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Gauging circadian variation in ketamine metabolism by real-time breath analysis

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Time-of-day of drug application is an important factor in maximizing efficacy and minimizing toxicity. Real-time *in vivo* mass spectrometric breath analysis of mice was deployed to investigate time-of-day variation in ketamine metabolism. Different production rates of ketamine metabolites, including the recently described anti-depressant hydroxynorketamine, were found at opposite circadian phases. Thus, breath analysis has potential as a rapid and 3Rs (Replacement, Reduction and Refinement) conforming screening method to estimate time-dependence of drug metabolism.

Mammalian physiology and behaviour are modulated by biological clocks preparing and adapting the organism to the 24 h cycles of day and night in the environment. These so-called circadian clocks are present in virtually all mammalian cells and modulate not only levels of endogenous metabolites in mice[1] and human beings,[2] ~~,~~but also the metabolism of xenobiotics.[3] For example, the hepatocyte clock is critically important for daily variation in CYP P450 dependent drug metabolism.[4] Chronotherapy aims to capitalize on these rhythms by maximizing drug efficacy and minimizing toxicity through adjusting dosing-time.[3] As a result, chronobiology is playing a growing role in drug development to address the ~~optimization~~ optimisation of the most beneficial time to administer drugs.[5] However, studying the impact of time-of-day of dosing in drug metabolism represents a challenge in the already congested pipeline of typical drug development workflows. Thus, the development of novel methods to rapidly assess timing in drug development is of high interest.

The use of sensitive gas-trace analysers to measure metabolites emitted by unrestrained mice is an attractive approach to study *in vivo* metabolism.[6] Recently, we have shown that secondary electrospray ionization (SESI)[7] coupled with high resolution mass spectrometry (HRMS) provides sufficient sensitivity and selectivity to track in vivo and in real time the metabolism of drugs in mice breath, which correlate with blood levels.[8] The proposed method enables capturing pharmacokinetic profiles of injected drugs in real time with a time resolution of 10 s from a single mouse in a comparably stressless procedure (Fig. S1, ESI†). Here, we show that the SESI-HRMS technique can also contribute to rapidly assessing the impact of dosing time drug metabolism. In view of recent findings suggesting that hydroxynorketamine (HNK), a metabolite of ketamine, is crucial for ketamine's immediate anti-depressant effects but lacks the psychotic side-effects of its parent compound,[9] we ~~payed~~ paid particular attention to the response of this metabolite.

The abundance of ketamine as well as its major metabolites was tracked in the breath of adult male C57BL/6J congenic (referred to as wild-type, WT) mice immediately after ketamine injection (30 mg/kg). Fig. 1 ~~normal~~shows the real-time pharmacokinetic profiles of HNK either during the rest phase (~~ZT22-2,~~ early morning), or early active phase (evening~~ZT10-14, early night~~) for two individuals from each phase~~in two individuals each~~. Of note, mice from morning and evening groups were measured in alternation to avoid potential confounding effects due to carry-over and instrument drifts with time. The time-to-peak for this particular dose was found to be around 25-30 min, which is fully consistent with our previous results.[8] In contrast, however, a clear difference in ketamine metabolism was observed. Evening injection of ketamine leads to two-fold higher HNK levels than in the morning. The same trend was observed for further ketamine metabolites (*i.e.*, norketamine, hydroxyketamine, and dehydronorketamine~~; Fig S2, ESI†~~).

The results shown in Fig. 1 suggest that ketamine metabolism is dependent on time-of-day. This is in line with previous observations on the time-of day variation of the hypnotic effects ("sleep time") of ketamine in rodents,[10] ~~;~~ early morning injection of ketamine led to longer sleep time compared to evening injection. In line with these results, we found higher

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levels of ketamine metabolites after evening dosing suggesting faster ketamine metabolism in the evening and therefore shorter sleep time.

