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Circadian asthma airway responses are gated by REV-ERBα

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Abstract

Background—Asthma is an inflammatory disease of the airway showing a strong time of day rhythm. Airway hyperresponsiveness is a dominant feature of asthma, but it is not known if this is under clock control. The circadian clock powerfully regulates inflammation. The clock protein REV-ERBα is known to play a key role as a repressor of the inflammatory response.

Objectives—To determine if allergy mediated airway hyperresponsiveness is gated by the clock protein, REV-ERBα.

Methods—After exposure to the intra-nasal house dust mite allergen challenge model at either dawn or dusk, airway hyper-responsiveness to methacholine was measured invasively in mice.

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Author's Contributions:

HJD conceived of the study, secured the funding, ran the study, analysed the results and prepared the manuscript.
KK performed Bioplex, PCR, histology, cell counts and lung slice experiments.
PM performed lung slice experiments.
NB performed Flexivent measurements and collected samples.
LG and CML advised on house dust mite model and FlexiVent measurements and helped to prepare the manuscript.
RJM ran statistical analysis and advised on the statistics used and prepared the manuscript.
ASIL, JG and JFB prepared the manuscript. JFB also advised on lung slice experiments.
DWR conceived the study, analysed the results and prepared the manuscript.

Conflict of Interest Statement

HJD, KK, PM, NB, RM, JEG, JFB, LG, CML, ASIL and DWR have no conflict of interests to declare.

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Main Results—Wild-type mice showed marked time-of-day differential responses of airway hyper-responsiveness (maximal at dusk, start of the active phase), both *in vivo* and *ex vivo* in precision cut lung slices. Hyper-responsive time of day effects were abolished in mice lacking the clock gene *Rev-erbα*, indicating that time-of-day effects on asthma responses are likely mediated via the circadian clock. We suggest that muscarinic receptors 1 and 3 (*Chrm 1, 3*) may play a role in this pathway.

Conclusions—We identify a novel circuit regulating a core process in asthma, potentially involving circadian control of muscarinic receptor expression, in a REV-ERBα dependent fashion.

Keywords

Body clock; airway hyperresponsiveness; muscarinic

Introduction

Asthma, is a chronic inflammatory disease of the airways and displays strong circadian rhythmicity (1–4). Asthma-associated mortality is strongly time-of-day dependent, peaking overnight between midnight and 08:00 (5). Airway hyper-responsiveness (AHR), a cardinal feature of asthma (6, 7) is increased sensitivity of the airways to bronchoconstrictor challenge, such as methacholine; clinically, AHR is useful in diagnosing asthma. There is diurnal variation in AHR in asthma, with a peak around 04:00, the time of maximal disease expression (8–13). Potential causes for this diurnal change in AHR in asthma remain undefined; but may be important for improved asthma treatment.

Circadian rhythms are generated by a molecular clock, expressed in virtually all cells. A central clock in the suprachiasmatic nucleus of the brain synchronises peripheral tissue clocks via neural and humoral mediators. The cellular circadian molecular clock consists of a positive arm — CLOCK and BMAL1 heterodimers — driving transcription of 2 inhibitory arms — PER/CRY and REV-ERBα/REV-ERBβ, which feedback to inhibit BMAL1/CLOCK heterodimer transactivation function (16). The circadian clock powerfully regulates inflammation (17–19). REV-ERBα plays a key role as a repressor of the inflammatory response (20).

Here we explored the biology of REV-ERBα and address whether this protein acts as a circadian mediator, gating AHR following allergic challenge. Using the house dust mite (HDM (19)) mouse model for allergic airways disease (20), and an *in-vitro* lung slice model, we investigated the role of airway smooth muscle in the circadian gating of AHR. We find that time-of-day effects in AHR following allergen challenge are ablated in REV-ERBα-deficient mice, as is rhythmic expression of key muscarinic receptor sub-classes, mediating cholinergic smooth-muscle responses. Thus, we identify a pathway linking the core cellular clock, through REV-ERBα to airway reactivity, smooth muscle tone, and airway narrowing.

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Methods

Animals

All experimental procedures were carried out in accordance with the Animals (Scientific Procedures) Act, 1986. Rev-erbα$^{-/-}$ mice were provided by Ueli Schibler (University of Geneva) (21). Wild type control C57/Bl6J mice and *Rev-erbα*$^{-/-}$ mice were individually housed in 12:12 light/dark cycles. Zeitgeber time (ZT) 0 is when lights are turned on in the animal house, and ZT 12 is when lights are switched off. Female C57/Bl6 mice aged 8-12 weeks were used in all experiments.

Asthma Protocol-House Dust Mite (HDM)

Mice were exposed intra-nasally (i.n), to 25μg of HDM (Citeq Biologics; Batch No. 15J02) protein in 25μl of PBS under anaesthesia for 5 days/week for 3 weeks (22). Control mice received i.n PBS. One group of mice received HDM/PBS at ZT11 (just before lights off/start of active phase); a second group received HDM/PBS at ZT23 (just before lights on/start of rest phase).

Measurement of AHR

Airway resistance was measured 24 h after the final HDM exposure, in response to increasing concentrations of methacholine (3-100mg/ml, Sigma, UK), using flexiVent small animal ventilator (SciReq (23)) as previously described (24).

Collection of Serum

Blood samples (BD®Microtainer, Becton, Dickinson and Company) were placed on ice for one hour, then centrifuged to derive serum (5minutes, 7000rpm).

Bronchoalveolar Lavage (BAL) and Lung Digest

BAL was performed immediately after measurement of AHR (25). The right inferior and post-caval lobes were taken for lung digest (26). Lung cells were analysed by flow cytometry (24).

Histology

Following BAL, the left lung was taken for histology (26). For H&E stained slides a semi-quantitative scoring system graded the size of lung infiltrates (27). Goblet cells were counted on Periodic Acid-Schiff (PAS) stained lung sections using an arbitrary scoring system (28).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RNA was extracted from the right middle lobe (ReliaPrep™ RNA Miniprep Systems (Promega, #Z6011)) and reverse-transcribed (GoScript™ Reverse Transcription System (Promega, #A5001), before qPCR analysis (KAPA SYBR FAST qPCR Master Mix (2×) Universal Kit (KAPA Biosystems, #KK4601)). Relative gene expression was determined via normalization to Gapdh. Primers used: Qiagen-ADRB1 (QT00258692), ADRB2 (QT00253967), Chrm 1 (QT00282527), Chrm 2 (QT00290297), Chrm 3, GAPDH (QT01658692)and primer sequences-Nr1d1 (F GTCTCTCCGTTGGCATGTCT, R

CCAAGTTCATGGCGCTCT) and Bmal1 (F CCAAGAAAGTATGGACACAGACAAA, R GCATTCTTGATCCTTCCTTGGT).

ELISA

Serum was analysed for anti-HDM IgE as per manufacturer's instructions (Thermo Fisher, #EMIGHE and Chrondrex, Inc #3037).

Bioplex

BAL fluid was analysed using Bio-Plex Pro ™ Mouse Chemokine Panel 33-Plex (Bio-Rad, #12002231), on Bio-Rad Bio-Plex 200 system.

Lung Slice Model

Precision-cut ectopic lung slices (175 μm) were prepared (29). Slices placed on cell culture inserts (Millicell) were imaged using Nikon Long-Term Time Lapse microscope (Eclipse Ti Inverted). Airways were imaged in response to methacholine (0-100μM). Airway size was quantified using ImageJ software (v1.41o). Airway contraction was measured as percentage decrease from baseline.

