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ORIGINAL ARTICLE

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The circadian clock regulates the diurnal levels of microbial short-chain fatty acids and their rhythmic effects on colon contractility in mice

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Abstract

Aim: The microbiota shows diurnal oscillations that are synchronized by the host's circadian clock and feeding rhythms. Short-chain fatty acids (SCFAs) produced by the microbiota are possible synchronizers of peripheral circadian clocks. We aimed to investigate whether faecal SCFAs show a diurnal rhythm that regulates the rhythm of SCFA receptor expression (FFAR2/3, OLFR78, HCAR2) and SCFA-induced colonic contractility. The role of the circadian clock was studied in mice lacking the core clock gene *Bmal1*.

Methods: Mice were sacrificed at 4-hour intervals. Faecal SCFA concentrations and SCFA receptor expression were determined. The effect of increasing concentrations of a SCFA mix on electrical field-induced neural responses in colon strips was measured isometrically.

Results: Diurnal fluctuations in faecal SCFA concentrations (peak 4 hours after lights on) were observed that were in phase with the rhythm of *Ffar2/3* expression in the colonic muscle layer. *Olfr78* expression was not diurnal and *Hcar2* was not detectable. The inhibitory effect of a SCFA mix on neural contractions in colonic smooth muscle strips showed a diurnal rhythm and oscillated in phase with faecal SCFA concentrations and *Ffar2/3* expression. In contrast, neither excitatory neural responses nor acetylcholine-induced smooth muscle contractions showed a diurnal rhythm. In *Bmal1*$^{-/-}$ mice, no fluctuations in faecal SCFA levels, *Ffar3* expression and neural responses to SCFAs were observed.

Conclusion: Diurnal microbial SCFA levels regulate the rhythm of *Ffar3* expression in the colonic myenteric plexus, which causes rhythmicity in SCFA-induced colonic motility. Deletion of *Bmal1* abolishes rhythmicity of SCFA levels and their downstream effects.

KEYWORDS
circadian clock, colon contractility, short-chain fatty acids

1 | INTRODUCTION

The circadian clock coordinates the timing of physiological processes with solar time. The circadian system consists of a master clock, located in the suprachiasmatic nuclei of the anterior hypothalamus, and several peripheral clocks in different organs that are synchronized by the master clock.[1]

At the molecular level, generation of circadian rhythms is dependent on the concerted co-expression of a set of core clock genes, such as *Clock* and *Bmal1*, non-redundant and essential components of the circadian clock system.[2,3] The clock components form several transcription-translation feedback loops, through which they not only regulate their own expression but also that of numerous downstream clock-controlled genes and clock genes, such as *Rorα* (activator) and *Reverbα* (repressor). Their transcription is driven by BMAL1 but in turn they activate and repress *Bmal1* transcription.[1]

The gastrointestinal tract contains a powerful circadian clock, which can be uncoupled from the master clock by altering feeding time or diet.[4–6] This desynchronization caused by, for example, rotating shift work and frequent time zone travelling likely favours the development of diseases such as metabolic syndrome and neurological or intestinal disorders.[7–11]

Recent studies show that the intestinal microbiota show diurnal oscillations that are synchronized by the host's circadian clock and feeding rhythms.[12–15] In addition, the faecal concentrations of microbial metabolites such as the short-chain fatty acids (SCFAs) acetate, propionate and butyrate display diurnal fluctuations, which were abolished in mice with a disrupted circadian clock and in mice fed a high-fat diet.[15,16] In turn, SCFAs could modulate clock gene expression in peripheral tissues (eg, the liver) after timed oral administration, which identifies SCFAs as possible *zeitgebers* (ZTs) or synchronizers of peripheral circadian clocks.[15,16]

Studies in rodents have shown that SCFAs can influence colon contractility, although there is some controversy whether their effects were direct or indirect, mediated via serotonin released from the mucosa.[17–20] In contrast, intracolonic or -caecal infusions of SCFAs in humans had no effect on colonic motility, although infusions in the terminal ileum stimulated the occurrence of ileal propulsive contractions.[21–24]

Short-chain fatty acids activate several receptors with distinct ligand specificities: free fatty acid receptor 2 and 3 (FFAR2 and FFAR3, also known as GPR43 and GPR41, respectively), hydroxycarboxylic acid receptor 2 (HCAR2) and olfactory receptor 78 (OLFR78). All receptors are present in the gut,[25–27] but especially FFAR3 and OLFR78 were expressed on neurons from the myenteric plexus.[25,28]

Furthermore, *Ffar3* is a clock-controlled gene, since *Ffar3* mRNA expression in ileal epithelial cells of mice was found to be regulated by competitive binding of the clock components RORα and REVERBα to its ROR response element, leading to diurnal fluctuations in *Ffar3* mRNA expression. In addition, *Ffar3* mRNA expression in ileal epithelial cells was decreased in antibiotic-induced microbiota-depleted mice. Taken together, these experiments revealed that FFAR3 expression might be regulated by both the circadian clock and the microbiota.[29] Finally, *Olfr78* mRNA expression has also been shown to display diurnal fluctuations in the distal colon of mice.[30]

This study aimed to investigate whether microbial SCFA levels show a diurnal rhythm that is in phase with the rhythm of SCFA receptor expression (*Ffar3*, *Ffar2*, *Olfr78* and *Hcar2*) and SCFA-induced changes in contractility in mouse colonic smooth muscle tissue. The role of the circadian clock in these 24-hour fluctuations was studied using *Bmal1*$^{-/-}$ mice.

2 | RESULTS

2.1 | Diurnal effects of short-chain fatty acids on colon contractility

2.1.1 | Faecal SCFA concentrations in the distal colon show diurnal rhythmicity

Short-chain fatty acid concentrations were measured in faecal pellets present in the distal colon of C57BL/6J mice, sacrificed over the course of 24 hours (4 hours interval). The average faecal acetate concentration (36.90 ± 1.15 mmol/L) was 10-fold higher compared to faecal propionate (3.78 ± 0.22 mmol/L) and butyrate concentrations (4.40 ± 0.56 mmol/L). Acetate, propionate and butyrate levels all showed significant diurnal rhythmicity (acrophase ZT 6h04 [$P_{\text{cosinor}} < 0.01$], ZT 7h28 [$P_{\text{cosinor}} < 0.05$], ZT 5h19 [$P_{\text{cosinor}} < 0.001$] respectively) (Figure 1).

