Time of day influences stress hormone response to ketamine

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Abstract
Over 50% of depressed patients show hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis. Conventional therapy takes weeks to months to improve symptoms. Ketamine has rapid onset antidepressant effects. Yet its action on HPA axis activity is poorly understood. Here, we measured the corticosterone (CORT) response to ketamine administered at different times of day in the Wistar–Kyoto (WKY) rat. In male rats, blood was collected every 10 min for 28 h using an automated blood sampling system. Ketamine (5/10/25 mg·kg⁻¹) was infused through a subcutaneous cannula at two time points—during the active and inactive period. CORT levels in blood were measured in response to ketamine using a radioimmunoassay. WKY rats displayed robust circadian secretion of corticosterone and was not overly different to Sprague Dawley rats. Ketamine (all doses) significantly increased CORT response at both infusion times. However, a dose dependent effect and marked increase over baseline was observed when ketamine was administered during the inactive phase. Ketamine has a robust and rapid effect on HPA axis function. The timing of ketamine injection may prove crucial for glucocorticoid-mediated action in depression.

KEYWORDS
circadian, cortisol/corticosterone, glucocorticoids, ketamine

1 | INTRODUCTION

Major depressive disorder (MDD) is a leading cause of the burden of disease globally. It is frequently characterized by abnormal neurotransmitter function, for instance serotonin, norepinephrine, and dopamine—evidenced by using wide-ranging antidepressants including selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs) and norepinephrine and dopamine reuptake inhibitors (NDRIs). However, these treatments are often unsuccessful, identifying an urgent need to understand the neurobiology underpinning MDD and to discover effective treatment strategies.

The hypothalamic–pituitary–adrenal (HPA) axis synthesizes and releases the steroid hormone cortisol (human) and corticosterone (rodent). Dysregulated HPA axis function, marked by abnormal CORT secretion and responses to stress, are hallmarks of several chronic conditions, as well as MDD. Endogenous models of MDD, such as the Wistar–Kyoto (WKY) rat provide valuable insights for investigating the pathophysiology of MDD. Originally bred as normative controls for the spontaneous hypertensive rat, they display behavioral and neurobiological phenotypes like those observed in clinical cases of MDD, such as learned helplessness, stress-induced ulcers, and reported elevated glucocorticoids, as well as resistance to common antidepressants.

Ketamine has long been used in clinical practice, progressing from an anesthetic induction agent to being used in the treatment of therapy resistant major depression. Although its use and action on HPA axis function have previously been studied in animals and...
humans,\textsuperscript{22,23} results have often been ambiguous, and less is known about ketamine’s action on HPA axis function at different times of day.

In this study, we firstly characterize the WKY rat endogenous circulating CORT profile and follow up with distinguishing the complex action and potential importance of time-of-day administration of ketamine for HPA axis responsiveness.

\section*{2 | METHODS}

Male Sprague Dawley (SD) and WKY rats (250 g, \textsim 3 months old; Envigo, UK) were used in experimental procedures. Animals were maintained in soundproof rooms in standard housing conditions under a 14:10 light/dark cycle. Food and water were available ad libitum. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 under PPL 30/3114.

Rats were anaesthetized with a combination of Isoflurane (100\% w/w liquid vapor [Merial, UK]) and medical air during right jugular vein cannulation and subcutaneous cannula placement. The cannula (Smith Medicals, UK) was exteriorized via a vascular access button and attached to a spring and swivel system. Postoperative analgesic 1 mg of carprofen (Rimadyl, Pfizer, UK), and 2.5 ml of glucose (5\%)/saline (0.9\%) were administered subcutaneously. Implanted cannulae were “flushed” daily and replaced with fresh heparinized saline (1:100) to maintain patency. All rats were given 5 days postsurgical recovery prior to subcutaneous infusion of ketamine.

\subsection*{2.1 | Automated blood sampling}

Blood samples were collected via an in-house automated blood sampler. Forty microliters blood samples were collected every 10 min for 28 h in 160 \mu l heparin-saline (1:100). Plasma was separated from whole blood by centrifugation at 4000 rpm, 4\°C and diluted 1 in 50 with citrate buffer, processed in triplicate and incubated overnight in 50 \mu l of \textsuperscript{125}I corticosterone tracer (Oxford Biolinnovation DSL Ltd, Oxford, UK) with 50 \mu l of rabbit anti-rat corticosterone antibody (kindly donated by G. Makara, Hungary). Free/bound separation was performed using charcoal dextran precipitation and centrifuged pellets \textsuperscript{125}I corticosterone levels were measured using a gamma counter (Wizard-2470, Perkin Elmer, MA). Concentrations of corticosterone in each plasma sample were interpolated from a standard curve. Blood volume was replaced with heparin-saline during each sampling period.