Statistical Analysis

Linear mixed effects modelling was used to determine how AHR changes with increasing doses of nebulised methacholine.

Other data was analysed by 1-way ANOVA, followed by Tukey's multiple comparison test and represented as median (± IQR). IgE data and change in AHR after 75mg/ml methacholine was analysed by Mann Whitney U tests. Serum IgE, AHR and PCR data is represented as mean (± SEM).

For the precision-cut lung slice model, methacholine dose response curves were fitted to a three-parameter sigmoidal dose-response curve. An extra sum-of-squares F-test was used to test whether one curve could adequately fit the data for ZT11 and ZT23.

Results

AHR varies by time of day of HDM allergen challenge

The time of day at which wild-type (WT) mice are challenged with HDM, significantly impacts the resultant AHR (Fig 1, a). WT mice were challenged with HDM at either ZT11 (just before lights off and the start of the active phase for mice), or at ZT23 (just before lights on and the start of the rest phase in mice). WT mice challenged with HDM at ZT11 (and in which maximal airway resistance was recorded 24 hours later at ZT11) showed a significant increase in the slope of the methacholine dose response curve compared to mice challenged at ZT23 (Fig 1, a, P= 0.005), indicating a significant time of challenge effect and suggesting increased sensitivity of the airway to the effects of methacholine after HDM challenge at ZT11 compared to at ZT23. This was also the case when airway resistance was measured as area under the curve (Rrs) (Supplementary Fig 1, a, P=0.005)

WT mice challenged with HDM exhibited increased airway resistance after 75mg/ml methacholine at both ZT11 and ZT23 compared to control mice treated with PBS (P =0.007 for ZT11 and P = 0.055 for ZT23). Maximal airway resistance was significantly higher after 75mg/ml of methacholine in WT mice challenged with HDM at ZT11 compared to at ZT23 (Fig 1, a, P=0.05). This was also the case for mean resistance (Rrs) (Fig 1, b, P=0.03). There were no differences in lung compliance between groups (Fig 1, c).

Airway and lung inflammation reveal no time of challenge differences

Next, we examined BAL to determine whether time of day differences in AHR were associated with airway inflammatory changes. There was a significant increase in BAL total cells from mice treated with HDM at ZT11, compared to controls, but not at ZT23. There was no difference by time of challenge (Table 1). BAL eosinophils significantly increased following HDM challenge at both ZT11 and at ZT23 compared to control. No time of challenge differences were seen for differential BAL cell types. BAL macrophages significantly reduced at ZT23 after HDM challenge. HDM-specific IgE significantly increased after HDM challenge in WT mice; there was no time of challenge difference, indicating similar sensitisation, and acquisition of adaptive immunity (Supplementary Fig1, b).

Next we analysed inflammatory cells present in lung digests. There was an increase in total immune cell content after HDM challenge, but this only reached significance at ZT23. There was no time of HDM challenge difference (Fig2, a). There was a significant increase in lung eosinophils after HDM challenge, but again no time of challenge difference (Fig2, b).

Histology revealed a significant increase in eosinophil infiltration around the bronchioles and blood vessels within the lung following HDM challenge, compared to control mice. However, there was no time of challenge difference (Fig2, c). There was increased mucus in PAS stained lung sections after HDM challenge compared to control, but no time of challenge difference in PAS scores (Fig2, d).

REV-ERBα is a negative repressor of AHR

We then investigated the function of two components of the molecular clock within the lungs of WT mice. We focussed on BMAL1, the only non-redundant clock component, and the major element of the positive arm of the clock. BMAL1 has been implicated in the circadian control of inflammation (22–27). We also studied REV-ERBα, a component of the negative arm of the clock, and a known regulator of inflammation (16, 18), and itself repressed by inflammation. *Bmal1* expression is in antiphase to *Rev-erbα* expression in PBS treated mice (Fig3, a, b). There was a significant time of day difference in *Rev-erbα* expression at baseline, with high levels of *Rev-erbα* expression at ZT11, close to the predicted circadian peak of expression, and low levels of expression at ZT23 (Fig3, a). After HDM challenge there is reduced expression of both *Rev-erbα* and *Bmal1*, and a loss of the time of day of expression within the lung (Fig3, a, b).

The change in *Rev-erbα* expression seen after HDM challenge, taken with the previous work showing a role for REV-ERBα in lung inflammation prompted us to investigate HDM responses in *Rev-erbα*$^{-/-}$ mice. There is an increase in AHR to 75mg/ml methacholine, after

HDM challenge in *Rev-erbα*$^{-/-}$ mice compared to control (P< 0.03 at ZT23 and P= 0.09 at ZT11), but no time of challenge effect (in contrast to WT mice) (Fig3, c and Supplementary Fig2, a). There was no difference in the slope of the methacholine dose response curves between *Rev-erbα*$^{-/-}$ mice challenged with HDM at either ZT11 or ZT23, in contrast to WT mice. We also noted a higher baseline AHR at ZT23 compared to ZT11 in PBS treated *Rev-erbα*$^{-/-}$ mice (Fig3, c); although this was not significant, this trend was in anti-phase to the effect seen in WT mice (Fig 1).

Furthermore, there was an increase in maximal effect of methacholine in *Rev-erbα*$^{-/-}$ mice compared to WT mice for both PBS (Supplementary Fig2, b) and HDM challenged mice (Fig3, d). This suggests that loss of REV-ERBα causes an exaggerated and clock-time independent AHR in response to methacholine challenge.

Airway and lung inflammation reveal no time of challenge differences in *Rev-erbα*$^{-/-}$ mice

There was a significant increase in the total number of cells in BAL and in the percentage of eosinophils following HDM challenge in *Rev-erbα*$^{-/-}$ mice, but with no time of challenge effect in either, as previously seen in WT mice (Fig3, e, f). There was a significant increase in total cells in the lung digest following HDM challenge in *Rev-erbα*$^{-/-}$ mice at ZT23, compared to control mice but no time of challenge difference (Fig4, a). Lung eosinophils also increased after HDM challenge (only reaching significance at ZT11) and there was no time of challenge differences (Fig4, b).

Histological analysis showed increased H&E staining around the bronchioles, blood vessels and within the interstitial spaces as well as increased PAS staining, at both challenge times after HDM challenge, with no time of day effect in the *Rev-erbα*$^{-/-}$ mice (Fig4, c, d). HDM specific IgE following HDM challenge in *Rev-erbα*$^{-/-}$ mice was increased, but no time of day difference was seen (Supplementary Fig2, c).

Genotype comparison of WT versus *Rev-erbα*$^{-/-}$ mice

BAL individual cell counts measured as a percentage of the total cell count, reveal no significant genotype differences (Supplementary Fig 3, a). Cytokine and chemokine analysis revealed no time of challenge differences (data not shown) and only CXCL13 showed a genotype difference (Table 2).

REV-ERBα action is through airway smooth muscle muscarinic receptor regulation

Since we did not find a convincing correlation between inflammatory parameters and AHR in our models, we next investigated bronchiolar smooth muscle function.