2.1.2 | SCFA receptor expression in the smooth muscle layer of the distal colon shows diurnal rhythmicity

The SCFA receptors *Ffar3*, *Ffar2* and *Olfr78* were all expressed in the smooth muscle layer of the distal colon of C57BL/6J mice, although *Ffar2* expression was very low and above threshold value in only 13 of 48 mice tested (Figure 2A). *Hcar2* mRNA expression was undetectable. *Ffar3* and *Ffar2* mRNA expression showed a diurnal rhythm ($P_{\text{cosinor}} < 0.05$), peaking at ZT 3h25 and ZT 4h10 respectively (Figure 2B,C). *Olfr78* mRNA expression did not show diurnal rhythmicity (Figure 2D).

Similar to the distal colon, *Ffar3*, *Ffar2* and *Olfr78* were all expressed in the smooth muscle layer of the proximal colon of C57BL/6J mice, *Ffar2* expression was very low (above threshold value in 22 of 48 mice tested) and *Hcar2* mRNA expression was undetectable (Supporting Information Figure S1A). *Ffar3* mRNA expression showed a diurnal rhythm ($P_{\text{cosinor}} < 0.05$) peaking at ZT 3h36, similar to the distal colon (Supporting Information Figure S1B). In contrast to the distal colon, *Ffar2* mRNA expression did

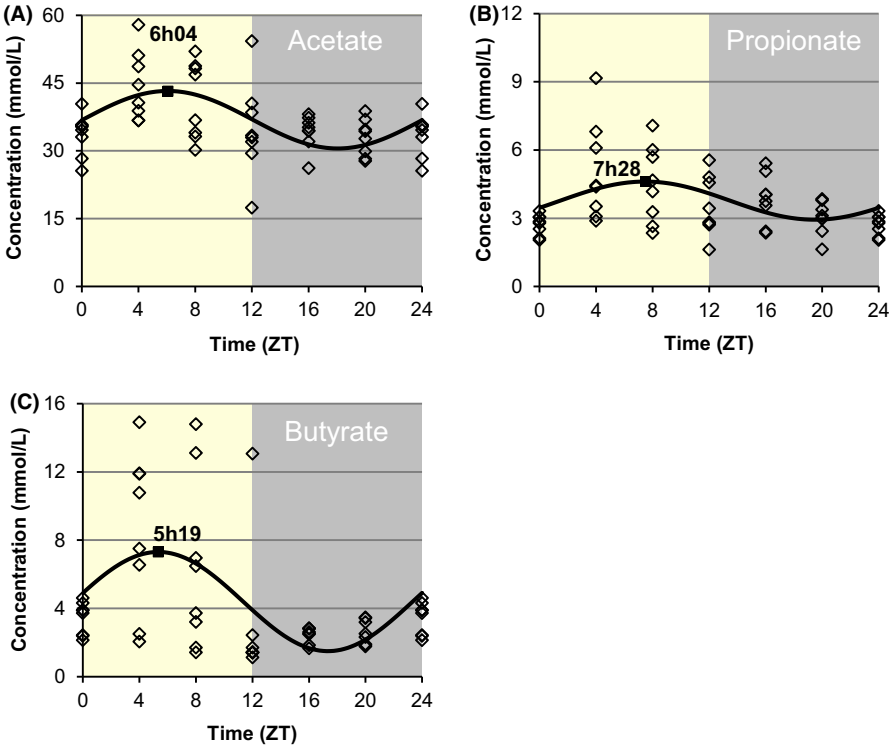

FIGURE 1 Faecal acetate, propionate and butyrate concentrations in the distal colon of C57BL/6J mice (N = 8 mice per time point). The fitted cosine curve and acrophase determined by cosinor analysis (period = 24 h) are shown. Light and dark phases are shaded in yellow and gray respectively

not show diurnal rhythmicity, while *Olfr78* mRNA expression showed a diurnal rhythm ($P_{cosinor} < 0.05$) peaking at ZT 15h39 (Supporting Information Figure S1C,D).

2.1.3 | Inhibition of neural contractions by SCFAs in the colon shows diurnal rhythmicity

Addition of L-NAME and MRS 2500 generated stable on-contractions, representing cholinergic excitatory responses since they were blocked by atropine (Figure 3A).

The in vitro effect of SCFAs on neural cholinergic excitatory responses elicited by electrical field stimulation (EFS) was investigated over the course of 24 hours at 4-hour intervals, in smooth muscle strips from the distal colon. A representative tracing of the inhibitory effect of the SCFA mix on the EFS-induced cholinergic responses is shown in Figure 3B. The inhibitory effect of the SCFA mix showed diurnal rhythmicity ($P_{cosinor} < 0.01$) at all concentrations (1-100 mmol/L) tested and peaked between ZT 3 and ZT 4 (Supporting Information Figure S2A-D; Figure 3C). The inhibition was concentration dependent, with an EC_{50} of 83.5 ± 5.0 mmol/L at ZT 4 (Figure 3D), and a maximal inhibition of 64% ± 4% at 100 mmol/L of the SCFA mix. Contractile responses to 0.1 mmol/L acetylcholine as well as the EFS-induced excitatory responses in the absence of SCFA mix did not show diurnal rhythmicity (Figure 3E,F).

In the proximal colon, similar results were obtained. The SCFA mix reduced the EFS-induced response in a concentration dependent (ZT 4: EC_{50} = 80.8 ± 6.9 mmol/L; maximal inhibition by 100 mmol/L SCFA mix = 69% ± 7%) and rhythmic manner ($P_{cosinor} < 0.05$, Supporting Information Figure S3A-D). Contractile responses to acetylcholine, as well as EFS-induced excitatory responses in the absence of SCFA mix showed no diurnal rhythmicity (Supporting Information Figure S3E,F).

2.1.4 | Faecal SCFA concentrations, *Ffar3* mRNA expression and neural contractile effects of SCFAs fluctuate in phase

To investigate whether faecal SCFA concentrations, *Ffar2* and *Ffar3* mRNA expression and the inhibitory effect of the SCFA mix on neural contractions in the distal colon of C57BL/6J mice fluctuated in phase, their acrophases were compared.

The acrophases of faecal acetate and butyrate concentrations, *Ffar2* and *Ffar3* mRNA expression and the inhibitory effect of the SCFA mix on neural contractions were not significantly different, indicating that they fluctuated in phase (Figure 4A).