We have previously shown in many publications that this is not associated with any change in HPA activity, although it does result in a small fall in hematocrit.

\subsection*{2.2 | Automated ketamine infusion}

Ketamine (5/10/25 mg · kg) (Ketalar; ketamine hydrochloride) or vehicle (0.9\% saline) was infused at two different times of day (AM–Zeitgeber 2; PM–Zeitgeber 16) as a bolus subcutaneous infusion (10 min) using a programmable pump (PHD Ultra Syringe Pump, Harvard Apparatus, USA).

\subsection*{2.3 | Statistical analysis}

Results are presented as mean ± SEM. All statistical analyses were performed using GraphPad Prism (version 9.1, GraphPad Software),
using restricted maximum likelihood (REML) mixed effects analyses, analyses of variance (ANOVA) or repeated measures ANOVA (RM ANOVA), followed by post hoc tests where appropriate. Two-way RM ANOVA was used to test if differences in corticosterone secretion were observed across time and in two different rat strains—SD and Wistar Kyoto (Figure 1). In A, C, and E (Figures 2 and 3), mixed effects analysis was used to assess if ketamine infusion affected corticosterone secretion across time. With automated blood sampling, samples being collected every 10 min can result in a poor sample draw. This prevented the use of two-way-RM ANOVA. In B, D, and F (Figures 2 and 3), two-way-RM ANOVA was used to identify if ketamine infusion affected corticosterone secretion across time immediately following infusion. In A and B (Figure 4), one-way ANOVA was used to compare the peak level of corticosterone within 120 min of

**FIGURE 2** Effect of ketamine on corticosterone secretion infused during the active period. (A) Automated blood sampling for 28 h of CORT secretion in WKY rats with a time automated infusion of ketamine (25 mg · kg) during the active period (at zeitgeber 16). Mixed effects analysis identified a significant effect of time ($F_{3,728,37.53} = 7.094, p < .0001$), no effect of treatment ($F_{1,8} = .071, p = .7966$), but an interaction ($F_{143,937} = 2.51, p < .0001$). (B) Corticosterone levels for 120 min following ketamine (25 mg · kg) infusion. Two-way ANOVA reported a significant effect of time ($F_{12,96} = 2.103, p = .0235$), a trend to significance with treatment ($F_{1,96} = 3.310, p = .0564$) and an interaction ($F_{12,96} = 4.065, p < .0001$). Sidak’s post hoc: 50 min: $p = .0046$; 60 min: $p = .0139$). (C) Twenty-eight hours of CORT secretion in WKY rats with an infusion of ketamine (10 mg · kg) during the active period. Mixed effects analysis detected a significant effect of time ($F_{3.208,12.03} = 5.176, p = .0148$), no effect of treatment ($F_{1,6} = .4216, p = .5402$), but an interaction ($F_{143,536} = 1.769, p < .0001$). (D) Corticosterone levels for 120 min following ketamine (10 mg · kg) infusion. Two-way ANOVA found a significant effect of time ($F_{12,60} = 3.956, p = .0002$), treatment ($F_{1,60} = 6.612, p = .0126$) and an interaction ($F_{12,60} = 3.603, p = .0005$). Sidak’s post hoc: 30 min: $p = .0004$; 40 min: $p = .0007$). (E) Twenty-eight hours of CORT secretion in WKY rats with an infusion of ketamine (5 mg · kg) during the active period. Mixed effects analysis detected a significant effect of time ($F_{3.874,18.88} = 5.972, p = .0003$), no effect of treatment ($F_{1,7} = 0.3886, p = .5528$), but an interaction ($F_{143,697} = 1.814, p < .0001$). (F) Corticosterone levels for 120 min following ketamine (5 mg · kg) infusion. Two-way ANOVA reported a significant effect of time ($F_{12,79} = 4.894, p < .0001$), no effect of treatment ($F_{1,79} = 1.03, p = .3132$) but a significant interaction ($F_{12,79} = 2.987, p = .0017$). Sidak’s post hoc: 30 min: $p = .0001$. Data are represented as mean ± SEM of 3–5 rats per treatment. Black bars indicate period of dark/active period in 24 h cycle. ANOVA, repeated measures-analyses of variance; WKY, Wistar–Kyoto.
ketamine infusion during the inactive and active periods, respectively. In A, Dunnett’s post hoc test was used to compare the peak levels to a control (vehicle) group. In C and D, mixed effects analysis (REML) was used to compare the effect of ketamine dose across time. Sidak’s post hoc test was used to compare effect of drug within time. Details of specific analyses are provided in the text and figure legends. Statistical significance was set at \( p < .05 \).