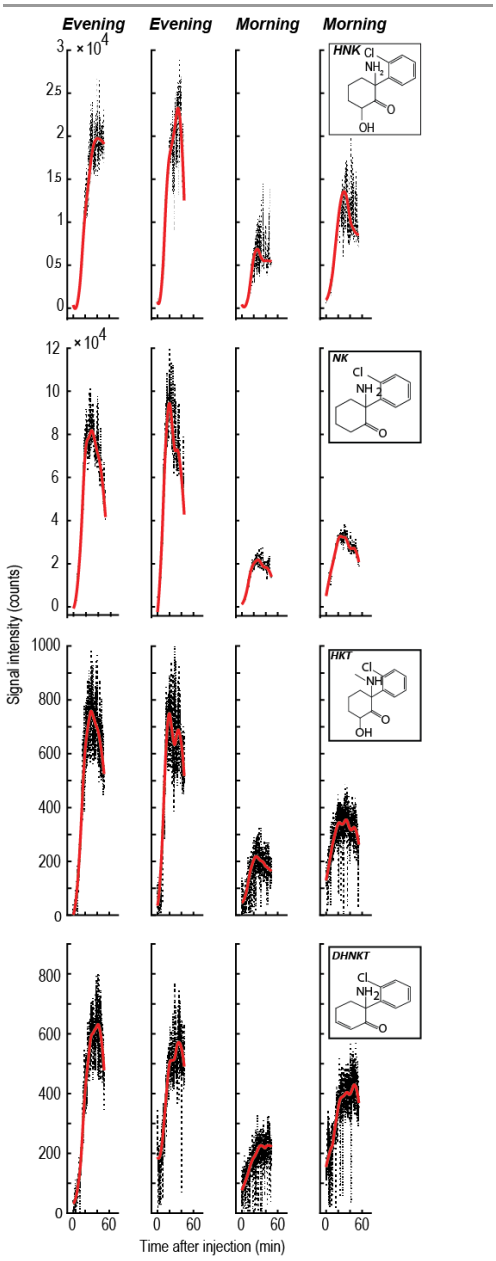
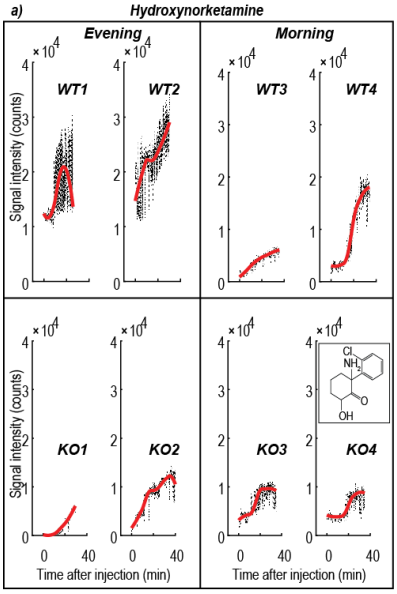


Fig. 1. Different ketamine metabolism depending on internal time. The traces correspond to exhaled hydroxynorketamine (~~structure displayed~~HNK), norketamine (NK),

hydroxyketamine (HKT) and dehydronorketamine (DHNKT) for four wild-type mice: two



of them measured in the evening and the other two in the opposite circadian phase. Black dots represent raw data (5 s time resolution) and red traces are smoothed curves.

Ketamine is metabolized by liver microsomal cytochrome (CYP) P450,[11] which are under circadian control.[12] However, it is unclear whether the daily variation in its hypnotic effect is dependent on circadian control of drug metabolism or, as hypothesised elsewhere, the availability of its receptor target in the brain.[10a] To confirm whether the liver clock is necessary to create the time-of-day effect (Fig. 1), we repeated the our measurements in wild-type (WT) and congenic in addition we included mice specifically lacking a functional liver clock, *i.e.* mice with liver-specific deletion of the core clock gene *Bmal1* ((referred to as knock-out, KO). Figure 2a shows the time traces of HNK in twowild-type and knock-out mice either with or without liver clock injected and measured either in the morning or evening. Consistent with Fig. 1, we observed a clear diurnal difference in drug metabolism in wild-type mice depending on the time of administration. In contrast, this difference was absent in liver clock deficient miceKO mice did not show injection time dependent differences indicating a loss of circadian control of metabolism. In fact, The same results are observed for the more abundant norketamine metabolite (Fig. 2b). Ketamine metabolism in knock-outliver specific BMAL1 deficient mice wshowedas similar to the lower morning levels in wild-type at all timesketamine metabolism and this suggests CYP P450 activity at all times, which has also been observed after liver specific deletion of its binding partner CLOCK. In line with previous studies[4], this suggests lower CYP P450 activity in knock-out mice.[4] The difference between WT and KO is even morealso clear for the more abundant norketamine metabolite (Fig. S32b). Thus, we conclude that the circadian metabolism of ketamine is likely modulated by the liver clock. Of note, our data do not exclude that the variation in the hypnotic effects of ketamine are also due to NMDA receptor availability rather than metabolism alone.[10a]

One of the main advantages of our method is that hundreds of exhaled metabolites are monitored simultaneously. Thus, apart from tracking the pharmacokinetic profiles of known xenobiotic metabolites (*e.g.*, HNK), it offers an opportunity to explore – in an untargeted fashion – endogenous metabolites either altered as a result of drug administration or representing the physiological state of the animal. A closer inspection of the mass spectra revealed that, apart from the noted differences observed for ketamine and its main metabolites, a set of compounds showed a marked variation with time-of-day of drug administration in wild-type mice. For example, Figureg. 3 shows the the time traces of one compound example compound displaying an inverse time-of-day dependent variation in abundance measured when measured at the same times as the data mice presented in Fig. 1. By HRMS, this compound was assigned a molecular formula of The high mass accuracy and resolution of the mass spectrometer enabled assigning the molecular formula for this compound as $C_{16}H_{28}N_2O_2$.