In the proximal colon of C57BL/6J mice, faecal acetate and butyrate concentrations, *Ffar3* mRNA expression and the inhibitory effect of the SCFA mix on neural contractions also fluctuated in phase (Figure 4B). *Olfr78* mRNA expression peaked when faecal acetate and butyrate concentrations, *Ffar3* mRNA expression and the inhibitory effect of the SCFA mix on neural contractions were minimal (Bathyphase *Olfr78* not significantly different compared to their acrophases).

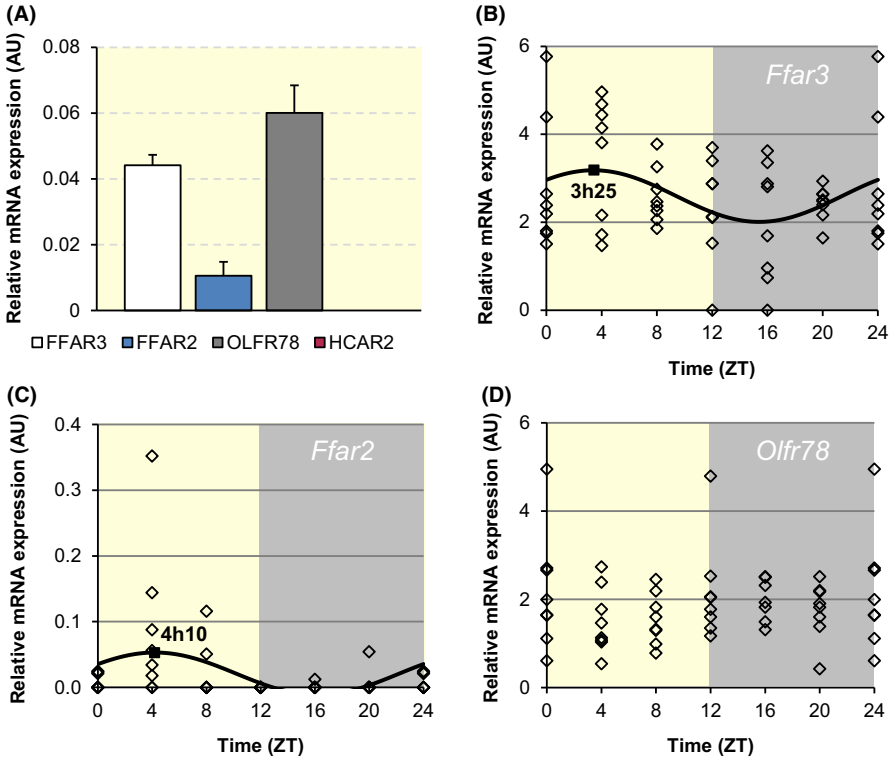

FIGURE 2 A, Average of *Ffar3*, *Ffar2*, *Olfr78* and *Hcar2* mRNA expression for all ZT in the smooth muscle of the distal colon of C57BL/6J mice (N = 48 mice). B-D, *Ffar3*, *Ffar2* and *Olfr78* mRNA expression in the smooth muscle of the distal colon of C57BL/6J mice (N = 8 mice per time point). The fitted cosine curve and acrophase determined by cosinor analysis (period = 24 h) are shown. Light and dark phases are shaded in yellow and gray respectively. AU, arbitrary unit

2.1.5 | A circadian clock system is present in the smooth muscle layer of the distal colon

To investigate whether a circadian clock system was present in the smooth muscle layer of the distal colon of C57BL/6J mice, the diurnal fluctuations in *Bmal1* mRNA expression were measured. *Bmal1* mRNA expression showed a significant ($P_{cosinor} < 0.001$) diurnal rhythm (acrophase ZT 1h59), peaking in phase with *Ffar3* mRNA expression (Figure 5A).

The mRNA expression of *Pparα*, a known integrator of microbial and circadian clock signals was measured as well.[29] The mRNA expression of *Pparα* showed a significant ($P_{cosinor} < 0.01$) diurnal rhythm (acrophase ZT 18h41), peaking when *Ffar3* mRNA expression was minimal (acrophase and bathyphase not significantly different) (Figure 5B). Deletion of *Bmal1* abolished rhythmicity in *Pparα* mRNA expression (Figure 5C).

2.2 | Role of the circadian clock in the diurnal effects of short-chain fatty acids on colon contractility

2.2.1 | *Diurnal fluctuations in faecal SCFA concentrations are no longer present in Bmal1$^{-/-}$ mice*

To investigate the role of the circadian clock on the parameters investigated, rhythmic changes were investigated in mice knockout for the core clock gene *Bmal1* and their wild type (WT) littermates at ZT 4 and 16.

Parallel to C57BL/6J mice, faecal SCFA concentrations in the distal colon of WT littermates were significantly ($P < 0.001$, two-way analysis of variance—planned comparisons) higher at ZT 4 compared to ZT 16. In *Bmal1$^{-/-}$* mice, SCFA concentrations were lower ($P < 0.01$) at ZT 4 compared to their WT littermates (Figure 6A). As a result, no significant difference between faecal SCFA concentrations at ZT 4 and 16 was observed in *Bmal1$^{-/-}$* mice.

2.2.2 | *Ffar3 mRNA expression is lower in Bmal1$^{-/-}$ mice*

Ffar3 mRNA expression in the smooth muscle of the distal colon of WT littermates tended to be higher at ZT 4 compared to ZT 16 but not in *Bmal1$^{-/-}$* mice. At ZT 4, *Ffar3* mRNA expression was lower in *Bmal1$^{-/-}$* mice compared to their WT littermates (Figure 6B).