### RESULTS

#### 3.1 Characterization of endogenous corticosterone secretion in SD and WKY rats

Endogenous CORT secretion exhibits a circadian and ultradian pattern of secretion. In rodents, CORT levels are low during the inactive phase...
The WKY rat is used as an experimental model for depression, often characterized by altered HPA axis regulation. To assess if the WKY rat HPA axis function is different to that of the commonly used SD rat, blood was sampled every 10 min for 28 h. Mean plasma CORT concentration of SD (Figure 1A)—nondepressive-like model, and the WKY rat (Figure 1B), are shown. Perhaps unsurprisingly, the WKY rat displays normal HPA axis function, with clear circadian and ultradian rhythm. However, to assess if there are differences in the circadian secretion of CORT between strains, epochs of active (dark phase) and inactive phases (light phase) were measured (Figure 1C). We show a clear increase in CORT in both SD and WKY strains—we report a main effect of time \( F_{3,12} = 49.25, p < .0001 \). Yet, to address the potential elevation in circulating CORT as a characteristic of the WKY rat, we measured average CORT secretion between active and inactive phases (Figure 1D). We found a main effect of time \( F_{1,10} = 54.38, p < .0001 \), but only a trend toward differences in strain \( F_{1,10} = 4.132, p = .0695 \). This is interesting, as although the data did not reach significance, the elevated circulating CORT shown in the WKY rat may contribute to the endogenous phenotype it possesses. The WKY rat shows similar behavioral and neurobiological phenotypes to depression. We therefore continued with this endogenous model to identify the action of ketamine at different doses on HPA axis activity.

**3.2 | CORT response to ketamine infusion during the active phase in WKY rats**

Comparable to above, we measured endogenous CORT secretion over 28 h in WKY rats. In addition, these rats also received a bolus (10 min) subcutaneous infusion of ketamine (5/10/25 mg · kg) during the active phase—line with clinical treatment times. Importantly, infusion of vehicle did not elicit a CORT response. However, ketamine infusion increased CORT secretion rapidly with all doses (Figure 2A, C, E). Peak levels were not dose-dependent; however, the maintenance of circulating CORT was different across doses. We measured the levels of circulating CORT for 120 min post infusion. A high dose of ketamine significantly increased CORT secretion compared to vehicle infusion.
ketamine (25 mg · kg) delayed the increase in CORT secretion above vehicle until 50 min post infusion, before returning to baseline by 70 min (Figure 2B—25 mg · kg: Time; $F_{12,96} = 2.103, p = .0235$: Treatment; $F_{1,96} = 3.73, p = .0564$: Interaction; $F_{12,96} = 4.065, p < .0001, 50$ min: $p = .0045, 60$ min: $p = .0139$). Surprisingly, ketamine (10 mg · kg) increased CORT levels above vehicle faster than the higher dose (30 min), and was back to baseline by 50 min (Figure 2D—10 mg · kg: Time; $F_{12,60} = 3.956, p = .0002$: Treatment; $F_{1,60} = 6.612, p = .0126$: Interaction; $F_{12,60} = 3.603, p = .0005, 30$ min: $p = .0004, 40$ min: $p = .0007$). This effect was also evident with ketamine (5 mg · kg), where an increase in CORT secretion was observed only at 30 min (Figure 2F—5 mg · kg: Time; $F_{12,79} = 4.894, p < .0001$: Treatment; $F_{1,79} = 1.03, p = .3132$: Interaction; $F_{12,79} = 2.987, p = .0017, 30$ min: $p = .0001$). These data indicate a dose dependent effect on the maintenance of circulating glucocorticoids and suggest a differential timing of activity on the glucocorticoid receptor (GR) with ketamine treatment during the active period.

