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REVIEW

M_4 Muscarinic Receptors and Locomotor Activity Regulation

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Summary

M_4 muscarinic receptors (M_4 MR) represent a subfamily of G-protein coupled receptors serving a substantial role in spontaneous locomotor activity regulation, cognition and modulation of cholinergic system. With increasing body of literature discussing the role of M_4 MR some controversies arose. Thus, we try here to summarize the current evidence regarding the M_4 MR, with the special focus on their role in locomotor activity control. We review the molecular function of M_4 MR in specific brain areas implicated in locomotor regulation, and shortly in other CNS processes that could be connected to locomotor activity. We also focus on brain areas implicated in locomotor activity biorhythm changes like suprachiasmatic nucleus, subparaventricular zone posterior hypothalamic area, striatum and thalamus. Gender-related aspects and differences in locomotor activity in males and females are discussed further.

Key words

M_4 Muscarinic receptor • Locomotor activity • Biorhythm • Suprachiasmatic nucleus

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Muscarinic receptors – prototypical member of G protein coupled receptors family

Muscarinic receptors (MR) are typical members of G protein coupled receptors (Kruse *et al.* 2013) and can be divided into 5 subtypes (M_1-M_5) (Eglen 2012), which activate different G proteins (G_q, G_i) – while odd-numbered subtypes activate G_q, even-numbered activate G_i protein (Kow and Nathanson 2012, Eglen 2012, Reiner and Nathanson 2012).

Respective MR subtypes have been assigned to different functions in CNS (Wess *et al.* 2007). Odd-numbered receptors are considered primarily as post-synaptic receptors, however, both M_2 MR and M_4 MR are localized both pre-synaptically and post-synaptically (Fig. 1). As cholinergic autoreceptors, M_2 and M_4 provide feedback control of acetylcholine release (Zhang *et al.* 2002, Shin *et al.* 2015). M_4 MR are coupled, as stated before, to $G_{i/o}$ G proteins (Mistry *et al.* 2005) and thus they are connected to the adenylyl cyclase/cyclic AMP signaling pathway. However, they are also able to couple with G_s (Nathanson 2000) what makes another level of signal fine tuning. M_4 MR are spontaneously active and can cause constitutive inhibition of adenylyl cyclase (Migeon and Nathanson 1994). M_4 MR was also shown to enhance Ca^{2+} currents *via* effect on Ca_{V1} channels (Hernández-Flores *et al.* 2014). Sequencing study on rat M_4 MR has shown the presence of cell type-specific silencer element in the promoter region (Mieda *et al.* 1996) and that expression of M_4 MR is regulated by the neuron-restrictive silencer element/ repressor element 1 (Mieda *et al.* 1997). Promoter region also contains cAMP response element (Migeon and Nathanson 1994). Other study identified that promoter region does not contain a TATA or CAAT box and has several consensus sequences for enhancer elements including five Sp-1 binding sites, one AP-2 site, one AP-3 binding site and two E-boxes within the proximal 600 bp (Wood *et al.* 1995).