2.2.3 | *Diurnal fluctuations in neural responses to SCFAs are no longer present in Bmal1$^{-/-}$ mice*

Short-chain fatty acids reduced EFS-induced neural excitatory responses in a concentration-dependent manner at ZT 4 in WT (EC_{50}: 142.8 ± 13.0 mmol/L) and *Bmal1$^{-/-}$* mice (EC_{50} 137.9 ± 4.5 mmol/L) (Figure 6C,D). Parallel to C57BL/6J mice, the inhibitory effect of the SCFA mix

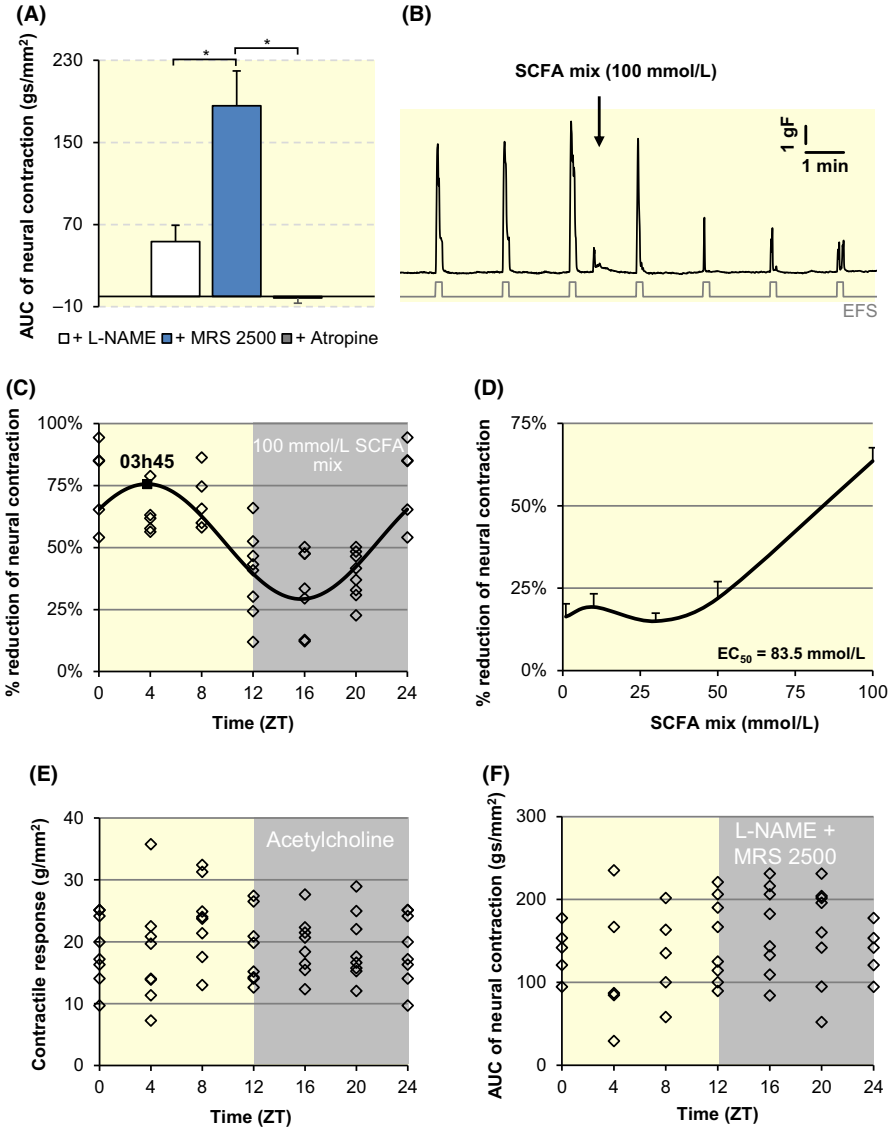

FIGURE 3 A, Effect of cumulative addition of 0.3 mmol/L L-NAME, 1 μmol/L MRS 2500 and 5 μmol/L atropine on the EFS-induced on-contraction of distal colonic smooth muscle strips of C57BL/6J mice at ZT 1h30. Repeated measures analysis of variance, followed by planned comparisons was used to compare the effect of L-NAME, MRS 2500 and atropine. $*P < 0.05$ (N = 8 mice, n = 10 strips). B, Representative tracing of the inhibitory effect of a SCFA mix (100 mmol/L) on the EFS-induced cholinergic response in distal colonic smooth muscle strips. C, Inhibitory effect of the SCFA mix (100 mmol/L) on the EFS-induced response in the distal colon of C57BL/6J mice (N = 5-8 mice, n = 6-12 strips per time point). The fitted cosine curve and acrophase determined by cosinor analysis (period = 24 h) are shown. D, Concentration-dependent inhibitory effect of the SCFA mix on the EFS-induced response at ZT 4 in the distal colon of C57BL/6J mice (N = 5 mice, n = 8 strips). E, Contractile response to acetylcholine (0.1 mmol/L) in distal colonic smooth muscle strips of C57BL/6J mice (N = 8 mice, n = 22-24 strips per time point). F, Cholinergic excitatory neural response, studied in the presence of L-NAME (0.3 mmol/L) and MRS 2500 (1 μmol/L), in distal colonic smooth muscle strips of C57BL/6J mice (N = 5-8 mice, n = 6-12 strips per time point). Light and dark phases are shaded in yellow and gray respectively. AUC, area under the curve

(100 mmol/L) on the EFS-induced contraction was significantly higher at ZT 4 compared to ZT 16 in WT littermates ($P < 0.001$, two-way analysis of variance—planned comparisons) but not in *Bmal1*$^{-/-}$ mice (Figure 6E).

Overall, the potency of SCFAs seemed to be lower in WT littermates compared to C57BL/6J mice (EC_{50} of 142.8 ± 13.0 mmol/L vs EC_{50} of 83.5 ± 5.0 mmol/L, at ZT 4), resulting in a maximal inhibition of the neural contractions of 33% ± 3% in WT littermates, compared to 64% ± 4% in C57BL/6J mice (effect of 100 mmol/L SCFA mix).

3 | DISCUSSION

The dynamic crosstalk between microbiota and the host is important for achieving and maintaining homoeostasis.[31]