Using precision cut lung sections in organotypic culture we quantified airway contraction in response to methacholine. We found a significant increase in the maximal effect to methacholine at ZT11 compared to at ZT23 (P=0.03) and a reduction in the EC50 for methacholine in HDM challenged lung slices at ZT11 (3.2μM) compared to ZT23 (6.2μM) (Fig5, a, b). We repeated these experiments in lung slices from HDM challenged *Rev-erbα*$^{-/-}$ mice. We found no time of day differences to methacholine challenge and similar EC50s (ZT11, EC50= 9.3μM and ZT23, EC50= 8.5μM) (Fig5, c). There were no changes by time

of day in PBS treated lung slices. We then investigated muscarinic receptor expression in the lungs. After saline challenge, we found Chrm 1 was more highly expressed at ZT11 rather than at ZT23, perhaps accounting for the physiological differences in AHR at baseline by time of day in WT mice (Fig5, d). We also found Chrm 3 expression was higher after HDM challenge at ZT11, but not at ZT23, potentially explaining the time of challenge difference in AHR. These time of day effects were lost in *Rev-erbα*$^{-/-}$ mice (Fig5, f). Chrm 2 expression showed no time of day or genotype differences after PBS or HDM challenge (Fig5, e). We also measured the expression of the muscle contractile apparatus genes smooth muscle actin (*Acta*), myosin light chain kinase (*mylk1*) and smooth muscle myosin (*sm-mhc*); none of these demonstrated a time of day response (Supplementary Fig4, a, b, c). Similarly, the beta adrenoceptors (*Adrb1* and *Adrb2*) although reduced in response to HDM challenge, importantly also showed no time of day effects (Supplementary Fig4d, e).

Discussion

We show that AHR is determined by time of day, an effect regulated through REV-ERBα. Allergen challenge at ZT11 (just before lights off/beginning of the active phase in mice; equivalent to early morning in humans) significantly increases the magnitude of AHR compared to allergen challenge at ZT23 (just before lights on/beginning of the rest phase in mice, equivalent to late afternoon/early evening in humans). This effect is abolished in *Rev-erbα*$^{-/-}$ mice, suggesting that AHR is regulated, or gated, by REV-ERBα. Despite the marked changes in AHR only modest changes in inflammatory mediators and cells were seen in the lungs, suggesting dissociation between the inflammatory response, and the airway constriction. Even *ex-vivo* the airways retain a time of day signature in response to methacholine, an effect which was lost in *Rev-erbα*$^{-/-}$ mice, prompting our analysis of the muscarinic receptor types. This revealed both time of day, and also REV-ERBα dependent changes in expression, especially of the M3 receptor in whole lung.

Nocturnal exacerbations of asthma, hospital admissions and deaths, remain an unmet medical need. The immune system lies under strong circadian control (28, 29), and lung inflammatory responses are strongly regulated by the circadian clock, and specifically by REV-ERBα (16, 18). Since asthma symptoms in humans peak in early morning at around 6am, we focussed on this time point (ZT11) and its anti-phasic time point ZT23 in our mouse studies. These time points have also been shown to be important for the lung innate immune response and in food allergy (26, 30) and also in our own work in human asthma (1). Using direct flexivent measurement of AHR and a physiologically relevant allergen, HDM, we found higher AHR at ZT11. In nocturnal mice, this time-point is equivalent to the transition from the rest-phase to activity, and is biologically comparable to early morning in humans.

The allergic inflammatory process recruits many specialised cells to the lung, resulting in a changed immune environment. We characterised the immune cell repertoire, both in BAL, and lung digests, and also measured inflammatory and immune mediators. Overall, the effects of time of day were dissociated from the consistent and marked changes in AHR, suggesting possible non-immune cell involvement. We acknowledge that there could have

been time of day differences at other time points. Our sampling was undertaken at 24 hours after the final allergen challenge, when AHR was predicted to be greatest (20).

The orphan nuclear receptor REV-ERBα has recently emerged as a major regulator of the lung immune response, mediating time of day changes to acute inflammatory challenges (16, 18). Moreover, REV-ERBα plays important roles in non-immune cells, regulating energy metabolism, and muscle function (31, 32). For these reasons we repeated the HDM challenges in *Rev-erbα*$^{-/-}$ mice and showed that the time of day AHR effect was abolished. Interestingly, we also found that HDM challenge in wild-type mice had a major inhibitory effect on *Rev-erbα* expression, identifying inflammation acting through both transcriptional, and post-translational mechanisms to repress *Rev-erbα* expression (18). Again, we saw no differences in immune cells infiltrating into the lungs between wild-type and *Rev-erbα*$^{-/-}$ mice, despite the loss of temporal control of AHR. This again suggests a non-immune cell, and non-inflammatory effector mechanism.

To examine the airway responses directly, we removed the lungs of HDM sensitised animals, prepared precision cut lung slices for organotypic culture, and measured airway responses to methacholine. Here, we saw an increase in the maximal effect to methacholine at ZT11, indicating greater methacholine sensitivity. Furthermore, when we repeated these experiments in *Rev-erbα*$^{-/-}$ mice, the time of day difference was abolished. This correlates with the *in-vivo* measurements, and indicates a lung-intrinsic mechanism of action. Methacholine acts on muscarinic receptors, with little effect on nicotinic receptors (33). Therefore, we examined the expression of muscarinic receptors, and identified major changes in both type M1 and M3 muscarinic receptors. Importantly, we found no changes in the expression of genes involved in the contractile apparatus of airway muscle, or in adrenoceptors, suggesting that the changes in muscarinic receptors in the lung by time of day were specific. The increase in M1 receptor expression at ZT11 in the PBS treated group suggests that this receptor is important for conferring time of day constrictor tone to the airway under basal conditions. Furthermore, Chrm1 contains transcription factor binding sites for the clock proteins BMAL:CLOCK and RORβ (34); suggesting that *Chrm1* is under direct clock control. In contrast *Chrm3* only acquires a time of day effect after HDM-inflammation, with peak expression at ZT11. Strikingly, this time of day change in *Chrm3* expression is completely lost in the *Rev-erbα*$^{-/-}$ mouse, providing an attractive explanation for the loss of temporal gating in AHR we observed. However, according to the circadian data-base of rhythmic gene expression (Circa DB, 35), the expression of *Chrm3*, oscillates in healthy mouse lung with maximal expression at 6pm (ZT11) and nadir of expression at 6am (ZT23). In our study, we identified similar time of day differences in *Chrm3* expression, but in our case, these differences were only apparent following stimulation with HDM and were not observed in baseline conditions. One potential explanation may be that our assay was insufficiently sensitive to detect low level changes in gene transcription in un-stimulated conditions. Bioinformatic analysis revealed no evidence of clock transcription factor binding sites in *Chrm3* (34), and we therefore postulate that *Chrm3* transcription may be under indirect clock control.

We acknowledge that the M3 receptor is not only expressed by airway smooth muscle cells but by multiple other cell types (36) including endothelial cells and inflammatory

cells. However, given the immediate and directly visualised contraction of the airway to methacholine during the lung slice experiments, the likely mechanism of action of methacholine is through the muscarinic receptors present in the airway smooth muscle. Although we have focussed on time of day changes in muscarinic receptors, it should be noted that the parasympathetic nervous system as a whole displays marked circadian rhythmicity (37). It is therefore likely that *in vivo* the diurnal variation in AHR would be affected by both neural and humoral circadian rhythms, as well as in rhythmic changes in receptor expression.

