Research Article

The Intergeniculate Leaflet Plays a Key Role in Sex Differences in the Biological Rhythm of Locomotor Activity in M_4 Muscarinic Receptor Knockout Mice

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Circadian locomotor activity rhythms are generally regulated by complex neural networks involving multiple brain regions and neurotransmitter systems. Previous research has established that the M4 muscarinic receptor (mAChR) subtype plays a crucial role in regulating the circadian rhythm across various brain structures. However, significant sex differences exist in how these rhythms respond to circadian disruption, with females showing changes in locomotor activity biological rhythms while males do not, yet the specific pacemaker structures responsible for maintaining consistent locomotor activity in wild-type and M4 muscarinic receptor knockout (M4 KO) males remain unclear. To address this gap, we compared mAChR densities between females and males under standard light/dark conditions across eight brain regions: the motor cortex, somatosensory cortex, striatum, thalamus, intergeniculate leaflet (IgL), suprachiasmatic nucleus, subparaventricular zone, and posterior hypothalamic area. We then examined the effects of constant darkness and constant light regimes on receptor density in locomotor rhythm-related brain areas in males only. Among all examined structures, only the IgL showed variations across the light/dark, constant darkness, and constant light regimes. Significant differences were observed between light/dark and constant light in wild-type, between light/dark and constant darkness in both wild-type and M4 KO, between constant darkness and constant light in M4 KO, and between wild-type and M4 KO under light/dark conditions. These findings indicate that the IgL is the key structure determining sex differences in the regulation of locomotor activity biological rhythms.

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1. Introduction

Locomotor activity reveals changes over time. Depending on whether the animal is diurnal or nocturnal, the peak of its locomotor activity differs. This biological rhythm is affected by multiple neurotransmitter systems. Among others, neuropeptide Y (NPY) and 5-HT[1], glutamate and dopamine[2], the cholinergic system[3], transforming growth factor α (TGF-α) and epidermal growth factor (EGF)[4] play an important role, and it is modulated by the estrogen/estrogen receptor[5][6].

Regarding the specific brain areas that contribute to the pattern of the locomotor biological rhythm, some structures have been implicated in this biological rhythm's regulation. In addition to the main body pacemaker, the suprachiasmatic nucleus [SCN,[4][7][8]], these structures comprise the subparaventricular zone [SPVZ,[4][7][9]], intergeniculate leaflet [IgL,[10]], posterior hypothalamic area[11], and the structures involved in locomotor pattern generation [thalamus[12], striatum[13][14], and motor cortex[14]].

In recent years, we have described that M4 muscarinic receptors (M4 mAcChRs) affect the biological rhythm of locomotor activity in females[15] but not in males, that the effects of muscarinic drugs differ in the morning and the evening[16], that these effects are caused by specific pacemaker structures[17] in the CNS (striatum, IgL, and thalamus), and that the key structure for rhythm determination is the striatum, where the M1 mAcChR subtype is also involved. No influence of cholinesterases (AChE, BuChE) on the biological rhythm was shown in this regulation.

As is widely known, the SCN receives light input via the retinohypothalamic tract [e.g., [18]]. The switch from the standard light/dark (LD) regime, usually 12 h of light and 12 h of dark, to constant darkness (dark/dark, DD regime, dark for 24 h) led to free-running activity in the SCN. Thus, this switch could be used to determine if the changes caused by experimental conditions originate in the pacemaker structures or are caused by light entrainment[19].

The constant light (light/light, LL regime, light for 24 h) causes chronodisruption of physiological processes. The use of the LL regime is a frequent study protocol for the induction of circadian disruption and its consequences, although this condition is not usually present in real life[18]. The LL regime led to the inhibition of circadian rhythm amplitudes, or even their elimination, as well as the disruption of rhythms. The disruption can appear within cyclic environmental conditions (external misalignment) or their phases among each other (internal misalignment)[20].

