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

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The 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 in opposite circadian phases. Thus, breath analysis has potential as a rapid and 3Rs (Replacement, Reduction and Refinement) conforming screening method to estimate the 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 optimisation of the most beneficial time to administer drugs.[5] However, studying the impact of the time-of-day of dosing on 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 mouse breath, which correlates with blood levels.[8] The proposed method enables capturing the 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 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^{-1}). Fig. 1 shows the real-time pharmacokinetic profiles of HNK either during the rest phase (early morning) or during the early active phase (evening) for two individuals from each phase. Of note, mice from morning and evening groups were tested 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 other ketamine metabolites (*i.e.*, norketamine, hydroxyketamine and dehydronorketamine).

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

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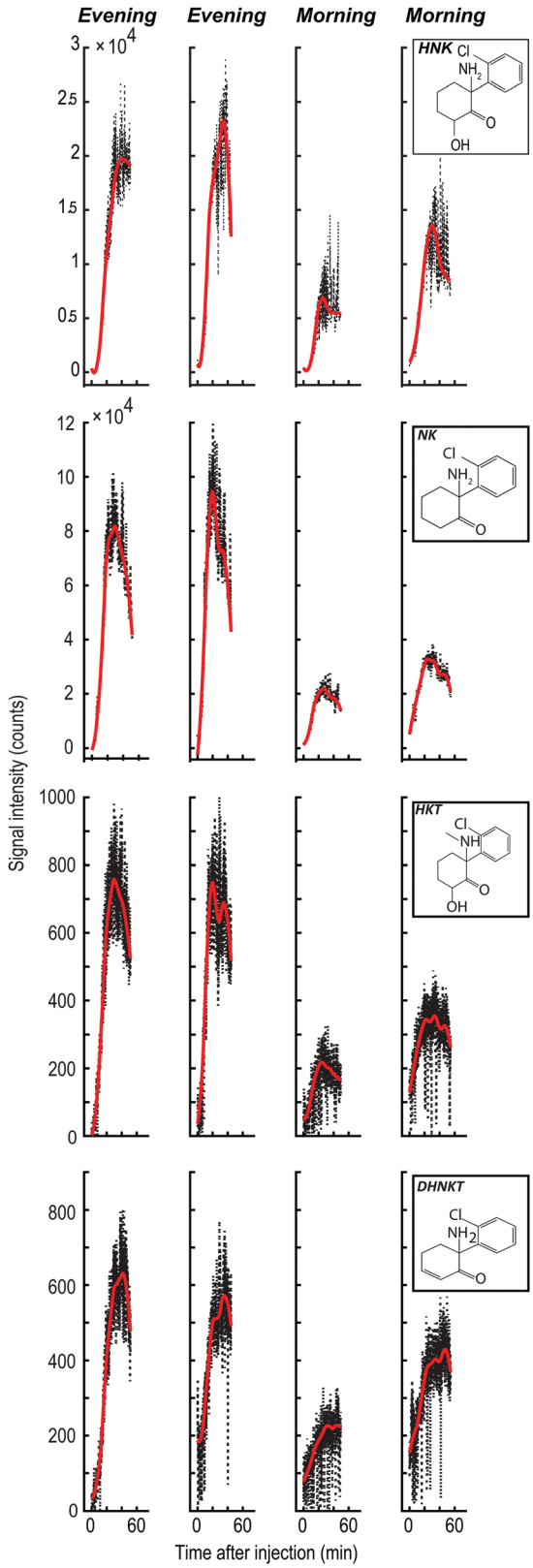