To our knowledge this is the first time that the molecular clock has been shown to be important in gating AHR. Furthermore, the discovery that muscarinic receptors might play a role is important for the treatment of asthma (38). The cholinergic system is functionally linked to the circadian system (37). Tiotropium bromide, a long-lasting M3 muscarinic-receptor antagonist is licensed for asthma (39). In the future, a short-acting drug antagonising both M1 and M3 might prevent AHR in asthma and its administration at the peak of receptor expression could significantly increase its efficacy, leading to novel chronotherapeutic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Implication

These insights suggest the importance of considering timing of drug administration in clinic trials, and in clinical practice; chronotherapy.

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Capsule Summary

REV-ERBα gates airway hyperresponsiveness by time of day; future asthma therapies should aim to dose anti-muscarinic agents at the most efficacious time of day (chronotherapy) and modulate the molecular clock.

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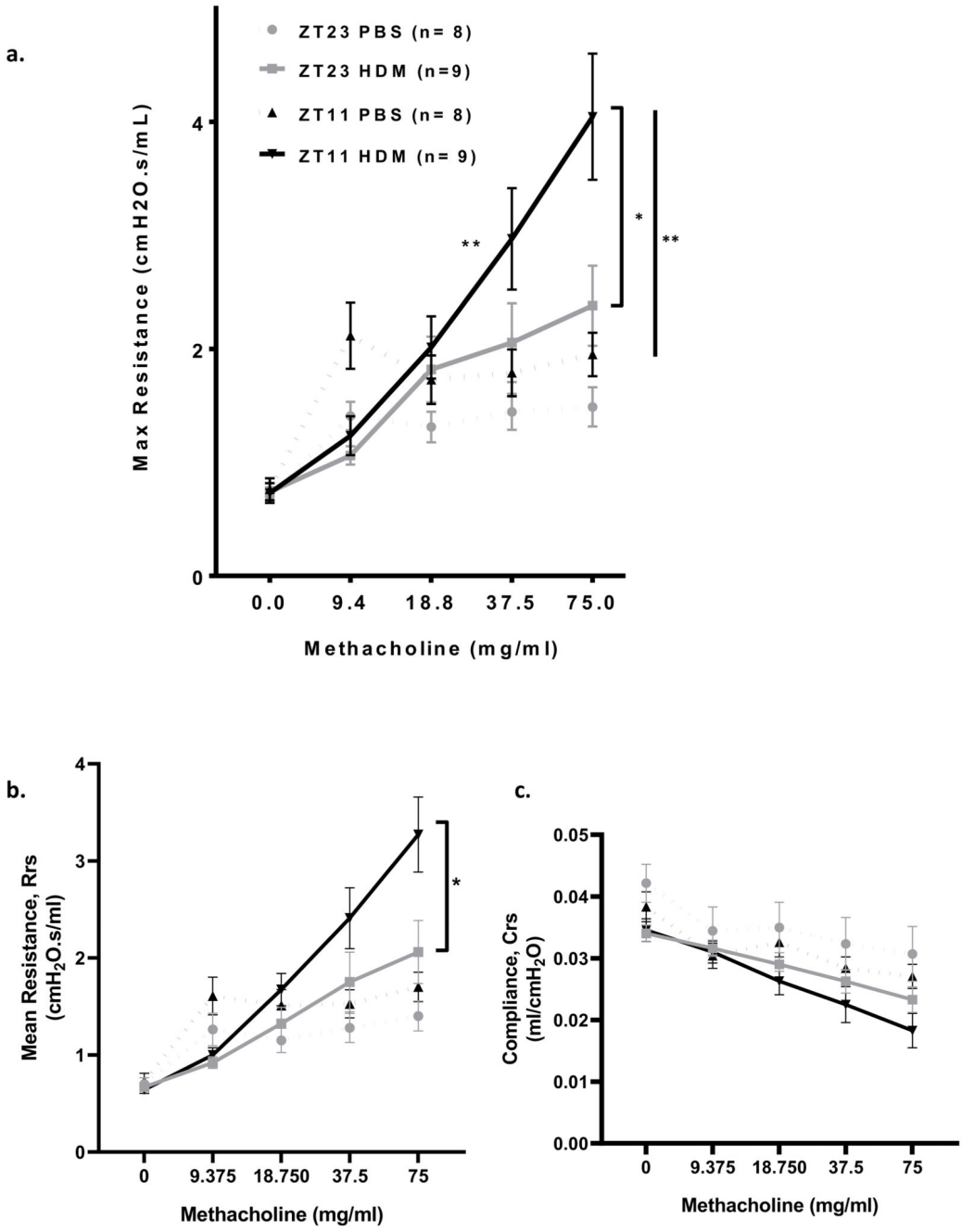

Fig 1. HDM challenge at ZT11 (just before start of the active phase) results in a significant increase in AHR compared to challenge at ZT23.

a. Airway hyperresposiveness to increasing doses of methacholine was measured as maximum airway resistance (mean ± SEM) using Flexivent in WT mice at ZT11 and ZT23. The slope of the dose response curve for methacholine is significantly increased after HDM challenge at ZT11 compared to at ZT23 (*P= 0.005), mixed linear modelling. WT mice challenged with HDM exhibited increased airway resistance after 75mg/ml methacholine at both ZT11 and ZT23 compared to control mice treated with PBS (‡P =0.007 for ZT11 and P = 0.055 for ZT23), Mann Whitney U. Maximal airway resistance was significantly higher

after 75mg/ml of methacholine in WT mice challenged with HDM at ZT11 compared to at ZT23 (†P=0.05), Mann Whitney U. Baseline airway resistance was higher in control (PBS treated) mice at ZT11 compared to at ZT23.

b. Airway hyperresponsiveness to increasing doses of methacholine was measured as mean reistance (Rrs) (mean ± SEM) in WT mice at ZT11 and ZT23. WT mice challenged at ZT11 exhibited significantly increased mean resistance after 75mg/ml methacholine, compared to at Zt23 (*P=0.03) Mann Whitney U.

c. Compliance (Crs) to increasing doses of methacholine was measured (mean ± SEM) using FlexiVent in WT mice at ZT11 and ZT23. There was a reduction in compliance in both HDM and PBS challenged mice. There was no time of day difference. Mann Whitney U. Solid lines-HDM treated; dotted lines-PBS treated mice. Grey lines-ZT23 and black lines indicate ZT11.