While females reveal changes in locomotor activity and biological rhythm, males do not; we would like to search for the nature of this difference. This fact raises the question of what the reason is for this variance, in

addition to the obvious difference in hormone levels, i.e., what pacemaker structure is responsible for the same locomotor activity biological rhythm in wild-type (WT) and knockout (KO) males? To determine the CNS area that is responsible for the sex differences, we have used circadian disruption (LL regime) to assess the specific CNS structure(s) involved in these differences. Furthermore, we also compare these receptor changes with changes in constant darkness (DD regime).

In these experiments, we compared the densities of muscarinic receptors between females and males (in standard light/dark regimes) in the motor cortex (MCx), somatosensory cortex (SSCx), striatum (CPu), thalamus (Th), intergeniculate leaflet (IgL), suprachiasmatic nucleus (SCN), subparaventricular zone (SPVZ), and posterior hypothalamic area (PHA). The effect of the light regime (constant darkness, constant light) on receptor density in brain areas connected with locomotor biological rhythm regulation (MCx, CPu, Th, IgL, SCN, SPVZ, and PHA) was then studied in males only.

2. Methods

2.1. Animals

The mice lacking the M4 muscarinic receptor were generated in Wess's laboratory and then bred in our animal facility (Prague, Czech Republic). Their genetic background was C57Bl/6NTac. Animals were treated following the legislation of the Czech Republic and the EU, 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-5939/2022-4. The wild-type line was the C57Bl/6NTac line. We studied fully backcrossed (16 generations) muscarinic M4-/- and M4+/+ littermates. The animals were maintained under controlled environmental conditions (12/12 light/dark cycle, 22±1°C, light on at 6:00 AM, relative humidity 55±10%) unless stated otherwise. Food and water were available ad libitum. A total of 12 females (weighing 20-26 g, age 3-6 months) and 48 males (weighing 20-26 g, age 3-6 months) were used in the study, of which there were 25 M4 KO animals and 35 WT. Please note that females were used for comparison in the LD regime only. Before the experiments, the mice were genotyped, and only homozygous mice were used. The females were housed separately from males, which suppressed the estrous cycle (anestrous), making the female group homogeneous in hormone levels. Moreover, no differences were seen in light microscopy of vaginal lavage and actograms in females for 15 consecutive days (control animals were not included in the experiment because of stressful procedures when acquiring lavage).

2.2. Autoradiography detection of muscarinic receptors

For receptor determination, autoradiography was performed in several brain areas: motor cortex [MCx], somatosensory cortex [SSCx], striatum [Cpu], thalamus [Th], suprachiasmatic nucleus [SCN], subparaventricular zone [SPVZ], posterior hypothalamic area [PHA], and intergeniculate leaflet [IgL]. Brains were rapidly removed (4 to 6 brains per group), frozen on dry ice, and then stored at -80 °C until cryostat sectioning. Sixteen-micrometer-thick sagittal sections were cut on a cryostat at -20°C and thaw-mounted on Superfrost® Plus glass slides (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and stored in storage boxes at -80°C until use. For binding to muscarinic receptors, the sections were allowed to thaw and dry for 30 min at 22 °C, and the density of receptors was determined as previously described[21][22]. Dried sections were pre-incubated for 30 min in 50mM Na/K phosphate buffer (pH 7.4) at room temperature (RT). Following pre-incubation, sections were transferred into fresh 50mM Na/K phosphate buffer containing 2nM [3H]-QNB and incubated for 2 hours at RT. Nonspecific binding was assessed on adjacent sections in the presence of 10µM atropine sulfate. After incubation, sections were washed two times for 5 min each in ice-cold buffer and shortly washed for 5 s and then dipped for 2 s in ice-cold water to remove buffer salts. Wet sections were immediately dried by a gentle stream of cold air. Dry sections were apposed to tritium-sensitive Fuji BAS imaging plates (GE Healthcare Europe GmbH, Freiburg, Germany) in Kodak Biomax autoradiographic cassettes (Carestream Health, Inc., Rochester, NY) for 5 days. To ensure the linearity of the signal and to convert photostimulated luminescence to radioactivity, tritium autoradiographic standards containing known quantities of radioactivity (American Radiolabeled Chemicals, Inc., St. Louis, MO) were exposed along with the samples to the screens and films. The film autoradiograms were scanned and the densitometry was done with PC-based analytical software, MCID analysis software (InterFocus GmbH, Mering, Germany). Measurements were taken and averaged from at least three sections for each animal and brain region.