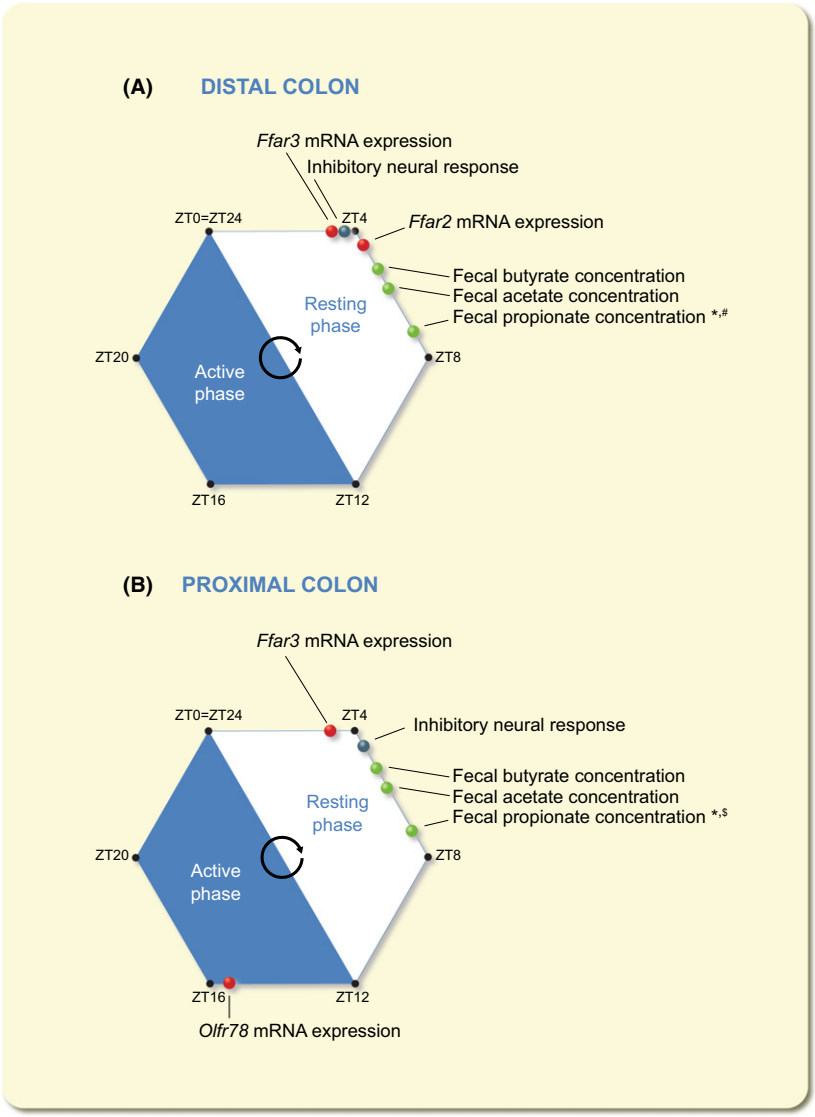

FIGURE 4 Comparison of acrophases of faecal acetate, propionate and butyrate concentrations, *Ffar2*, *Ffar3* and *Olfr78* mRNA expression and the inhibitory effect of the SCFA mix on neural contractions in the distal and proximal colon of C57BL/6J mice. Acrophases were calculated by cosinor analysis (period = 24 h). Acrophases and bathyphases were compared using non-linear regression analysis. $^{*}P < 0.05$ vs acrophase of *Ffar3* mRNA expression, $^{\#}P < 0.05$ vs acrophase of the inhibitory neural response induced by the SCFA mix. $^{\$}P < 0.05$ vs bathyphase of *Olfr78* mRNA expression. Light and dark phases are shaded in white and blue respectively

Recent evidence suggests that a bidirectional interaction between the microbiota and the host's circadian clock plays an important part in maintaining circadian rhythmicity. The host's circadian clock generates diurnal oscillations in composition and function of the microbiota that are abolished in mice with a disrupted circadian clock.[12,13] In turn, signals from the microbiota are able to regulate the circadian clock system of the host.[29] Indeed, SCFAs were identified as possible synchronizers of peripheral circadian clocks.[15,16] Therefore, SCFAs might be important in the bidirectional interaction between microbiota and their host's circadian clock system, maintaining homoeostasis and synchronizing the circadian clock system.

In this study, we showed that the concentration-dependent inhibitory effect of SCFAs on cholinergic excitatory responses in the colon displays diurnal rhythmicity, peaking around ZT 4. Interestingly, this rhythm was in phase with rhythmic *Ffar3* mRNA expression and faecal SCFA concentrations in the colon.

Ffar3 expression has been demonstrated in cholinergic and nitrergic neurons of the myenteric plexus of the rat colon and in the myenteric plexus of the mouse small intestine.[25,32] The inhibitory effect of SCFAs was observed in the presence of L-NAME and MRS 2500, indicating that it is not mediated via the release of nitric oxide (NO) or purines acting on the $P2Y_1$ receptor. Our findings therefore suggest a direct FFAR3-mediated suppression of cholinergic activity. Other neurotransmitters such as vasoactive intestinal peptide (VIP) might however still be involved, since FFAR3 has been shown to colocalize with VIP in submucosal ganglia of the mouse small intestine.[25] Since mucosa-free muscle strips were used, it can be excluded that the effect is mediated via mucosal release of serotonin or PYY, which have been shown to possibly influence SCFA-mediated colon contractility.[19,33,34]

Ffar2 mRNA expression was very low, so it is unlikely that the observed effect is mediated via FFAR2 activation, which is in line with the findings of Dass et al,[18] who

