areas, but the amount of M₄ MR binding in autoradiography was particularly high in the striatum. A relatively good correlation between in situ hybridization and autoradiography was found for M₁ MR.

Cholinergic neurons have been shown to be present in the posterior hypothalamic area (Casini et al. 2018), although in carp (Cyprinus carpio). Our results showed MRs in the posterior hypothalamic area but no M₄ MRs (Valuskova et al. 2018b), and the density of M₁ MRs was low.

On the other hand, M₁ MRs and M₄ MRs are both present in the striatum (Felder et al. 2018), and both receptor types are important in locomotor regulation. In the thalamus, M₄ MRs were shown to modulate glutamatergic transmission in the corticostriatal pathway (Pancani et al. 2014). In agreement with this finding, we found that approximately 50% of MRs in this region are M₄ MRs (Valuskova et al. 2018b).

Taken together, our results suggest that the core clock output is changed in M₄-deficient mice. The structures involved in biological rhythm regulation in WT and KO mice are most likely the same. The main area that affects the M₄ MR-directed locomotor biological rhythm is likely the striatum together with coordinated interconnection with the thalamus and intergeniculate leaflet.

Fig. 6 The sample actograms of WT and M₄ KO mice. Top: the actograms in mice where the LD regime was switched on the 9th day to the DD regime. Bottom: the actograms in mice where the LD regime was switched on the 9th day to the DD regime and where the light pulse was applied on day 9. WT wild type mice, KO M₄ KO mice, LD light/dark regime (see “Methods”), DD: dark/dark regime (see “Methods”).
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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval Animals were treated in accordance with the EU legislation (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [Council of Europe No. 123, Strasbourg 1985]), and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the 1st Medical Faculty, Charles University, Prague and by the Ministry of Education of the Czech Republic under No. MSMT-2409/2017–3.

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Fig. 7 The densities of M1 MRs in the areas implicated in locomotor biological rhythm regulation. Ordinate: relative density (nCi/mg). Striatum (Str), thalamus (TH), suprachiasmatic nucleus (SCN), subparaventricular zone (SPVZ), posterior hypothalamic area (PHA), and intergeniculate leaflet (IGL). See legend for symbol explanation. ***p < 0.001, difference from WT animals
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