2.3. Histology

Nissl staining was used for SCN, SPVZ, IgL, and PHA identification in MR autoradiography determination. Briefly, the parallel sections were obtained using a cryostat (the appropriateness of the section was controlled using a mouse atlas[23]), and the sections were collected and divided into 4 sets. The first section from the set was placed on the first glass slide and used for Nissl staining, while the remaining 4 sections from the set were placed on other glass slides (3 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 0.2mol/l acetic acid+ 0.2mol/l sodium acetate, 4:1, pH=3) for 20 minutes, then washed twice 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 minutes. Then the sections were incubated for another 45 minutes in xylene (p.a., Penta, Czech Republic) and mounted using DPX (Sigma-Aldrich, Czech Republic) with a coverslip.

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

2.4. The effect of constant darkness and constant light

The male mice were first exposed to a standard LD regime (the same as described above, i.e., 12/12 light/dark cycle, light on at 6:00 AM) for eight consecutive days and then they were exposed to constant darkness (for 24 hours/day, DD regime) for another 63 days. In another experiment, after classical exposure to the standard LD regime, the mice were exposed to constant light (20 to 50 lx) for 56 days.

2.5. Statistical analysis

Three-way ANOVA was used for the comparison of differences between sex (males, females, i.e., first factor), brain areas (MCx, SSCx, CPu, Th, SCN, SPVZ, PHA, and IgL, i.e., second factor), and genotype (WT, KO, i.e., third factor). Two-way ANOVA with Tukey's post-hoc correction was used for the specific determination of differences in specific brain areas under different light regimes (first factor: light regime [LD, DD, LL], second factor: genotype [WT, KO]). Generally, values of $p<0.05$ were considered significant. A specific test with factor significance and/or interaction significance is given in the Results section. The statistics were calculated using the GraphPad Prism program.

3. Results

3.1. Differences between males and females

The three-way ANOVA (genotype: wild type/knockout; brain area: MCx, SSCx, Cpu, Th, SCN, SPVZ, PHA, and IgL; sex: males/females) revealed a significant main effect (Figure 1) in the respective brain area (FDFn, DFd, where DFn is degrees of freedom numerator, and DFd is degrees of freedom denominator: $F_{7,152}=320.2$, $p<0.0001$), a main effect of sex ($F_{1,152}=28.8$, $p<0.0001$), and a main effect of genotype ($F_{7,152}=229.2$, $p<0.0001$). There was also a significant interaction between all three factors: brain area × genotype × sex ($F_{7,152}=3.78$, $p=0.0008$). In addition, a significant interaction was shown between brain area and genotype ($F_{7,152}=24.64$, $p<0.0001$), and brain area × sex: $F_{7,152}=10.68$, $p<0.0001$). The interaction between genotype and sex was not significant ($F_{7,152}=1.39$, $p=0.24$). It can be deduced from Figure 1 that there are substantial differences in MCx between WT males and females and between female WT and KO. In SSCx, there was a significant difference

between WT males and females and between female WT and KO. In the striatum, there was a significant difference between WT males and females, male WT and KO, and between female WT and KO. Also, there was a significant difference in the IgL between male WT and KO.