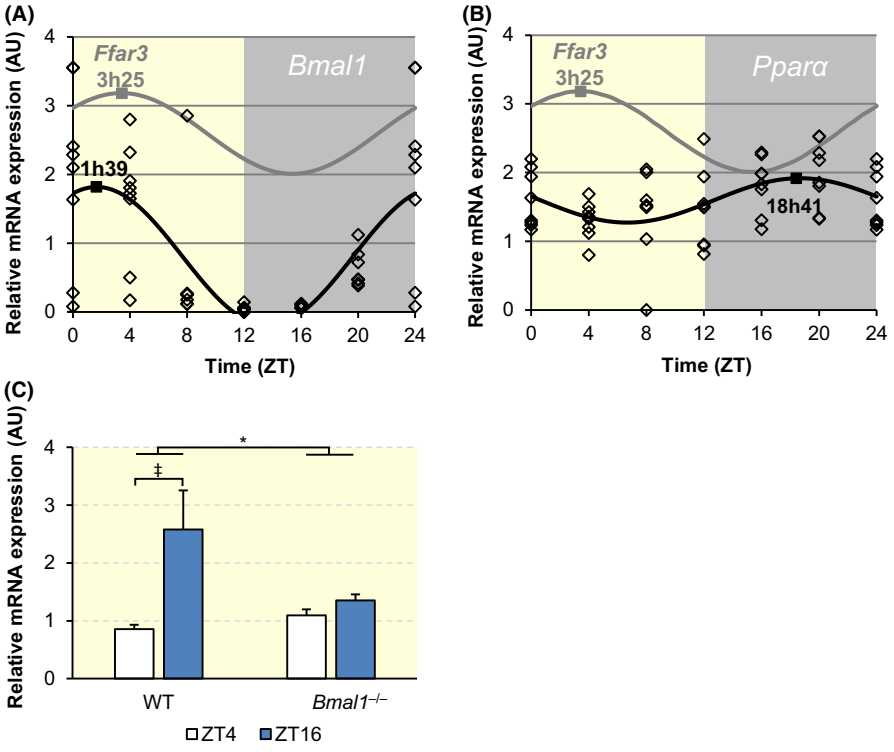

FIGURE 5 A, B, *Bmal1* and *Pparα* mRNA expression in the smooth muscle of the distal colon of C57BL/6J mice (N = 8 mice per time point), compared to *Ffar3* mRNA expression shown in gray. The fitted cosine curve and acrophase determined by cosinor analysis (period = 24 h) are shown. C, *Pparα* mRNA expression in the smooth muscle of the distal colon of WT and *Bmal1*$^{-/-}$ mice at ZT 4 and 16 (N = 5-7 mice per time point and genotype). Two-way analysis of variance was used to compare different ZTs and genotypes, followed by planned comparisons. *$P < 0.05$, ‡$P < 0.001$. AU, arbitrary unit

observed a similar inhibition of neuronal colonic contractile responses by SCFAs in both *Ffar2*$^{-/-}$ and WT mice.

Olfr78 was expressed in the smooth muscle layer of the distal colon, but showed no diurnal fluctuations. Interestingly, *Olfr78* mRNA expression did show diurnal fluctuations in the proximal colon, peaking when faecal SCFA concentrations and *Ffar3* mRNA expression were minimal. OLFR78 and FFAR3 have previously been shown to cooperate in small resistance vessels to maintain blood pressure. Activation of FFAR3 by low concentrations of propionate induced a hypotensive response, while activation of OLFR78, which is activated at higher concentrations of propionate, induced a hypertensive response.[28] It would be interesting to investigate whether FFAR3 and OLFR78 show a similar interplay in the diurnal regulation of colon contractility.

Colonic contractile responses and faecal output have been shown to display diurnal fluctuations.[35,36] However, we neither found rhythmicity in excitatory neural responses nor in acetylcholine-induced smooth muscle contractions. The observed diurnal inhibitory effect of SCFAs is therefore not caused by rhythmic changes in muscarinic receptor expression or diurnal release of excitatory neurotransmitters.

Our findings confirm the presence of a circadian clock system in the smooth muscle layer of the distal colon, since expression of the core clock gene *Bmal1* showed clear diurnal rhythmicity. We showed that genetic deletion of *Bmal1* abolished rhythmicity of microbial SCFA levels, *Ffar3* mRNA expression and the inhibitory effect of SCFAs on cholinergic excitatory responses, demonstrating that an intact circadian clock system is necessary to maintain rhythmicity in faecal SCFA concentrations and their functional effects.

In addition, the mRNA expression of *Pparα*, a known integrator of signals of the circadian clock and the microbiota, also showed a diurnal rhythm that was abolished in *Bmal1*$^{-/-}$ mice. Since *Pparα* mRNA expression was minimal at the peak of *Ffar3* mRNA expression, it would be interesting to investigate whether SCFAs produced by the microbiota inhibit *Pparα* mRNA expression through the activation of FFAR3. In turn, changes in PPARα might regulate *Ffar3* expression by affecting the competitive binding of the clock components RORα and REVERBα for which the *Ffar3* promotor has a binding site.[29] However, this is only one of the putative hypotheses and other clock genes or integrators of the clock and the microbiota might be involved as well.

Deletion of *Bmal1,* a non-redundant and essential circadian clock component, not only disrupts the host's circadian clock, but also disrupts the diurnal rhythm in food intake present in WT mice (peak between ZT 16-20).[13,37] Therefore, it remains to be determined whether disrupted oscillations in faecal microbiota[13] and the disrupted rhythmicity of microbial SCFA levels in *Bmal1*$^{-/-}$ mice is the result of the altered feeding pattern due to clock disruption. Restricted feeding of arrhythmic *Per1/2*$^{-/-}$ mice in the dark or light phase restored diurnal patterns in microbiota.[12] However, mice subjected to constant intravenous parenteral nutrition still showed diurnal fluctuations in microbiota,