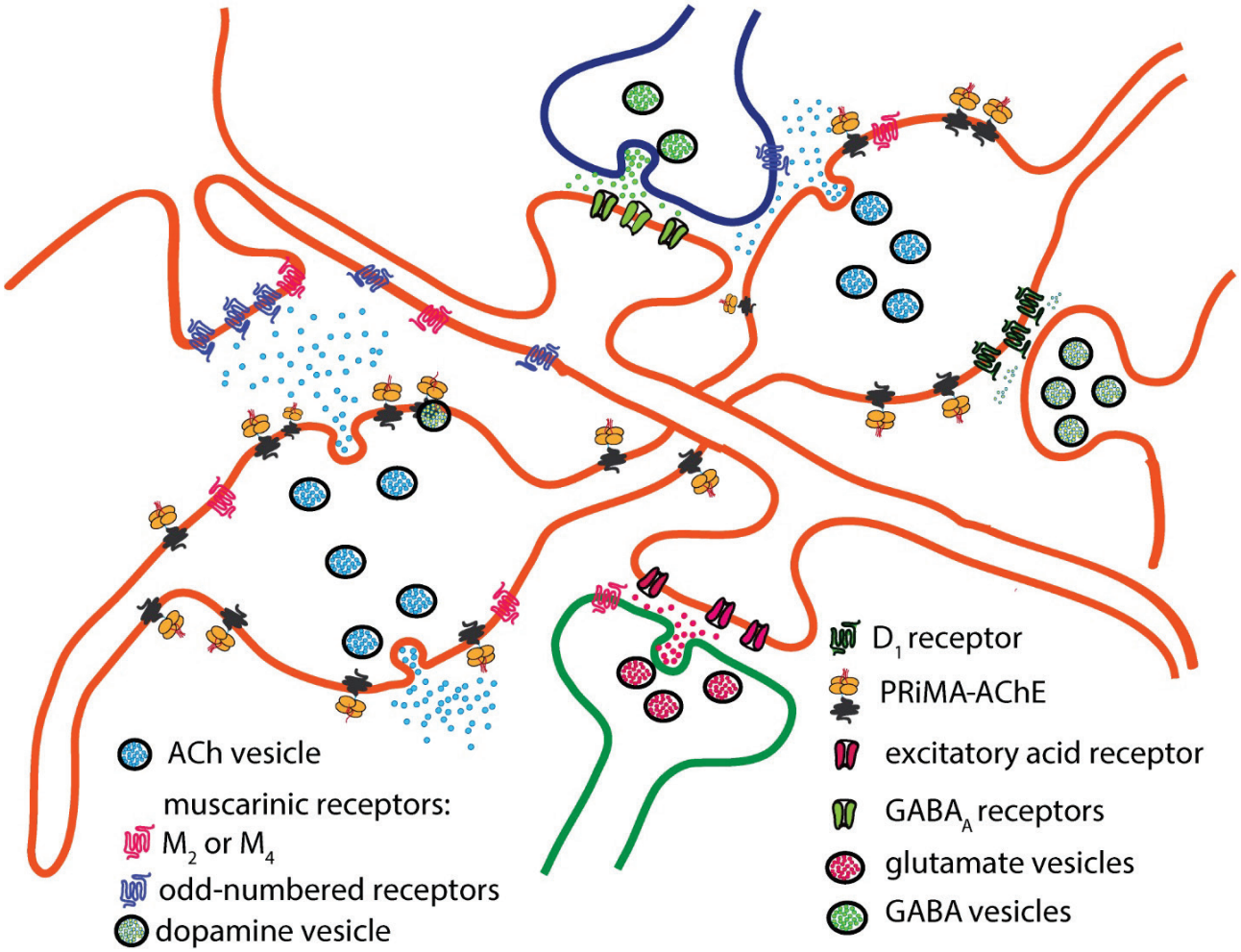

Fig. 1. Schematic possible neurotransmitter-receptor interactions in the striatum. Each neurotransmitter is marked by different color (GABA – green, acetylcholine – blue, glutamate – red, dopamine – yellow). Please note also different color for target muscarinic receptors (red for even-numbered and blue for odd-numbered receptors). PRiMA-AChE is the main heteromer breaking down acetylcholine (this is acetylcholinesterase anchored in the membrane by anchoring protein PRiMA – proline-rich membrane anchor).

M_4 muscarinic receptor function

M_4 MR have been associated with different organism function during past years. Initially, the role of MR can be elucidated only by means of pharmacological studies. These studies have indicated an important role of MR in several brain processes including learning and memory (Anagnostaras *et al.* 1995), attention (Chen *et al.* 2004, Mirza and Stolerman 2000), locomotion (Sipos *et al.* 1999), thermoregulation, sleep and wakefulness (Sanford *et al.* 2006), food intake (Pratt and Blackstone 2009) and reward (Crespo *et al.* 2008). However, the limited selectivity of commonly used MR ligands and the often overlapping pattern of expression of individual MR subtypes had made it difficult to define MR subtype specific roles.

The current understanding of the role of M_4 receptors has been advanced by the use of genetically modified mice with either overall lack of M_4 MR or in individual neuronal subpopulations (Wess *et al.* 2007, Jeon *et al.* 2010). These studies have revealed implication of M_4 MR in several brain processes and have helped to clarify the molecular mechanisms by which M_4 MR govern neuronal circuits. Thus M_4 MR are assigned to have role in different physiological events, like behavior (Bubser *et al.* 2014), social behavior (Koshimizu *et al.* 2012), learning, memory and addiction (Thomsen *et al.* 2012).