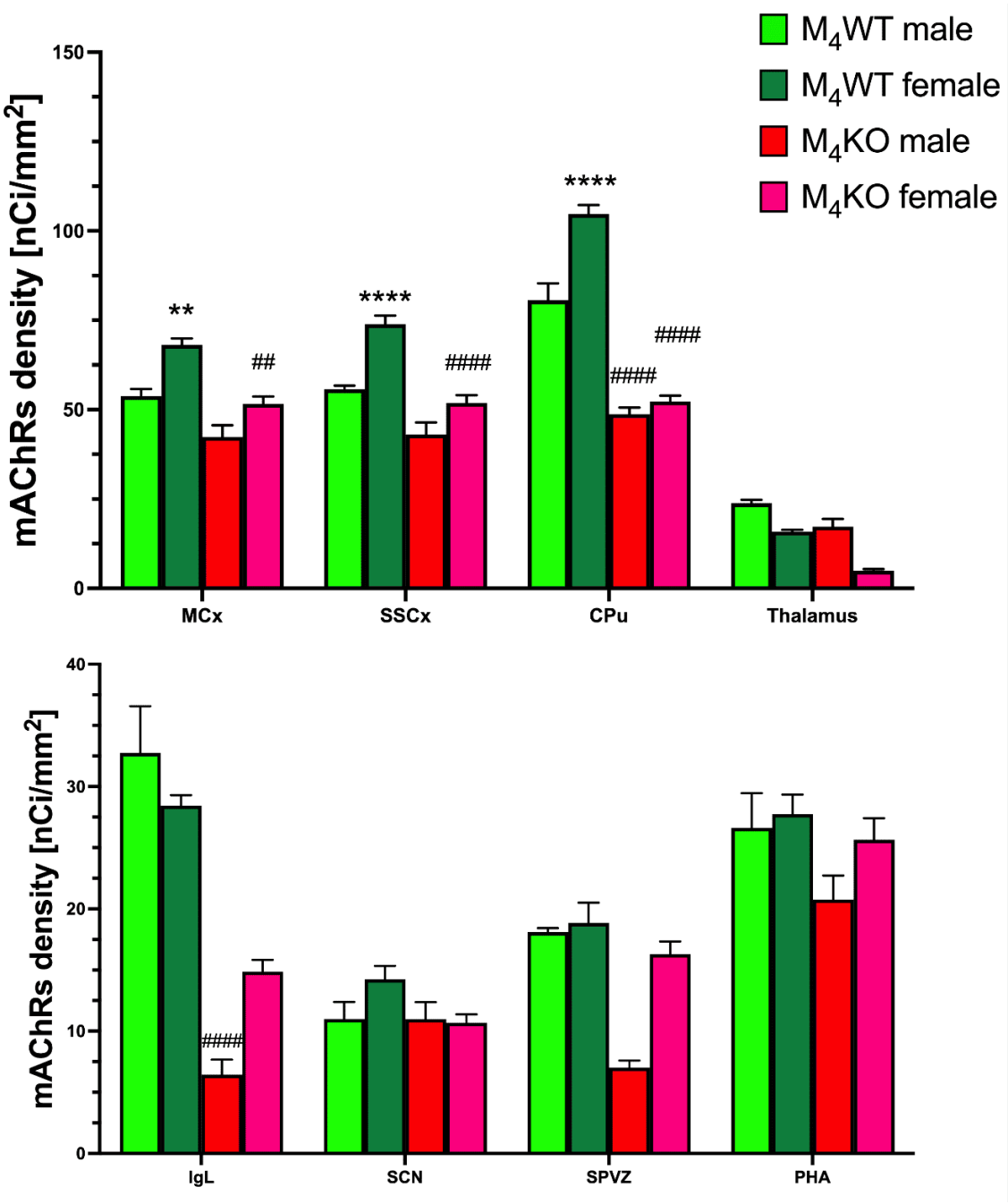

Figure 1. The differences between male and female total muscarinic receptor density in specific brain areas. Top: Motor cortex - MCx, somatosensory cortex - SSCx, striatum - Cpu, thalamus – Th. Bottom: intergeniculate leaflet – IgL, suprachiasmatic nucleus - SCN, subparaventricular zone - SPVZ, and posterior hypothalamic area. **p<0.01 (between male and female), ****p<0.0001(between male and female), ##p<0.01 (between WT and KO),#### p<0.0001(between WT and KO).