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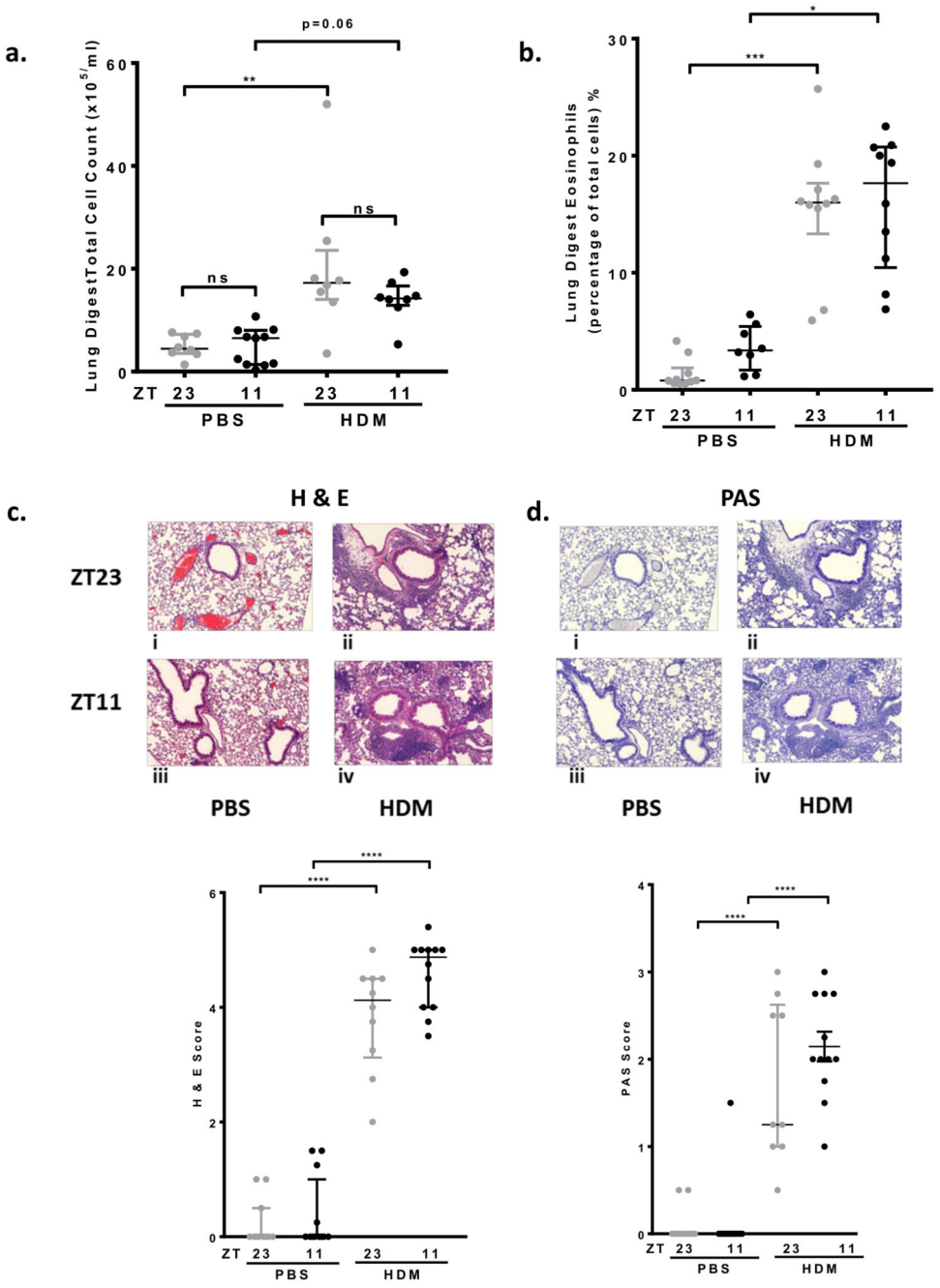

Fig 2. Eosinophilic lung inflammation increases after HDM challenge; but no real time of challenge differences.

a. Total cell count from lung digests in WT mice. There was an increase in total cell count after HDM challenge, compared to control (** $P < 0.01$ at ZT23, and $P=0.06$ at ZT11). There was no time of challenge difference after PBS or HDM challenge. Data is presented as median ± IQR. 1 way ANOVA followed by Tukey's multiple comparison test, (n=8-11 per treatment group).

b. Eosinophils in lung digests from WT mice significantly increased after HDM challenge (*** $P < 0.001$ at ZT23 and $P < 0.05$ at ZT11); there was no time of challenge difference after PBS or HDM challenge. Data is presented as median ± IQR. 1 way ANOVA, followed by Tukey's multiple comparison test, (n=8-11 per treatment group).
c. HDM challenge at ZT11 and ZT23 caused predominantly eosinophilic inflammation around the bronchioles and blood vessels (haemoatoxylin and eosin (H&E) staining, i-iv) compared to PBS challenge (**** $P < 0.0001$ at ZT23 and ZT11). There was no time of challenge difference after PBS or HDM challenge. Data is presented as median ± IQR. 1 way ANOVA, followed by Tukey's multiple comparison test, (n=10-12 per treatment group).
d. Periodic Shift Staining (PAS) shows increased mucus present on the bronchial epithelium in the lungs of WT mice treated with HDM at both ZT11 and ZT23. There was no PAS staining seen in PBS treated mice (i-iv). There was significantly increased PAS scores in HDM challenged mice (**** $P < 0.001$ at ZT23 and ZT11), compared to controls, but no time of challenge differences. Data is presented as median ± IQR. 1 way ANOVA, followed by Tukey's multiple comparison test, (n=10-12 per treatment group).

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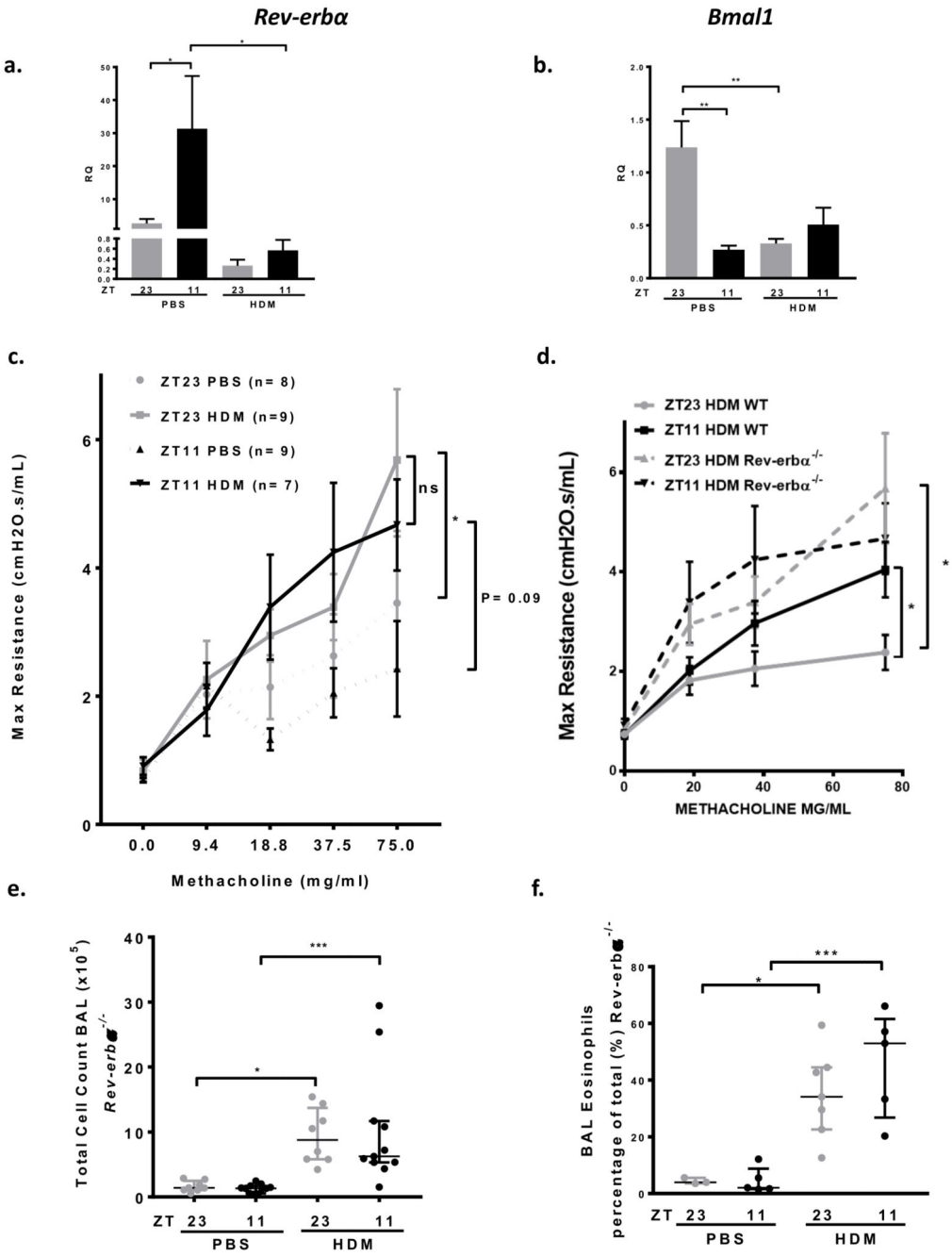