Locomotor activity regulation

Locomotion, in the widest definition, is any of a variety of movements or methods that animals use to move from one place to another. The locomotor control, in general, includes hierarchical network (circuits) of CNS areas including cortex, basal ganglia, thalamus,

cerebellum, reticular formation, vestibular apparatus and spinal medulla. This control of locomotion can be influenced by CNS structures involved in biorhythm activity changing (or anticipating) the needs of organism according to internal and external conditions.

These circuits are affected by wide range of receptors as neurotransmitter targets. Besides, muscarinic/nicotinic acetylcholine receptors, dopamine receptors, GABA receptors and excitatory amino acid receptors are considered as means of locomotor control and balance between these receptors (and neurotransmitters targeting to receptors) is key factor for effective locomotor regulation (Tzavara *et al.* 2004, Gomeza *et al.* 1999, Carr and Surmeier 2007). The role of receptors and/or neurotransmitters in locomotor regulation is discussed below (see part M_4 MR molecular function).

In addition to general effects of specific brain circuits in locomotor activity, there are other CNS areas, that are specialized on the biorhythmic coordination that can help the organism to cope with actual need in locomotor regulation. These structures and the role of receptor/neurotransmitter systems are discussed further.

M_4 MR molecular function

M_4 MR are able to modulate the neuronal circuits directly (post-synaptically) or indirectly (through regulation of acetylcholine (ACh) tone) by several mechanisms, including the regulation of neurotransmitter release, neuronal excitability, transcription and translation (Brown 2010). The modulation of neurotransmitter release has been linked to the ability of M_4 MR to trigger long-term changes of synaptic strength (Bonsi *et al.* 2008, Wang *et al.* 2006) – the synaptic plasticity – which is considered to be the molecular mechanism underlying learning and memory (Malenka and Bear 2004).

Direct regulation

The direct regulation of neuronal excitability is largely mediated by postsynaptic MR coupled to PLC such as M_1 MR, albeit M_4 MR can provide postsynaptic inhibition of neuronal excitability *via* direct activation of G-protein gated inwardly rectifying potassium channels (GIRK) by $G_{\beta\gamma}$ (Brown 2010, Lüscher and Slesinger 2010) or increase neuronal excitability by enhancing Ca^{2+} currents through Ca_{V1}-channels (Hernández-Flores *et al.* 2014).

Indirect regulation

A well established role of M_4 MR is the feedback control of ACh release (Zhang *et al.* 2002). Consistent with the control of ACh release by M_4 MR, the lack of M_4 MR leads to increased levels of ambient ACh in several brain regions, as determined by *in vivo* microdialysis (Tzavara *et al.* 2004, Tzavara *et al.* 2003). In spite of the well recognized role of M_4 MR in the autoinhibition of ACh release, less clear are the underlying mechanisms how the control of ACh release is exerted. This might involve the inhibition of N and P/Q calcium channels *via* a membrane-delimited pathway, presumably by direct action of $G_{\beta\gamma}$ dimer on ion channels (Brown 2010, Yan and Surmeier 1996) as well as the activation of G protein-coupled inwardly-rectifying potassium channels (GIRK), resulting in hyperpolarization of cholinergic neurons (Calabresi *et al.* 1998, Bonsi *et al.* 2008). Another mechanism might lie downstream to the calcium entry and involve direct interaction with the exocytotic machinery (Blackmer *et al.* 2001, Kupchik *et al.* 2011).

M_4 MR are powered to control neuronal excitability indirectly through regulation of ACh tone at M_1 MR. M_1 MR stimulation provides the postsynaptic excitation by suppression of several potassium currents including the voltage and time-dependent K^+ current (I_M/KCNQ channel current), the Ca^{2+} activated K^+ current that generates after-hyperpolarization (I_{AHP}), a leak K^+ current (I_{leak}) (Brown 2010) and inwardly rectifying potassium channels Kir_2 (Shen *et al.* 2007). Activation of M_1 MR also can induce depolarization by increasing the mixed Na^+/K^+ hyperpolarization-activated current (I_h) and Ca^{2+} dependent nonspecific cation current (I_{cat}) (Fisahn *et al.* 2002).

The role of M_4 MR on other neurons

M_4 MR can be also found as heteroreceptors at glutamatergic and GABAergic terminals (Hersch *et al.* 1994) and their activation can alter the release of glutamate and GABA (Carr and Surmeier 2007, Koós and Tepper 2002).