Fig. 2. Hydroxynorketamine (a) and norketamine (b) breath levels for WT measured in the evening (upper-left), WT measured in the morning (upper-right), KO measured in the evening (bottom-left) and KO measured in the morning (bottom-right). Each curve per metabolite corresponds to one mouse (n = 8). The data suggests that the time-dependent sensitivity to ketamine is dictated by the liver clock. Black dots represent raw data and red traces the corresponding smoothed curves

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In total, we observed 24 compounds not reported, to the best of our knowledge, as ketamine metabolites that showed daily variations similar or in anti-phase to that of ketamine. Figure 3 provides an overview of the temporal evolution variation in of these compounds. It shows the heatmap and hierarchical cluster analysis for these 24 compounds found to have a distinct behaviour depending on the circadian phase with diurnal regulation. The Cluster analysis revealed 11 compounds increased in the morning mice and 13 compounds relatively increased in evening mice. Interestingly, among the compounds relatively elevated in in the mice injected with ketamine in the morning, we found a clear series of compounds clustering closely together. For example, $C_{16}H_{28}N_2O_2$, $C_{21}H_{38}N_2O_2$, $C_{21}H_{38}N_2O_3$ and $C_{17}H_{30}N_2O_3$. Table S1 lists the measured accurate masses and Figure S2 shows some representative mass spectra. $C_6H_6N_2O_2$, $C_{15}H_{26}N_2O_2$, $C_{16}H_{26}N_2O_2$, $C_{16}H_{28}N_2O_2$, $C_{16}H_{30}N_2O_2$, $C_{21}H_{38}N_2O_2$, $C_{17}H_{30}N_2O_3$ and $C_{21}H_{38}N_2O_3$. Similarly, families of metabolites (*e.g.*, aminoacids metabolism), have been shown to be altered shortly after ketamine administration.[13] However, while the molecular formulae in our study can be assigned with high confidence, positive identification of these altered metabolites remains to be accomplished.

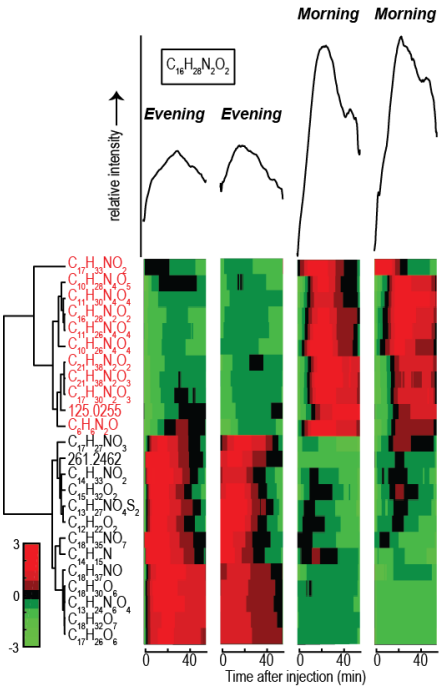

Fig. 3 The levels of other metabolites apart from those showed in Fig. 1 and S2 also showed a circadian behaviour. The heatmap provides an overview of 24 compounds found to have a distinct behaviour depending on time-of-dose. On the top the actual (smoothed) time traces of one such compound with molecular formula $C_{16}H_{28}N_2O_2$ are shown. In contrast with hydroxyketamine, this compound was produced in higher abundance in mice injected in the morning. Note also the series of compounds with closely related molecular formulas showing similar responses.

In conclusion, SESI-HRMS has potential not only to assess pharmacokinetic profiles by analysis of exhaled breath in mice, but also to further investigate the impact of time of-day on drug behaviour, *i.e.*, pharmacokinetics and metabolism. Our results suggest that ketamine does exhibit circadian variation in metabolism, which leading is especially apparent in to the widely differing levels of metabolites observed at different circadian times. Furthermore, these differences are liver clock dependent, because animals that lack a functional hepatocyte clock lack this variation. Interestingly, hydroxynorketamine has recently been shown to have an immediate anti-depressant effect like ketamine but without any of the psychotic side-effects.[9] Our data suggest that time-of-dosing should be considered in its future clinical development. In addition, 24 other unidentified endogenous metabolites showed a marked circadian response. Our method shows potential as a rapid and animal-friendly screening method, for example, to optimise chronotherapeutic regimes and monitor drug and metabolite levels in multiple dosing schedules in a single animal. It significantly advances two of the 3Rs[14], *i.e.*, *refinement* because the blood sampling is circumvented and *reduction* because multiple measurements can be taken from one animal.

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