Fig 3. REV-ERBα is a negative repressor of AHR.

a. Expression of *Rev-erbα* in WT murine lung tissue, relative to the expression of *Gapdh*. There was a time of day difference in the expression of *Rev-erbα* in PBS challenged mice; *Rev-erbα* expression at ZT11 was significantly greater than at ZT 23 (* $P < 0.05$). This time of day difference was lost after HDM challenge; there was a reduction in expression of Rev-erbα at both challenge times (* $P < 0.05$ at ZT 11). 1 way ANOVA, followed by Tukey's multiple comparison test. Data is presented as mean ± SEM (n=5-7 per treatment group, in duplicate). Grey bars-ZT23, Black bars ZT11.

b. Expression of *Bmal1* in WT murine lung tissue, relative to the expression of *Gapdh*. There was a time of day difference in the expression of *Bmal1* in PBS challenged mice; *Bmal1* expression at ZT23 was significantly greater than at ZT11 (** $P < 0.01$), in anti-phase to *Rev-erbα* expression. This time of day difference was lost after HDM challenge; there was a reduction in expression of *Bmal1* at both challenge times (** $P < 0.01$ at ZT23). 1 way ANOVA, followed by Tukey's multiple comparison test. Data is presented as mean ± SEM (n=7-11 per treatment group, in duplicate).

c. Airway hyperresponsiveness to increasing doses of methacholine was measured in *Rev-erbα*$^{-/-}$ mice, as maximum airway resistance using Flexivent. There was no difference in the slopes of the methacholine dose response curves between *Rev-erbα*$^{-/-}$ mice challenged with HDM at either ZT11 or ZT23 (mixed linear modelling). There was no difference in the maximal AHR measured after 75mg/ml of methacholine in *Rev-erbα*$^{-/-}$ mice after challenge at ZT11 and ZT23 (Mann Whitney U). Mice challenged with HDM exhibited increased airway resistance after 75mg/ml methacholine at ZT11 ($P=0.09$) and at ZT23 (* $P=0.03$) compared to control (PBS challenged) mice, Mann Whitney U test. In *Rev-erbα*$^{-/-}$ mice baseline airway resistance is higher in control (PBS treated) mice at ZT23 compared to ZT11. This is in anti-phase to WT mice (Fig 1). Data is presented as mean± SEM. Grey lines-ZT23, HDM challenged, grey dotted line-ZT23, PBS control, black line-ZT11, HDM challenged and black dotted line-ZT11, PBS control.

d. AHR was significantly increased in *Rev-erbα*$^{-/-}$ mice after challenge with HDM compared to WT controls. After HDM challenge at ZT23 there was a significant increase in the maximal response to methacholine in *Rev-erbα*$^{-/-}$ mice compared to WT mice (* $P < 0.05$) mean ± SEM (n=7-9 per treatment group), 1 way ANOVA, followed by Tukey multiple comparison adjustment. Grey lines-ZT23 HDM challenged WT, black line-ZT11, HDM challenged WT, grey dotted line-HDM challenged ZT23 *Rev-erbα*$^{-/-}$, and black dotted line-HDM challenged ZT11 *Rev-erbα*$^{-/-}$.

e. Total cells recovered from BAL fluid from *Rev-erbα*$^{-/-}$ mice significantly increased after HDM challenge at both times, compared to control, PBS challenged mice (* $P < 0.05$ ZT23, ** $P < 0.01$ ZT11). There was no time of challenge difference in total cells in BAL in either PBS challenged or HDM challenged groups. 1 way ANOVA, followed by Tukey's multiple comparison test, (n=8-12 per treatment group). Data is presented as median ± IQR.

f. BAL eosinophils (measured as a percentage of the total) significantly increased after HDM challenge at both times in *Rev-erbα*$^{-/-}$ mice, compared to PBS challenged control mice (* $P < 0.05$ ZT23, *** $P < 0.001$ ZT11). There was no time of challenge difference in percentage eosinophils in BAL in either PBS challenged or HDM challenged groups. 1 way ANOVA, followed by Tukey's multiple comparison test, (n=8-12 per treatment group). Data is presented as median ± IQR.