Very importantly, M_4 MR inhibit striatal and mesolimbic dopamine release affecting locomotor coordination (Tzavara *et al.* 2003) as M_4 MR are the most abundant striatal MR subtype (Chapman *et al.* 2011). When focusing on neurotransmitter/receptor map in locomotion regulation, striatonigral pathway expresses

both D_1 dopamine receptors and M_4 MR (Bernard *et al.* 1999). Activation of D_1 dopamine receptors increases locomotor activity, whereas M_4 MR activation has opposite effects. In addition, M_4 KO mice responded excessively to the non-selective dopamine agonist apomorphine and the D_1 dopamine receptor agonist SKF 38393 but not the D_2 dopamine receptor agonist quinpirole (Bymaster *et al.* 2003) indicating tight interconnection between muscarinic and dopaminergic system. This is also in agreement with finding that D_1 dopamine receptor-mediated locomotor stimulation is enhanced in M_4 KO mice (Gomeza *et al.* 1999). By contrast, this study indicated that M_4 MR are not involved in muscarinic receptor-mediated analgesia, tremor, hypothermia and salivation (Gomeza *et al.* 1999). Others have also demonstrated the dependence of dopaminergic neurotransmission on M_4 MR function. M_4 KO mice had elevated dopamine basal levels and enhanced dopamine responses to psychostimulants such as D-amphetamine and phencyclidine (PCP, angel dust) in the nucleus accumbens alongside with increased basal ACh efflux in midbrain (Tzavara *et al.* 2004). M_4 MR have also been shown to negatively regulate the reinforcing effects of cocaine (Schmidt *et al.* 2011). M_4 KO mice showed increased cocaine self-administration, cocaine-induced dopamine efflux in the nucleus accumbens as well as cocaine-induced hyperlocomotion (Schmidt *et al.* 2011). To specify the neuronal population of M_4 MR crucial to modulation of dopamine-dependent behaviors, mice with more selective lack of M_4 MR were generated.

Besides the control of dopaminergic transmission, M_4 MR have been shown to be major MR subtype mediating the cholinergic inhibition of corticostriatal glutamatergic input on striatonigral and striatopallidal medium spiny neurons through regulation of glutamate release (Pancani *et al.* 2014). Furthermore, M_4 MR are (together with M_1 MR) the major MR subtypes responsible for direct cholinergic modulation of the excitatory hippocampal circuit (Dasari and Gulledge 2011).

The role of M_4 MR in locomotor activity regulation

The role of M_4 MR in the control of locomotor activity is a subject of discussion as contradictory findings have been reported. Concerning the role of M_4 MR in locomotor control, the initial knockout study (Gomeza *et al.* 1999) strongly indicated that M_4 MR exert inhibitory action on the overall animal locomotor activity and these studies also showed slight effect on salivation (Bymaster *et al.* 2001) and no effect on tremor and hypothermia. The increased locomotion of M_4 KO mice has been attributed to the enhanced dopaminergic signaling at D_1 dopamine receptors (Gomeza *et al.* 1999) and thus likely increased activity of the striatonigral pathway which promotes movement (Kravitz *et al.* 2010). Consistent with this early finding, a recent report demonstrated locomotor hyperactivity of M_4 KO mice in open field and light/dark transition (Koshimizu *et al.* 2012), further supporting the role of M_4 MR in the control of locomotor activity. By contrast, several other studies have reported contradictory findings in terms of the involvement of M_4 MR in the control of spontaneous locomotion (Turner *et al.* 2010, Schmidt *et al.* 2011, Fink-Jensen *et al.* 2011, Woolley *et al.* 2009). These studies have reported the same locomotor activity in M_4 KO mice as in WT mice (Turner *et al.* 2010, Schmidt *et al.* 2011, Fink-Jensen *et al.* 2011, Woolley *et al.* 2009) as well as no change in diurnal pattern of locomotor activity in M_4 KO animals (Turner *et al.* 2010). Therefore the role of M_4 MR in the control of spontaneous locomotion remains puzzling. Even though all above-mentioned studies employed M_4 KO mice, there were substantial differences in experimental conditions that might account for different observations. Apart from differences in approach to measure locomotor activity and gender of experimental mice, the genetic background of M_4 KO mice appears to be central to different outcomes.

The hyperactive phenotype has been reported in M_4 KO mice bred on mixed 129SvEv/CF-1 or pure 129SvEv genetic backgrounds (Koshimizu *et al.* 2012, Gomeza *et al.* 1999). When the founder mouse line was extensively backcrossed to the C57BL/6NTac or C57Bl/6J inbred strain, the enhanced locomotion of M_4 KO mice has disappeared (Turner *et al.* 2010, Schmidt *et al.* 2011, Fink-Jensen *et al.* 2011, Woolley *et al.* 2009).