3.3 | CORT responses to ketamine infusions during the inactive phase in WKY rats

In addition to dose, the timing of ketamine delivery may prove critical for the regulation and activity of HPA axis function.25 For this, we assessed the response to the same doses of ketamine but this time during the inactive period (when endogenous circulating glucocorticoids are low). Again, importantly, and similar to above, infusion of vehicle did not induce a CORT response. However, ketamine increased CORT rapidly, maintained levels for an extended period and resulted in a dose dependent increase (Figure 3A,E). Interestingly, the CORT response was not only faster, but was maintained longer when ketamine (25 mg · kg) when infused during this period—increasing CORT above vehicle at 40 min, and not returning to baseline until 90 min (Figure 3B—25 mg · kg: Time; $F_{3,49522.72} = 5.936, p = .0027$: Treatment; $F_{2,9} = 37.87, p = .0002$: Interaction; $F_{12,78} = 6.218, p < .0001, 40$ min: $p = .0105, 50$ min: $p = .0217, 60$ min: $p = .0034, 70$ min: $p = .0084, 80$ min: $p = .0027, 90$ min: $p = .0015$). This was also evident with ketamine (10 mg · kg) (Figure 3D—10 mg · kg: Time; $F_{12,61} = 5.336, p < .0001$: Treatment; $F_{1,61} = 47.15, p < .0001$: Interaction; $F_{12,61} = 5.172, p < .0001, 20$ min: $p = .0004, 30$ min: $p = .0001, 40$ min: $p < .0001, 50$ min: $p = .0136, 60$ min: $p = .0028$) and (5 mg · kg) (Figure 3F—5 mg · kg: Time; $F_{12,48} = 7.241, p < .0001$: Treatment; $F_{1,48} = 32.79, p < .0001$: Interaction; $F_{12,48} = 6.940, p < .0001, 20$ min: $p = .0004, 30$ min: $p < .0001, 40$ min: $p < .0001, 50$ min: $p = .0143$).

3.4 | Time of day elicits differential CORT response to ketamine infusion

To identify if time of day influences the responsiveness of the HPA axis, we compared the peak CORT responses to ketamine during the active and inactive phases. We found during the inactive phase, ketamine increased maximal CORT in a dose dependent manner (Figure 4A—$F_{2,12} = 78.89, p < .0001$: 0 vs. 5: $p = .0182$; 0 vs. 10: $p = .0055$; 0 vs. 25: $p < .0001$). Interestingly, this was not evident across any dose during the active period (Figure 4B—$F_{2,13} = 1.469, p = .2637$: 0 vs. 5: $p = .2374$; 0 vs. 10: $p = .3205$; 0 vs. 25: $p = .9339$), suggesting that not only the dose, but the timing of ketamine infusion may be important. Toward this, we found that the peak CORT concentration was only different with ketamine (25 mg · kg) when comparing active and inactive period data (Figure 4C—Treatment; $F_{3,14} = 16.71, p < .0001$: Interaction; $F_{3,11} = 19.24, p < .0001, 25$ mg · kg: $p = .0001$). However, to delineate the action of ketamine on the CORT response, we needed to remove the underlying circadian regulation of CORT secretion. Here, we measured the concentration of CORT as a percentage of time-matched baseline and found that only when ketamine was infused during the inactive phase did it significantly increase the CORT response (Figure 4D—Time; $F_{1,25} = 216.6, p < .0001$: Treatment; $F_{3,25} = 85.53, p < .0001$: Interaction; $F_{3,25} = 85.74, p < .0001$: 5 mg · kg: $p = .0025; 10$ mg · kg: $p = .0005; 25$ mg · kg: $p < .0001$). This implies ketamine's effects on the HPA axis and GR activity may only be actionable if infused during the inactive period, when circulating endogenous glucocorticoids are low.

4 | DISCUSSION

In this study, we investigated HPA axis activity in response to ketamine treatment at different times of day in an endogenous model of depression. In over 30% of clinically depressed patients, antidepressants are ineffective.26–28 However, ketamine, an anesthetic since 1970, has rapid onset antidepressant effects in a cohort of treatment-resistant patients,29,30 yet its actions on HPA axis function are not fully resolved.