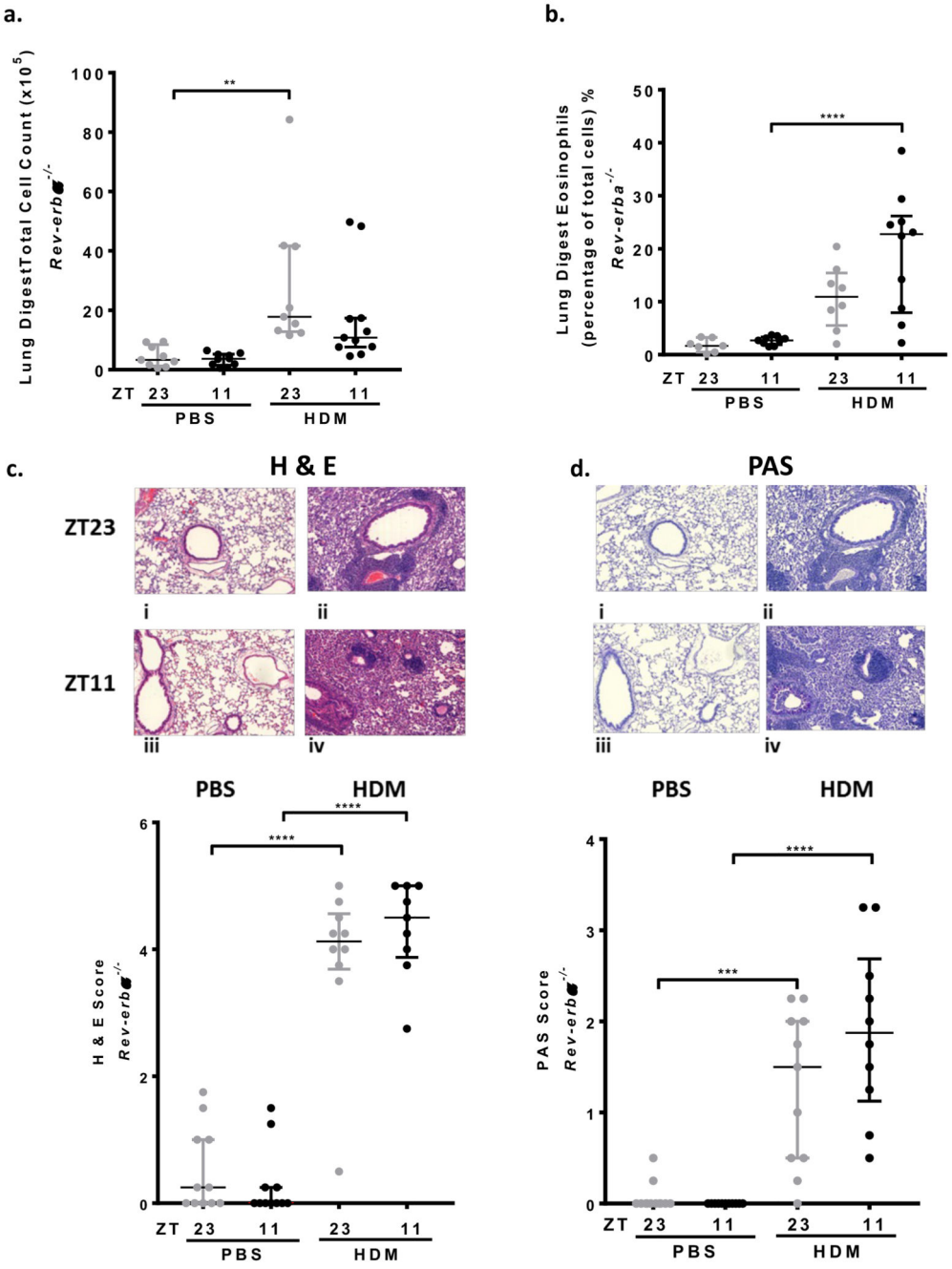

Fig 4. Lung inflammation in *Rev-erbα*$^{-/-}$ mice after HDM challenge show no time of challenge difference.

a. Total cell count from lung digests increased after HDM challenge, compared to control, although only reached significance at ZT23 (** $P < 0.01$). There was no time of challenge difference. Data is presented as median ± IQR (n=8-11 per treatment group) and analysed using a 1 way ANOVA, followed by Tukey's multiple comparison test.

b. Eosinophils in lung digests increased after HDM challenge, only reaching significance at ZT11 (**** $P < 0.0001$); there is no time of challenge difference. Data is presented as

median ± IQR (n=8-11 per treatment group) and analysed using a 1 way ANOVA, followed by Tukey's multiple comparison test.

c. HDM challenge at ZT11 and ZT23 caused predominantly eosinophilic inflammation around the bronchioles and blood vessels (haemoatoxylin and eosin staining (H&E)) compared to PBS challenge, histology sections i-iv and scatter plot, **** $P < 0.0001$ at ZT23 and ZT11. There was no significant time of challenge effect in PBS or HDM challenged groups. Data is presented as median ± IQR and analysed using a 1 way ANOVA, followed by Tukey's multiple comparison test, (n=9-11 per treatment group).

d. Periodic Shift Staining (PAS) shows increased mucus present on the bronchial epithelium in the lungs of WT mice treated with HDM at both ZT11 and ZT23. There was no PAS staining seen in PBS treated mice (i-iv) and scatter plot, *** $P < 0.01$ at ZT23 and **** $P<0.0001$ at ZT11). There was no time of challenge differences after PBS or HDM challenge. Data is presented as median ± IQR; data analysed using 1 way ANOVA, followed by Tukey's multiple comparison test, (n=9-11 per treatment group).

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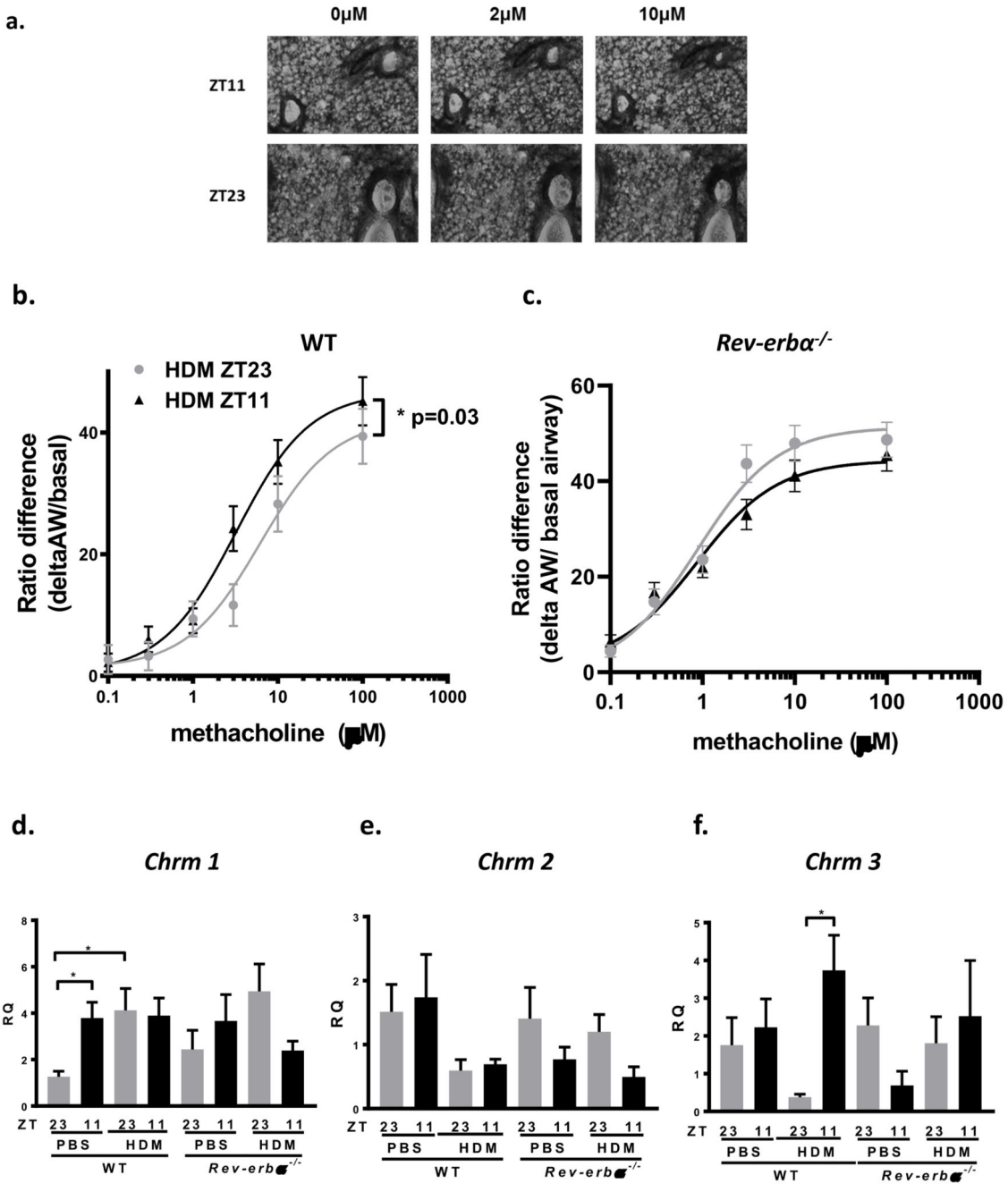

Fig 5. Time of HDM challenge determines magnitude of contraction of airways to methacholine in lung slice model; muscarinic receptor expression is regulated by time of day and also by HDM challenge and REV-ERBα.

a. Representative images of precision cut lung slice experiment showing contraction of airways to increasing concentrations of methacholine in mice challenged with HDM at ZT11 or ZT23.

b. Concentration-response curves to increasing concentrations of methacholine. There is a significant increase in the maximal effect to methacholine in mice challenged with HDM

at ZT11 compared to ZT23 (* P= 0.03). The EC50 for WT mice challenged at ZT11 was 3.2μM, and 6.2μM at ZT23.

c. Concentration-response curves to increasing concentrations of methacholine in *Rev-erbα*$^{-/-}$ mice challenged with HDM at ZT11 and ZT23. There was no difference in the effects of methacholine by time of day. The EC50 for ZT11 was 9.3μM and for ZT23 was 8.5μM.