Knockout studies were initially considered as optimal method for detection of gene function (Bymaster *et al.* 2003). The flanking allele effect were not sometimes considered as important factor for behavior determination (Crusio *et al.* 2009). It is therefore important to pay attention to genetic background when interpreting the results, or when comparing the results form studies with inconsistency in genetic background. It is recommended to backcross the mice at least for 10 generations with aim to eliminate the flanking allele effect (Crusio *et al.* 2009). Furthermore, the phenotype of gene targeted animals strongly depends on background genes and not solely on the mutations in the targeted gene

as it has been demonstrated in acetylcholinesterase knockout mice made on different genetic background (Duysen and Lockridge 2006).

In addition to the flanking allele problem, it is necessary to stress that mice are nocturnal animals (Roedel *et al.* 2006) and thus experiments performed in their non-active phase can be affected by this fact.

Brain areas implicated in locomotor activity biorhythmic changes

In addition to general effects of specific brain areas on locomotor activity, there are some data on the biorhythmic coordination in locomotor activity. It may be quite obvious that modification of the circadian rhythms may affect sleep and wakefulness and therefore circadian activities. Despite that, there are some data on brain areas that can effectively influence biorhythmic activity. The main biorhythm regulatory circuits are not deeply understood. It seems that multiple brain areas drive biorhythmic coordination in locomotor activity. The most prominent structure is, of course, suprachiasmatic nucleus (SCN). Other structures have been also implicated in these effects. There are areas with near proximity to SCN, like subparaventricular zone (SPVZ), dorsomedial nucleus and posterior hypothalamic area and tuberomammillary nucleus (Abrahamson and Moore 2006). Striatum and thalamus are another areas with locomotor biorhythmic effects. The schematic diagram showing these areas is on Figure 2.