The HPA axis is a neurohormonal system that utilizes feed-forward and feedback loops to regulate glucocorticoid hormone levels. Within the hypothalamus, the paraventricular nucleus is highly responsive to external physiological stimuli such as the light/dark cycle, owing to the classically observed circadian release of glucocorticoids in timing with the day cycle.9 In addition to this, glucocorticoids can be released from the adrenal gland rapidly, through sympathetic splanchic nerve stimulation, often a result of acute stress. It is well established that glucocorticoids mediate the body's response to stress.9 CORT is the most common biomarker studied in depression, with evidence suggesting that depression is associated with attenuated circadian variation of cortisol, as well as hypercortisolemia.12,31,32

The WKY rat—initially developed as a control for the spontaneous hypersensitive rat—has been used as a model of depression because of its hyper-responsiveness to stress;16 yet the circadian variation and endogenous basal levels of corticosterone have not been determined.

We found no distinct differences in circadian release of CORT in the WKY strain (low in the inactive period, high in the active period). However, when we compared the levels to that of the commonly used SD rat, we saw a trend to an overall increase in circulating corticosterone. It is surprising that we did not find the reported increased baseline levels of corticosterone, however we employed an automated blood sampling system that allowed for blood collection from undisturbed rats.24,33 This suggests that the significant increase in CORT levels previously seen may be due to hyperactivity of the HPA axis.
when handling and/or blood sampling. That being said, studies have reported altered density of GRs in the WKY rat and decreased GR function contributes to HPA axis hyperactivity and the development of depressive symptoms, which may account for the depressive-like phenotype seen in these rats.

Studies have investigated the interaction of ketamine with the HPA axis, and although ketamine is regularly used to treat depression, the importance of timing—something critical to HPA axis function—has been overlooked. Ketamine acts as a rapid clinical antidepressant, as fast as 30 min following injection at 0.5 mg·kg in humans (10 mg·kg in rat). Yet whether the responsiveness of classical depression biomarkers such as CORT and HPA axis function differs across time is unknown. Studies suggest antidepressants modulate the GR. For instance, the therapeutic action of antidepressants can restore GR function.

Importanty, stressors can stimulate the sympathetic nervous system to rapidly activate the HPA axis, release noradrenaline from the locus coeruleus, and increase heart rate. A large body of work has studied the effects of ketamine on the sympathetic nervous system, suggesting that ketamine's action on corticosterone secretion may also be mediated via sympathetic nervous action, although in these studies anaesthetic doses of ketamine were used. Dissociating the actions of subanesthetic doses of ketamine on sympathetic action and its influence on HPA axis function warrants further investigation.

In this study, using repeated blood sampling in undisturbed, freely behaving rats, we report robust secretion of corticosterone in rats infused with subanesthetic ketamine doses. Interestingly, our findings support the response being a direct result of HPA axis activity, rather than sympathetic action on adrenal activity as our data show a clear delay in CORT response—with a significant increase first observed 20 min following infusion. However, in our study, ketamine is delivered subcutaneously, and the delay in HPA axis response may be the result of a delay in ketamine reaching circulation. Yet, the differential time of day CORT response suggests this is not the case.

Crucially, our data suggests that the timing of ketamine administration may be critical to HPA axis activity. We found that with equivalent doses of ketamine, corticosterone levels increased substantially if ketamine was administered during the inactive period. During this period, glucocorticoids are not routinely bound to the receptor. Several studies report that depressed patients have GR resistance (i.e., reduced GR function). Indeed, most antidepressants increase GR-mediated gene transcription and GR function, which in turn is associated with enhanced GR-mediated negative feedback by glucocorticoids. Therefore, the sudden increase in circulating CORT during the inactive period (or when circulating levels are low) may act beneficially to bind to GRs to mediate negative feedback inhibition of the HPA axis to maintain low glucocorticoid levels under normal physiological conditions.

In summary, interactions between cortisol and ketamine have been reported yet these studies measure responses over short periods of time, and potentially miss long term activity of the HPA axis. In this study, we found that the HPA axis response to ketamine was far stronger when ketamine was administered during the inactive period. These findings suggest that the timing of ketamine administration may be an important factor to consider when evaluating treatments for depression.

**AUTHOR CONTRIBUTIONS**
Matthew Birnie: Conceptualization; data curation; formal analysis; methodology; writing – original draft; writing – review and editing.
Alen V. Eapen: Data curation.
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**CONFLICT OF INTEREST**
The authors declare no competing interests.

**PEER REVIEW**
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**DATA AVAILABILITY STATEMENT**
Raw data were generated at the University of Bristol, UK. Derived data supporting the findings of this study are available from the corresponding author [MTB] on request. cd_value_code="text

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