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3.2. The effects of constant darkness and constant light in males

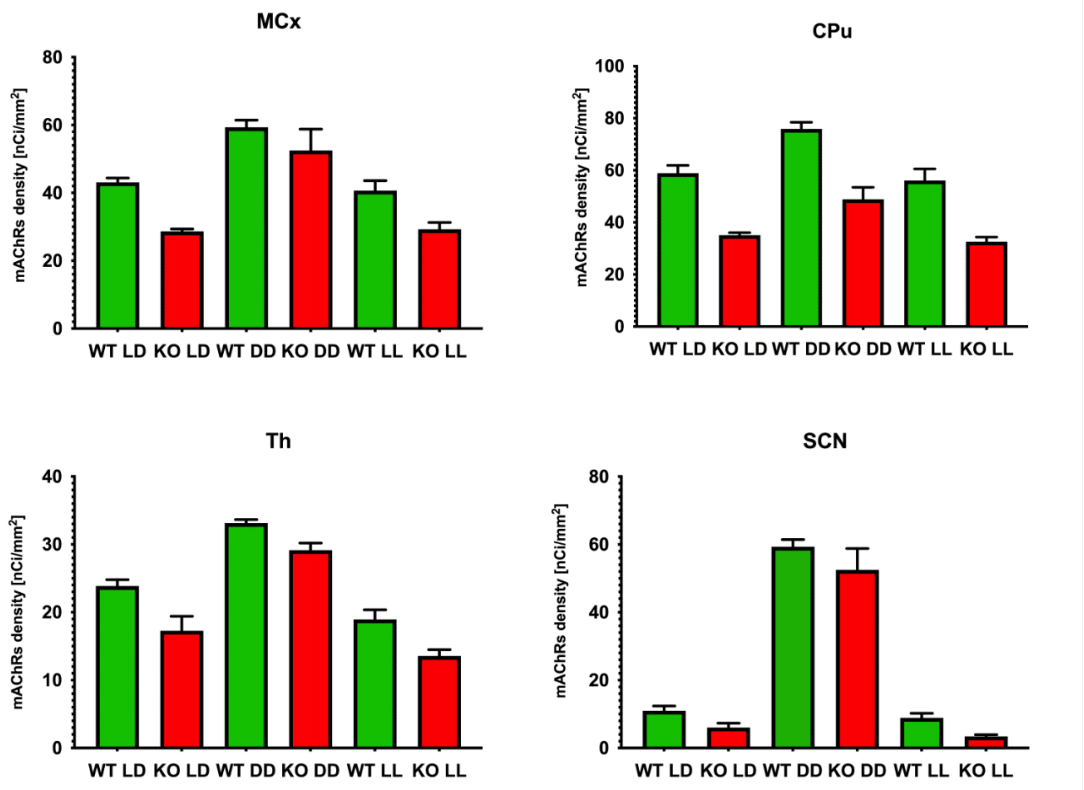

Figure 2. The differences between regime/genotype in the MCx, CPu, Th, and SCN. Ordinate, the density of total muscarinic receptors. Abscissa, specific groups (WT green, M4KO red) in specific light regimes (LD light/dark; LL light/light, constant light; DD dark/dark, constant darkness).