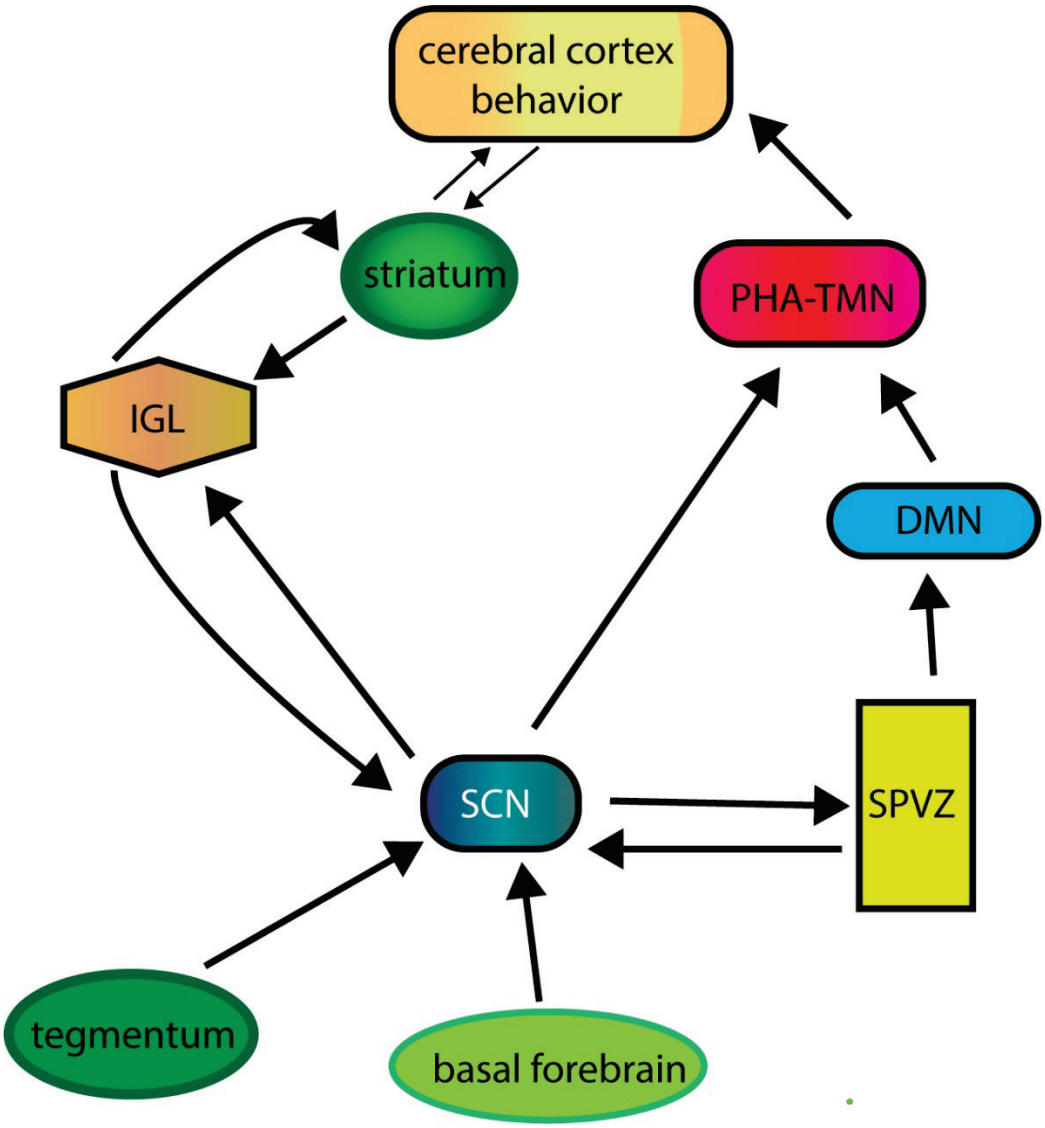

Fig. 2. Possible interactions between brain areas involved in locomotor activity biorhythm coordination. SCN – suprachiasmatic nucleus, SPVZ – subparaventricular zone, DMN – dorsomedial nucleus (hypothalamus), PHA – TMN (posterior hypothalamic area – tuberomammilar nucleus), IGL – intrageniculate leaflet (thalamus). Cerebral cortex behavior means that there are more cortex areas that determine the behavior affected by circadian rhythmicity. Please see details in the text.

It is important to stress that majority of these areas have cholinergic input or are intrinsically cholinergic and thus can be potential target for M_4 MR regulation of circadian locomotor activity.

Suprachiasmatic nucleus – main circadian pacemaker

Main circadian pacemaker is localized in hypothalamic suprachiasmatic nuclei. SCN is 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). There are species differences in the presence of cholinergic neurons in SCN among rat, hamster and mouse (Hut and Van Der Zee 2011). Thus, carbachol (i.c.v. injection) was able to bring about phase shifts but was not able to induce these effects when inject directly to SCN (Buchanan and Gillette 2005) in mice, while in hamsters the phase shifts were remarkable both after injection into ventricle or SCN (Bina and Rusak 1996). Muscarinic receptor subtype expression in SCN is still matter of debate. Initial paper used autoradiography with aim to determine the presence of MR in SCN (Bina *et al.* 1998). These authors revealed that the MR density in SCN is very low, mainly when compared to striatum. Another report indicated the presence of MR (generally) using immunohistochemistry (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 carbachol inhibitory effects (carbachol hyperpolarization) in SCN and found both M_4 and M_1 receptors are involved (Yang *et al.* 2010).

The number of studies aimed at identification of the functional role of MR subtype in SCN is limited. Carbachol induced phase advance in circadian rhythm of spontaneous neuronal activity (Gillette *et al.* 2001) what was assigned to M_1 MR. Another recent data suggest the role of M_4 MR in biorhythm regulation: the M_4 positive allosteric modulator LY2033298 enhanced oxotremorine (muscarinic agonist) inhibitory effect on light-induced phase delays but had no effect alone (Gannon and Millan, 2012). Paradoxical sleep was decreased in single M_3 KO but not in double M_2/M_4 KO mice (Goutagny *et al.* 2005).

The circadian rhythmicity, directed by suprachiasmatic nucleus (SCN), affect the rhythmicity of local pacemakers in peripheral organs, like heart, liver or pancreas (Schroeder and Colwell 2013). Each of these organ systems apparently has its own clockwork to regulate the transcription of genes that are important to the specific target organ. Recently, we have studied the heart biorhythm changes in M_2 KO animals and shown difference between light and dark phases (day and night mean, mesor and other) (Benes *et al.* 2012). It is therefore plausible that SCN could regulate circadian locomotor activity *via* similar secondary pacemakers that could be localized in CNS (see the discussion below).