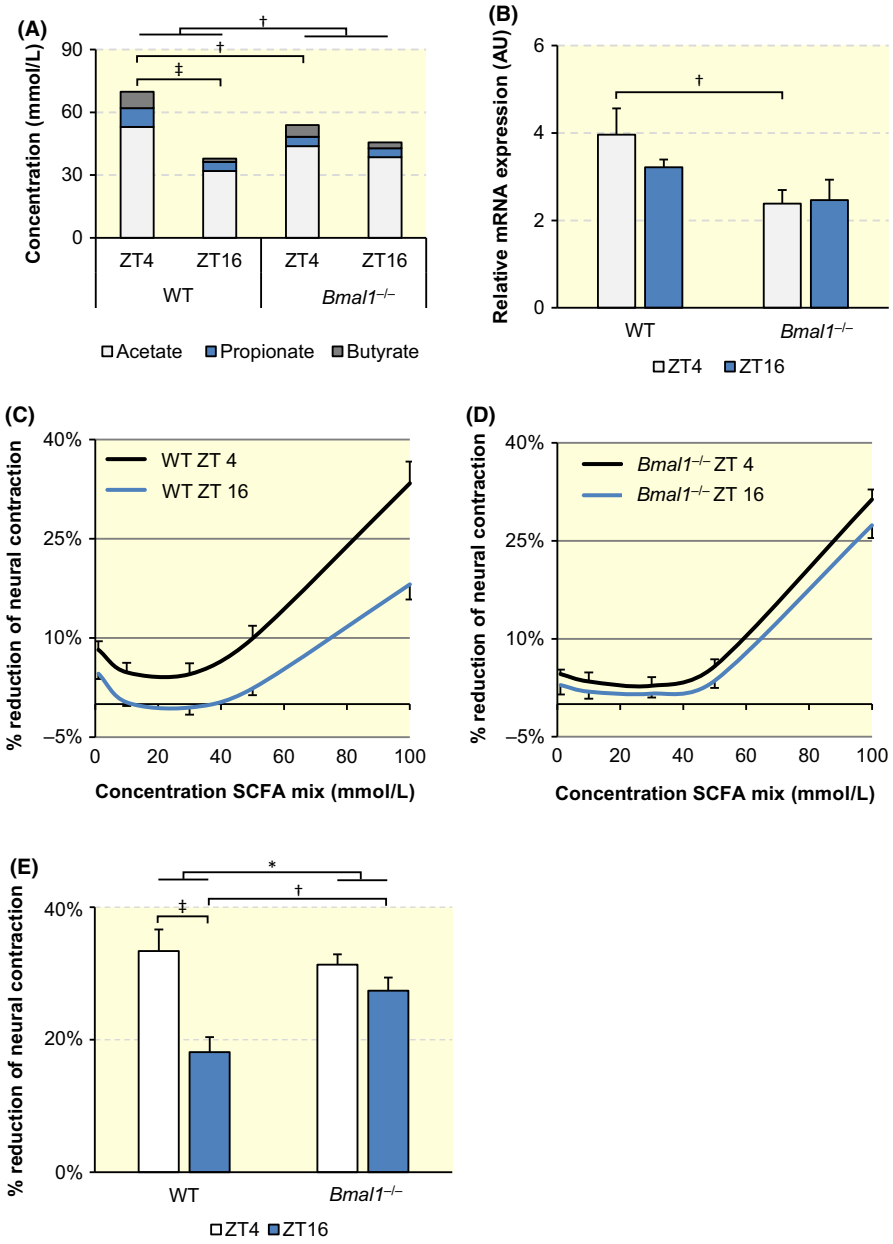

FIGURE 6 A, Faecal acetate, propionate and butyrate concentrations in the distal colon of WT and *Bmal1*$^{-/-}$ mice at ZT 4 and 16 (N = 6-7 mice per time point and genotype). B, *Ffar3* mRNA expression in the smooth muscle of the distal colon of WT and *Bmal1*$^{-/-}$ mice at ZT 4 and 16 (N = 4-7 mice per time point and genotype). C, D, Concentration-dependent inhibitory effect of the SCFA mix on the EFS-induced cholinergic excitatory response of circular smooth muscle strips of the distal colon at ZT 4 and 16 in WT and *Bmal1*$^{-/-}$ mice (N = 6-8 mice, n = 17-24 strips per time point and genotype). E, Inhibitory effect of 100 mmol/L of the SCFA mix on the EFS-induced response of circular smooth muscle strips of the distal colon at ZT 4 and 16 in WT and *Bmal1*$^{-/-}$ mice (N = 6-8 mice, n = 17-24 strips per time point and genotype). Two-way analysis of variance was used to compare different ZTs and genotypes, followed by planned comparisons. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$

despite significant changes in microbial community composition.[15] Hence, it seems likely that a combination of both the circadian clock and feeding time regulate the rhythm in microbiota and possibly in microbial SCFA levels.

Although several animal studies confirm that SCFAs can influence colon contractility,[17–20] SCFAs were shown to have no effect on colon motility in humans.[21–23] However, all studies were performed during the day, the active feeding phase in humans. Our findings showed that in mice, faecal SCFA concentrations and the effects of SCFAs on colon contractility were maximal during the day, the resting phase in mice. It seems plausible that in humans effects would also be maximal during their resting phase, and therefore minimal during the day. Further studies should therefore be aware of possible time-dependent effects of SCFAs and should consider testing at a time point when faecal SCFA concentrations are expected to be high, since SCFA receptor expression might only be upregulated during this time period.

It remains to be determined whether SCFA receptors expressed at multiple sites in the body such as white adipose tissue, immune cells and enteroendocrine cells show diurnal fluctuations.[38] Furthermore, our study raises the question whether circadian clock disruption by, for example, shift work or frequent flying, disrupt rhythms in SCFA receptor expression, as we observed in *Bmal1*$^{-/-}$ mice, and might be responsible for some of the symptoms observed in this population.[7–11]

In conclusion, this study shows that inhibition of colonic motility by SCFAs shows a clear diurnal rhythm

that peaks during the resting phase, possibly regulated by the diurnal levels of microbial SCFAs that in turn coordinate the rhythm of *Ffar3* expression in the myenteric plexus of the colon. Disruption of the circadian clock system by deletion of the core clock gene *Bmal1* abolishes rhythmicity of microbial SCFA levels and their downstream effects. It remains to be determined whether this is due to direct or indirect effects via disruption of feeding rhythms by the disturbed circadian clock.

4 | MATERIAL AND METHODS

4.1 | Materials

L-NAME hydrochloride and MRS 2500 were obtained from Tocris (Abingdon, UK). Acetylcholine, atropine, sodium acetate, sodium propionate and sodium butyrate were purchased from Sigma Aldrich (St. Louis, MO). SCFA mixtures were freshly prepared in Krebs buffer and osmolarity was adjusted to 285 mOsm/L by modifying NaCl concentrations.

4.2 | Mice

Wild type C57BL/6J mice were purchased from Janvier Labs (Le Genest Saint Isle, France). *Bmal1*$^{+/-}$ mice (gift R. Lijnen, KU Leuven, Leuven, Belgium)[39] were bred to generate WT and *Bmal1*$^{-/-}$ mice in the animal facility of the KU Leuven and genotyped by PCR on total genomic DNA from the ear. Mice were housed in a temperature-controlled environment under a 12-hour/12-hour light/dark-cycle (where ZT 0 is lights on by convention) and had ad libitum access to chow and drinking water. All experiments were approved by the Ethical committee for Animal Experiments of the KU Leuven and carried out in accordance with the approved guidelines.

4.3 | Experimental design

C57BL/6J mice (male, age 12-15 weeks) were sacrificed over the course of 24 hours at 4-hour intervals, unless otherwise stated. *Bmal1*$^{-/-}$ and their WT littermates (male, age 12-15 weeks) were sacrificed at ZT 4 and 16. The faeces from the distal colon was collected and processed for SCFA quantification. The colon was divided into proximal and distal colon and the mucosa was removed, after which 3 muscle strips were cut along the circular axis of the proximal and distal colon for contractility studies. The remaining smooth muscle tissue was stored in RNAlater (Qiagen, Hilden, Germany) and processed for quantitative real-time PCR (qRT-PCR).