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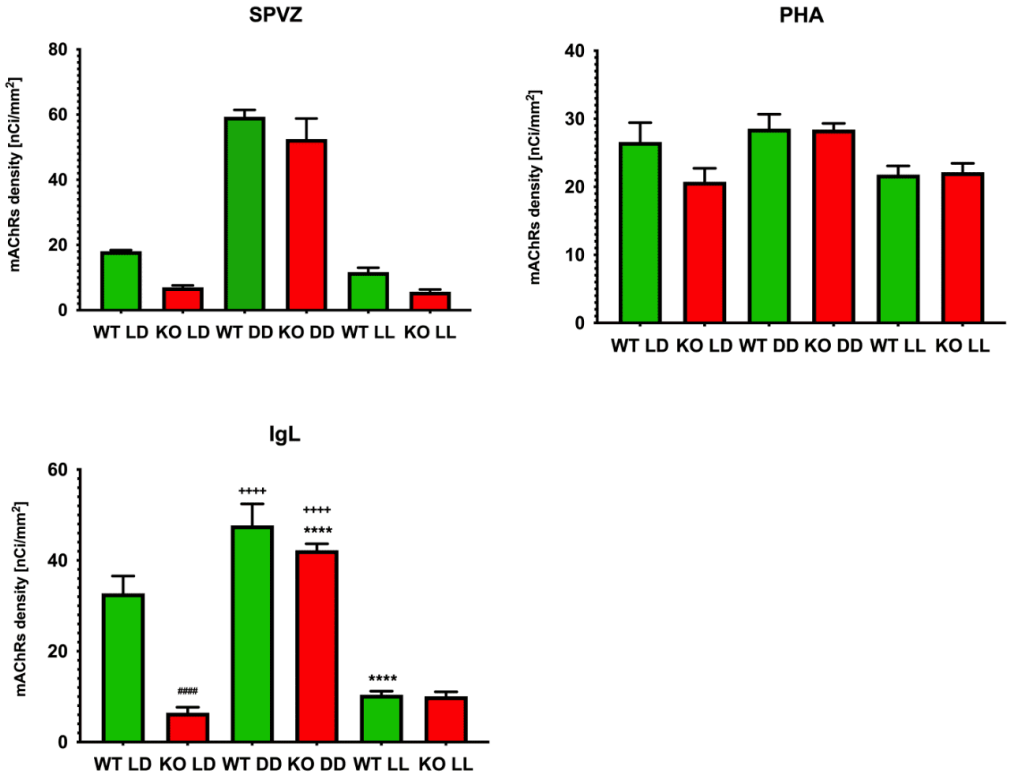

Figure 3. The differences between regime/genotype in the SPVZ, PHA, and IgL. Ordinate: the density of total muscarinic receptors. Abscissa: specific groups (WT green, M4KO red) in specific light regimes (LD light/dark; LL light/light, constant light; DD dark/dark, constant darkness). ****p<0.0001, difference from LD regime; #### p<0.0001, difference from WT animals; ++++ p<0.0001, difference from LL regime.

3.2.1. Motor cortex

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) did not show an effect of interaction ($F_{2,42}$= 0.80, p=0.46). However, there was a general effect of genotype ($F_{1,42}$=20.51, p<0.0001) and of the light regime ($F_{2, 42}$=31.62, p<0.0001). See Figure 2.

3.2.2. Striatum

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) did not show an effect of interaction ($F_{2,43}$=0.55, p=0.58). However, there was a general effect of genotype ($F_{1,43}$=55.32, p<0.0001) and of the light regime ($F_{2, 43}$=12.56, p<0.0001). See Figure 2.

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3.2.3. Thalamus

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) did not show an effect of interaction ($F_{2,40}=0.45$, $p=0.64$). However, there was a general effect of genotype ($F_{1,40}=25.33$, $p<0.0001$) and of the light regime ($F_{2, 40}=69.01$, $p<0.0001$). See Figure 2.

3.2.4. SCN

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) did not show an effect of interaction ($F_{2,23}=1.67$, $p=0.21$). However, there was a general effect of genotype ($F_{1,23}=14.3$, $p=0.001$) and of the light regime ($F_{2, 23}=119.5$, $p<0.001$). See Figure 2.

3.2.5. SPVZ

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) did not show an effect of interaction ($F_{2,20}=0.93$, $p=0.41$). However, there was a general effect of genotype ($F_{1,20}=28.67$, $p<0.0001$) and of the light regime ($F_{2, 20}=48.16$, $p<0.0001$). See Figure 3.

3.2.6. PHA

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) did not show an effect of interaction ($F_{2,33}=2.0$, $p=0.15$), and there was also no effect of genotype ($F_{1,33}=3.65$, $p=0.065$). However, the light regime revealed a significant general effect ($F_{2, 33}=8.34$, $p=0.0012$). See Figure 3.