Hypothalamic subparaventricular zone

Although SCN has been identified as main circadian pacemaker, the mechanism of locomotor activity circadian changes is more complicated. Locomotor activity can be also considered as non-photic entrainer of circadian rhythmicity (Hughes and Piggins 2012). There are some other brain areas that have been shown to affect the circadian rhythmicity from which the most prominent is SPVZ. Lesion of ventral part of this zone led to the loss of rest-activity circadian rhythm (Abrahamson and Moore 2006). Another study identified EGF as important molecule in this regulation (Kramer *et al.* 2001). However, projections from lower subparaventricular zone to SCN are cholinergic (Castillo-Ruiz and Nunez 2007) similarly to efferent SCN projections that are responsible for activation of arousal-promoting cells (Deurveilher and Semba 2005).

Posterior hypothalamic area

Lesion of posterior hypothalamic area (PHA) was able to decrease rest-activity circadian rhythm (Abrahamson and Moore 2006) but was not able to disrupt circadian rhythm. This area is tightly connected with tuberomammilar nuclei and is essential for waking state, what has been described in thirties and forties of 20^{th} century (Abrahamson and Moore 2006). However, studies performed in nineties have doubted these conclusions (Denoyer *et al.* 1991). Similarly, some new data reported no effect on the rest-activity rhythm after tuberomammillary nucleus ablation (Gerashchenko *et al.* 2004). As demonstrated by (Abrahamson and Moore 2006) SCN projections *via* SPVZ to PHA maintain normal level of arousal. It is therefore possible that all intact pathways are necessary for arousal coordination and that lesion of one of them has no fatal effect in rest-activity cycle.

Striatum

The only cholinergic input to striatum is provided by sparse cholinergic interneurons. Cholinergic neurons account to only 1-2 % of overall striatal neurons in rodents. However by their diverse branching and arborization impinge on large number of striatal neurons (Kawaguchi *et al.* 1995, Contant *et al.* 1996). The principal neurons of striatum accounting to 95 % are medium spiny sized neurons (MSNs). MSNs receives glutamatergic stimulation from cortex and other brain region as well as dopaminergic projections from VTA and SN (Sesack and Grace 2010). MSNs are GABAregic neurons projecting to output structures of basal ganglia (Bolam *et al.* 1984). Despite small in number cholinergic interneurons exert strong influence of striatal neuronal circuits (Kaneko *et al.* 2000, Morris *et al.* 2004, Wang *et al.* 2006). Striatal cholinergic interneurons are characterized by autonomous pacemaker activity and are referred to as tonically active neurons TANs (Bennett and Wilson 1999, Bennett *et al.* 2000) (Fig. 1).

In striatum, the most prominent site of M_4 MR expression, M_4 MR are co-localized with D_1 dopamine receptors in a subset of GABAergic projection neurons that constitute the so-called striatonigral or direct pathway (Bernard *et al.* 1992, Ince *et al.* 1997) that facilitates movement (Kravitz *et al.* 2010). Therefore mice with selective lack of M_4 MR in this neuronal population were generated, D_1-M_4-KO mice (Jeon *et al.* 2010). Similarly to whole body knockout – M_4 KO mice, D_1-M_4-KO mice showed increased psychostimulant-induced behavioral sensitization, hyperlocomotor activity and enhanced basal dopamine efflux the nucleus accumbens. In addition, at the molecular level M_4 MR inhibited D_1 DR mediated cAMP stimulation in striatum. These results suggest that a distinct subpopulation of neuronal M_4 MR is central to the dopamine-dependent behaviors (Jeon *et al.* 2010).

It has been shown that stimulation of paleostriatal structure, globus pallidus externa, is able to endorse sleep (Qiu *et al.* 2016).

Thalamus

Thalamic intergeniculate leaflet is also contributor to circadian rhythm regulation (Morin 2013). This nucleus has great level of complexity, connects abundantly throughout the brain (to approximately 100 regions) and to another thalamic nuclei: centromedian, centrolateral thalamus, and nucleus reuniens. It can provide feedback regulation (or fine tuning) of locomotor activity influencing SCN (Hughes and Piggins 2012).