4.4 | Analysis of total SCFA concentrations in faecal samples

Faecal samples (100 mg) were suspended in 1 mL of saturated NaCl (36%) solution. An internal standard (50 μL 2-ethylbutyric acid) was added and the samples were homogenized using glass beads. SCFAs were extracted with ether (3 mL) in the presence of H_2SO_4 (150 μL). The ether layer was collected and dried by Na_2SO_4 (50 mg). Analysis was done by gas chromatography-flame ionization detector (Agilent, Santa Clara, CA), with an injection volume of 0.5 μL. The resulting chromatograms were processed using the Xcalibur software (Thermo Fischer Scientific, Waltham, MA).

4.5 | Quantitative real-time PCR

Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen) and reverse transcribed to cDNA using

TABLE 1 Primers used in qRT-PCR

Gene	Forward primer	Reverse primer
β-actin	GATCTGGCACCACACCTTCTAC	TGGATGGCTACGTACATGGCTG
Bmal1	CGTTTCTCGACACGCAATAGAT	TCCTGTGGTAGATACGCCAAAA
Ffar2	CCCTGTGCACATCCTCCTGC	GCGTTCCATGCTGATGCCCG
Ffar3	TGTCCAATACTCTGCATCTGT	AGGTCCGAAATGGTCAGGTT
Hcar2	GCACAACCAGAAGTATTCCAG	CCAAATCGCCTCTCCAG
Hmbs	CTGAAGGATGTGCCTACCATAC	AAGGTTTCCAGGGTCTTTCC
Olfr78	GCTAAGACCAAACAGATCAGAAC	GGTGATAGGATGGTAAGGGTC
Pparα	CTTCCCAAAGCTCCTTCAAAAA	CTGCGCATGCTCCGTG
Ppib	GGAGATGGCACAGGAGGAAA	CCCGTAGTGCTTCAGCTTGAA
Reverbα	CCCTGGACTCCAATAACAACACA	GCCATTGGAGCTGTCACTGTAG
Rorα	GAGGTATCTCAGTCACGAAG	AACAGTTCTTCTGACGAGGACAGG
Tbp	AGGATGCTCTAGGGAAGAT	TGAATAGGCTGTGGAGTAAGT

SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed using the Lightcycler 480 (Roche Diagnostics, Basel, Switzerland) with the Lightcycler 480 Sybr Green I Master mix (Roche Diagnostics). Primer sequences are shown in Table 1. Results were expressed relative to the geometric mean of the normalized expression[40] of three stable housekeeping genes (*Hmbs*, *Ppib* and *Tbp* or *β-actin*), that did not show diurnal rhythms in expression levels. To compare expression levels of different genes, $2^{-\Delta Ct}$ values were used. To compare time-dependent changes in expression levels of one gene, $2^{-\Delta\Delta Ct}$ values were used.

4.6 | In vitro contractility studies

Proximal and distal colonic smooth muscle strips freed from mucosa (width: 2 mm, length: colon circumference) were suspended along their circular axis in tissue baths filled with Krebs buffer equilibrated with 5% CO_2/95% O_2. Contractile responses were measured using an isometric force transducer/amplifier (Harvard Apparatus, South Natick, MA) and analysed using the Windaq data acquisition system and a DI-2000 PGH card (Dataq Instruments, Akron, OH). After 30 minutes equilibration at optimal stretch (0.25 g), strips were calibrated by inducing a maximal contraction with 0.1 mmol/L acetylcholine. The tension of the strips was adjusted to ¼ of the maximal contractile response. This calibration step was repeated 3 times. Next, neural responses were elicited by EFS applied via two parallel platinum rod electrodes using a Grass S88 stimulator (pulse duration 0.5 ms, train duration 10 seconds, amplitude 10 V, frequency 8 Hz). Each consecutive pulse train was followed by a 90 seconds interval.

The EFS-induced responses were pharmacologically characterized by adding a NO synthase inhibitor (0.3 mmol/L L-NAME hydrochloride), $P2Y_1$ receptor antagonist (1 μmol/L MRS 2500) and muscarinic acetylcholine receptor antagonist (5 μmol/L atropine) cumulatively to the tissue baths. The tension (gF) for all responses was corrected for the weight (g) and cross-sectional area of the strip (mm^2) and the average of the area under the curve (AUC) for 12 consecutive on-contractions was calculated.

The effect of SCFAs on neural responses was studied in the presence of 0.3 mmol/L L-NAME and 1 μmol/L MRS 2500, which generated stable excitatory on-contractions. Increasing concentrations (1-100 mmol/L) of a SCFA mix (acetate, propionate and butyrate with a molar ratio of 3:1:1) were successively added to the tissue bath every 10 minutes. The average AUC for six consecutive on-contractions was calculated and expressed relative to the average AUC of the on-contractions before addition of SCFAs.

4.7 | Statistical analysis

Results are presented as mean ± SEM. Diurnal rhythm analysis was performed using the free Cosinor software (version 3.1, R. Refinetti, Boise State University, Boise, ID, USA), which calculates the best-fitting cosine curve for a data set using the cosinor procedure as described by Nelson et al.[41] Probability values for the best fitting cosine curve are indicated as $P_{cosinor}$. A fixed period of 24 hours was chosen for all parameters, to allow phase comparisons between parameters. Acrophases and bathyphases (the time point where the fitted cosine curve reaches its maximum or minimum, respectively) of different data sets were compared using non-linear regression analysis (SAS Studio University Edition 9.4, SAS Institute Inc., Cary, NC, USA). Data used for comparisons between ZT 4 and 16 were assessed for normality of distribution. Since data obtained in WT and *Bmal1*$^{-/-}$ mice were distributed in a non-normal and/or non-homogeneous manner, log-transformed data were used. Two-way analysis of variance was used to compare different ZTs and genotypes, followed by planned comparisons (Statistica 13; Statsoft, TIBCO Software Inc., Palo Alto, CA, USA). Repeated measures analysis of variance, followed by planned comparisons was used to compare the effects of antagonists for neurotransmitters on the on-contractions (Statistica 13). Significance was accepted at the 5% level.

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CONFLICT OF INTEREST

None declared.

AUTHORS' CONTRIBUTION

AS, KV, JT and ID conceived and designed the experiments. AS, LD and TT performed the experiments. AS, LD, TT and ID analysed the results. AS and ID wrote the manuscript. All authors reviewed the manuscript.

ETHICAL APPROVAL

Ethical Committee for Animal Experimentation of the KU Leuven.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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