3.2. IgL

The two-way ANOVA (genotype: wild type/knockout; light regime: LD, DD, LL) revealed a significant effect of interaction ($F_{2,32}=14.47$, $p<0.0001$), a main effect of genotype ($F_{1,32}=16.74$, $p=0.0003$), and a main effect of the light regime ($F_{2, 32}=84.84$, $p<0.0001$). The post-hoc analysis (Bonferroni) showed differences between the LD and LL regimes in WT ($p<0.0001$), between the LD and DD regimes in WT ($p<0.0001$), between the LD and DD regimes in M4 KO ($p<0.0001$), between the DD and LL regimes in M4 KO ($p<0.0001$), and between WT and M4 KO in the LD regime ($p<0.0001$). See Figure 3. To better demonstrate how deep the changes are in M4 mAcChRs, we express the changes in the IgL as M4 mAcChR density (Figure 4). A one-way ANOVA has shown $F_{2,11}=19.77$, $p=0.0002$. Significant differences between the DD and LD regimes ($p= 0.001$) and between the LL and LD regimes ($p=0.0002$) in the post-hoc analysis (Tukey) were observed.

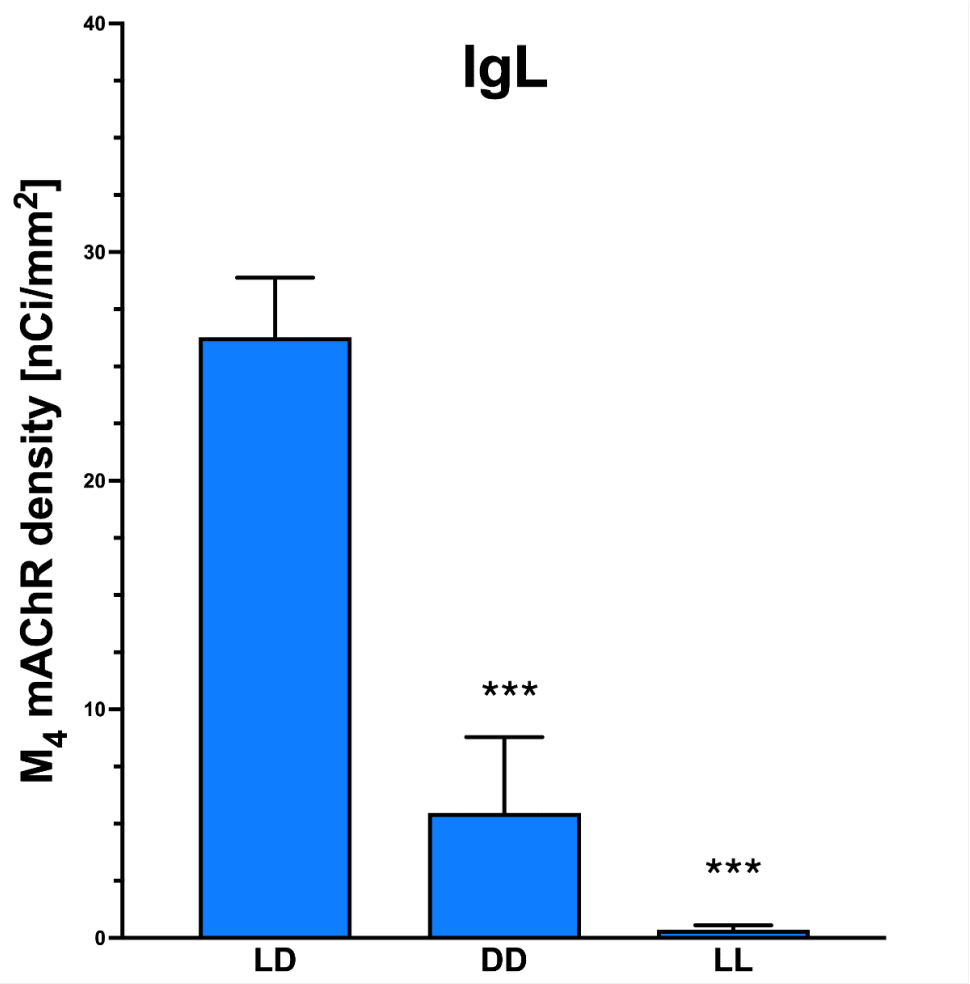

Figure 4. Changes in M4 mAcChRs in the IgL in different light regimes (LD, DD, LL). ***p<0.001 against the normal LD regime.

4. Discussion

Here, we have compared the densities of mAcChRs in CNS structures potentially involved in the biological rhythm of locomotor activity.

Importantly, the reduction of the total number of receptors, i.e., the number of M4 mAcChRs, was comparable in almost all CNS structures investigated in males and females. The only exception was the IgL, where the total number of mAcChRs was significantly lower in males but not in females.

In the MCx, CPu, and Th, there was a general effect of genotype and of the light regime. The effect of genotype is obvious, as the KO mice lack the M4 mAcChR. Interestingly, the PHA did not differ in the effect of genotype

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(i.e., there was no difference between WT and KO animals in receptor density, suggesting that in the PHA, there are no M4 mAcChRs).

As an important structure involved in differences in locomotor activity biological rhythm regulation, we can define the IgL. There was a significant effect of interaction, a main effect of genotype, and a main effect of the light regime. The post-hoc analysis showed differences between the LD and LL regimes in WT, between the LD and DD regimes in WT, between the LD and DD regimes in M4 KO, between the DD and LL regimes in M4 KO, and between WT and M4 KO in the LD regime. It is thus plausible that the IgL is the key structure determining the differences between males and females in the regulation of locomotor activity biological rhythm.