Sex differences in circadian rhythms

It has been previously shown that locomotor activity in running wheel, light-dark transition test, elevated plus maze and open-field is affected by sex steroid hormones (Kuljis *et al.* 2013, Blizard *et al.* 1975, Morgan and Pfaff 2001, Ogawa *et al.* 2003). This effect on locomotor activity is mediated *via* estrogen receptor α (Ogawa *et al.* 2003). A large body of evidence has demonstrated the impact of sex hormones on central cholinergic system. Estrogens in CNS control expression and activity of several cholinergic markers such as cholineacetyltransferase (ChAT) (Gibbs 2000, Luine *et al.* 1986, McMillan *et al.* 1996), high affinity choline uptake, ACh release (Gibbs 2000, O'Malley *et al.* 1987) and acetylcholinesterase (AChE) ((Luine *et al.* 1986). Moreover morphological sex differences have been shown in cholinergic cell size (Westlind-Danielsson *et al.* 1991), in the volume of medial pre-optic area and SCN (Gorski *et al.* 1978) and in the number of MR binding sites (Fragkouli *et al.* 2006).

Moreover, at the cellular level in situ hybridization/immunocytochemistry studies showed co-localization of estrogen receptors and ChAT in the adult mouse and/or rat basal forebrain cholinergic neurons (Shughrue *et al.* 1997, Toran-Allerand *et al.* 1992).

Despite the gender differences exist in terms of locomotor activity, the vast majority of studies use mainly male rodents as it is in case of M_4 KO studies. Thus besides genetic background, gender differences in experimental conditions might contribute to contradictory results regarding the role of M_4 MR in the control of locomotion. In the initial M_4 KO study experiments were done in both sexes, showing no sex differences in terms of locomotion and thus results presented in this paper were pooled. Here, M_4 MR were assigned to play an important role in regulation of locomotor activity (Gomeza *et al.* 1999). However, other studies which used only male M_4 KO and WT mice found no significant differences in basal locomotor activity between genotypes (Fink-Jensen *et al.* 2011, Schmidt *et al.* 2011). Similarly, in the study of (Turner *et al.* 2010) no change of locomotor activity diurnal pattern was observed

between M4 KO and WT male mice.

Importantly, the sex hormones have been shown to affect M_4 MR (El-Bakri *et al.* 2002). Ovariectomy up-regulated M_4 MR in hippocampal (dentate gyrus, CA1, CA3), hypothalamic structures and in frontal cortex. Estrogen substitution led to restoration of M_4 MR initial levels. Progesterone treatment had no effect on the ovariectomy-induced up-regulation of M_4 receptors. In addition, ovariectomy decreased the exploratory (i.e. locomotor) activity of the rats that was restored by estrogen treatment.

In addition, some studies proved that circadian rhythmicity can be affected by sex hormones (Bailey and Silver 2014), that are, *per se*, also the subject of rhythmicity. In eighties, (Wollnik 1985) observed an obvious sex differences in the daily pattern of locomotion in laboratory rats. Hormonal and genetic differences between males and females also influence development of locomotor activity circadian rhythm (Diez-Noguera and Cambras 1990). Similarly, estradiol has been shown to influence the level and distribution of daily locomotor activity, the response to light pulses behavior, and the time-span of the free-running period (Blattner and Mahoney 2014). Sex hormones are able to affect response to isoproterenol in pineal glands what is probably caused by biorhythmic changes in β-adrenoceptors (Yie and Brown 1995). Circadian rhythmicity exists also in plasma and liver enzyme activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) and this rhythm can be affected by sex hormones (Alves-Amaral *et al.* 2010). While plasma and liver AChE activity showed no differences between genders and were not influenced by castration, BuChE plasma activity was higher in females and this gender difference in enzyme activity was abolished by castration. The nature of sex differences is not clear to date but hypothetically can also arise from higher androgen receptor (AR) expression in SCN in males (Bailey and Silver 2014).

Concluding remarks

M_4 MR are able to affect locomotor activity. Despite the gender differences exist in terms of locomotor activity, the vast majority of studies use mainly male rodents. However, gender differences in experimental conditions might contribute to contradictory results regarding the role of M_4 MR in the control of locomotion. Also, the locomotor activity differs in active and inactive phase of the day, suggesting the biorhythmic effects on locomotor activity. Reviewing available data, it is plausible that SCN is not the key CNS structure that is responsible for M_4 MR determined changes in locomotor activity and that these effects are caused by M_4 MR signaling changes in other brain areas. This can be supported by finding that M_4 MR are (not uniquely but together with M_1 MR) responsible for carbachol hyperpolarization in SCN (Yang *et al.* 2010)

Conflict of Interest

There is no conflict of interest.

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