Methacholine dose response curves were fitted to a three parameter sigmodal dose-response curve. An extra sum-of-squares F-test was used to test whether one curve could adequately fit the data for ZT11 and ZT23. Black triangles-HDM challenge at ZT11 and grey circles-HDM challenge at ZT23

d. Quantitative PCR for muscarinic receptor *Chrm1. Chrm1* expression is significantly increased in PBS challenged mice at ZT11 compared to ZT 23 (* $P < 0.05$). This time of day difference is not apparent after HDM challenge. There is a significant increase in expression of *Chrm1* after HDM challenge at ZT23 compared to control, PBS challenged mice (* $P < 0.05$). There was no time of challenge difference seen in *Rev-erbα*$^{-/-}$ mice (n=5-9 per treatment group, in duplicate).

e. Quantitative PCR for muscarinic receptor *Chrm2. Chrm2* expression showed no time of challenge differences in WT or *Rev-erb*$^{-/-}$ mice (n=7-10 per treatment group, in duplicate). There were no time of genotype differences.

f. Quantitative PCR for muscarinic receptor *Chrm3. Chrm3* expression is significantly increased after HDM challenge at ZT11 compared to ZT 23 (* $P < 0.05$). There is no time of day difference in *Rev-erbα*$^{-/-}$ mice (n=5-9 per treatment group, in duplicate).

All data presented as mean ± SEM and analysed by 1 way ANOVA, followed by Tukey's multiple comparison test. All QPCR data is compared to expression of the housekeeping gene *Gapdh* in WT PBS challenged mice at ZT23. Black bars indicate challenge at ZT11 and grey bars indicate challenge at ZT23.

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Table 1

Bronchoalveolar lavage fluid differential cell counts after HDM challenge at either ZT11 or ZT23 reveal no time of challenge differences.

BAL Immune Cell type	Challenge	ZT11	ZT23	Adjusted P value
Eosinophils % of total cells Median (IQR)	**PBS**	2.36 (0.55-6.28)	3.1 (2.49-4.39)	1.0
	HDM	25.5 (19.6-50.8) *	46.5 (18-50.2) *	1.0
Neutrophils % of total cells Median (IQR)	**PBS**	8.7 (1.35-11.58)	3.29 (.05-5.62)	1.0
	HDM	25.65 (14.53-48.45)	32 (15.6-50.8)	1.0
Macrophages % of total cells Median (IQR)	**PBS**	28.6 (17.4-46.05)	67 (63.35-73.6)	1.0
	HDM	7.03 (5.45-12.45)	5.52 (1.11-9.59) *	1.0
Lymphocytes % of total cells Median (IQR)	**PBS**	36.5 (26.48-48.33)	20.0 (14.58-22.13)	1.0
	HDM	21.7 17.35-27.15)	17.3 (11.72-19.9)	1.0
Total Cells $\times 10^5$/ml Median (IQR)	**PBS**	0.75 (0.46-1.32)	1.134 (0.72-2.83)	0.97
	HDM	4.36 (3.48-15.2) **	4.09 (2.73-8.12)	0.94

* PBS v HDM, p≤ 0.05

** PBS v HDM P≤0.01

BAL total cells increased after HDM at ZT11, compared to controls, but not at ZT23. There was no difference by time of challenge (P≤ 0.01). BAL eosinophils significantly increased following HDM challenge at both ZT11 and at ZT23 compared to PBS control (P≤0.05 and P≤ 0.05) No time of challenge differences were seen for differential BAL cell types. There was a significant reduction in BAL macrophages at ZT23 after HDM compared to PBS (P≤ 0.05). Asterixes indicate difference between HDM and PBS challenge. Median ± IQR; 1 way ANOVA, followed by Tukey multiple comparison adjustment, n=9-11 per treatment group.

Table 2

Cytokine/chemokine detection in bronchoalveolar lavage fluid (BALF) after HDM challenge at ZT11 and ZT23 in WT and Rev-erbα-/- mice.

BAL cytokines/chemokines increased in response to HDM	ZT11 (pg/ml) Mean (± SEM)		Adjusted P value	ZT23 (pg/ml) Mean (± SEM)		Adjusted P value
	WT	*Rev-erbα*$^{-/-}$		WT	*Rev-erbα*$^{-/-}$	
ENA79/CXCL5	1708 (175.5)	2193 (273.5)	0.68	2097 (299)	2012 (424.7)	1.00
IP-10/CXCL10	1418 (498.6)	2017 (549.6)	0.88	1793 (593.6)	1798 (626.6)	1.00
SDF-1a/CXCL-12	59.33 (9.92)	158.7 (69.29)	0.53	72.58 (16.32)	120.2 (40.5)	0.98
BCA-1/CXCL13	2158 (448)	3088 (780.7)	0.76	**2025 (310.6)**	**4670 (1179)**	**0.03**
SCYB16/CXCL16	298.2 (51.95)	157.9 (25.75)	0.14	250.9 (37.11)	204.8 (48.24)	0.87
RANTES/CCL5	49.91 (14.27)	55.28 (27.11)	0.055	36.95 (6.43)	25.62 (7.14)	0.56
MCP3/CCL-7	12.65 (2.99)	12.48 (3.98)	1.0	20.25 (3.26)	16.06 (4.82)	0.86
CCL-17	4430 (1681)	5023 (1789)	0.61	6006 (2560)	3318 (1693)	0.49
MIP3b/CCL-19	94.13 (16.04)	110.3 (26.16)	0.96	99.79 (14.22)	133.6 (30.55)	0.76
MIP-3a/CCL20	129.3 (32.64)	102.9 (30.86)	0.84	117.9 (17.49)	165.2 (53.54)	1.00
MDC/CCL-22	347.8 (63.46)	274.3 (81.15)	0.93	498.1 (111.3)	291.5 (77.17)	0.31
Eotaxin 2/CCL24	16520 (3078)	25488 (8946)	0.69	24546 (5616)	15254 (9173)	0.17
IL-1b	76.98 (14.17)	82.18 (13.68)	0.99	97.59 (9.86)	89.1 (18.32)	0.97
IL-6	17.82 (3.41)	12.48 (2.23)	0.68	19.04 (3.17)	17.74 (4.64)	0.99
IL-16	178.8 (21.3)	175.2 (23.79)	1.00	239.7 (36.24)	279.8 (69.31)	0.90

Cytokines/chemokines that increased significantly in BALF after intranasal HDM challenge are shown in the table. Cytokine/chemokine concentrations (pg/ml) for WT and Rev-erbα$^{-/-}$ mice after HDM challenge at ZT11 and ZT23 are shown as mean ± SEM. BCA-1/CXCL13 showed a genotype effect and was increased in Rev-erbα$^{-/-}$ mice at ZT23 compared to WT mice (* $P < 0.03$). P values are given in the table. 1 way ANOVA, followed by Tukey multiple comparison adjustment, n=8-12 per treatment group.