As early as the mid-1990s, it was shown that the response of the circadian system to the LL regime can be affected by monosodium glutamate in the cells within the IgL/vLGN (ventrolateral geniculate nucleus) region[24]. Further, the IgL has been shown to be activated (determined via Fos production) in the LL regime[25] in intervals from 1 hour to 3 weeks of light exposure. Although the aim of the latter study was different from biological rhythm regulation, it has also been shown that estrogen receptor β and progesterone receptor mRNA are present in the IgL[26]. As reviewed by Moore[27], the intergeniculate leaflet has a primary role in integrating photic and non-photic information together with the geniculohypothalamic tract.

There are also studies trying to determine the neurotransmitters involved in the function of the IgL. The discharge activity of IgL neurons with monosodium glutamate in vitro showed either an excitatory, a biphasic, or an inhibitory response, while the application of NMDA induced an excitatory response[28]. The selective antagonist of NMDA receptors (AP-5) reduced the response. Bicuculline application ($GABA_A$ agonist) did not influence the observed inhibitory effects; thus, the involvement of $GABA_A$ receptors in the AP-5 response reduction can be excluded. Importantly, mesopontine cholinergic neurons, activated by arousing stimuli, can activate the IgL and, via a geniculohypothalamic projection, lead the signal to the SCN. Carbachol injected into the IgL during the subjective day induced phase advances[29]. Pre-injections of atropine (muscarinic/nicotinic antagonist) reduced carbachol-induced phase advances. In further work[30], the interconnection between glutamate and muscarinic transmission was studied. However, the results differ from the previous study[29]. In the presence of the cholinergic agonist, glutamate-induced activity was decreased in 32% and increased in 13% of the investigated cases. Carbachol failed to evoke any change in glutamate-induced activity in 55% of the recording cells. The IgL has also been shown to be one of the key nuclei mediating light-induced sleep in mice via GABAergic neurons[10]. This is in good agreement with our results showing the IgL as a key structure in sex differences and sensitively responding to the LL regime.

We can therefore conclude that the IgL represents a morphological substrate for sex differences in the biological rhythm of locomotor activity.

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