Fig. 1 Metabolism of different ketamine metabolites depending on internal time. The traces correspond to exhaled hydroxynorketamine (HNK), norketamine (NK), hydroxyketamine (HKT) and dehydronorketamine (DHNKT) for four wild-type mice: two of them measured in the evening and the other two in the opposite 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 is under circadian control.[12] However, it is unclear whether the daily variation in its hypnotic effect is dependent on the 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 our measurements in wild-type and congenic 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). Fig. 2a shows the time traces of HNK wild-type and knock-out mice injected either in the morning or in the evening. Consistent with Fig. 1, we observed a clear diurnal difference in drug metabolism in wild-type mice. In contrast, KO mice did not show injection time dependent differences. The same results are observed for the more abundant norketamine metabolite (Fig. 2b). Ketamine metabolism in knock-out mice was similar to the lower morning levels in wild-type mice at all times. In line with previous studies,[4] this suggests lower CYP P450 activity in knock-out mice. 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 is 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. Fig. 3 shows the time traces of one example compound displaying a time-of-day dependent variation in abundance when measured at the same times as the mice presented in Fig. 1. Using HRMS, this compound was assigned a molecular formula of $C_{16}H_{28}N_2O_2$.

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. Fig. 3 provides an overview of the temporal variation in these compounds. It shows the heatmap and hierarchical cluster analysis for these 24 compounds with diurnal regulation. Cluster analysis revealed that 11 compounds increased in the morning-injected mice and 13 compounds relatively increased in the evening-injected mice. Interestingly, among the compounds relatively elevated 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 (ESI†) lists the measured accurate masses and Fig. S2 (ESI†) shows some representative mass spectra. Similarly, families of metabolites (*e.g.*, aminoacid 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,

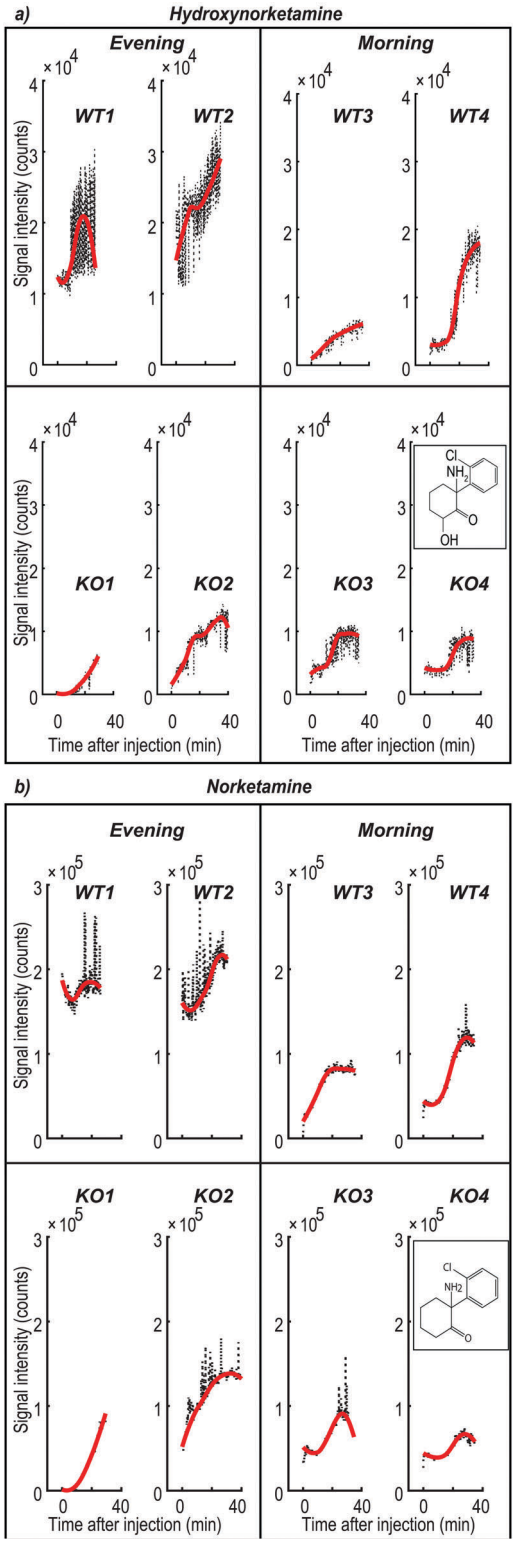

Fig. 2 Hydroxynorketamine (a) and norketamine (b) breath levels for WT measured in the evening (top-left), WT measured in the morning (top-right), KO measured in the evening (bottom-left) and KO measured in the morning (bottom-right). Each curve per metabolite corresponds to one mouse (n = 8). The data suggest 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.

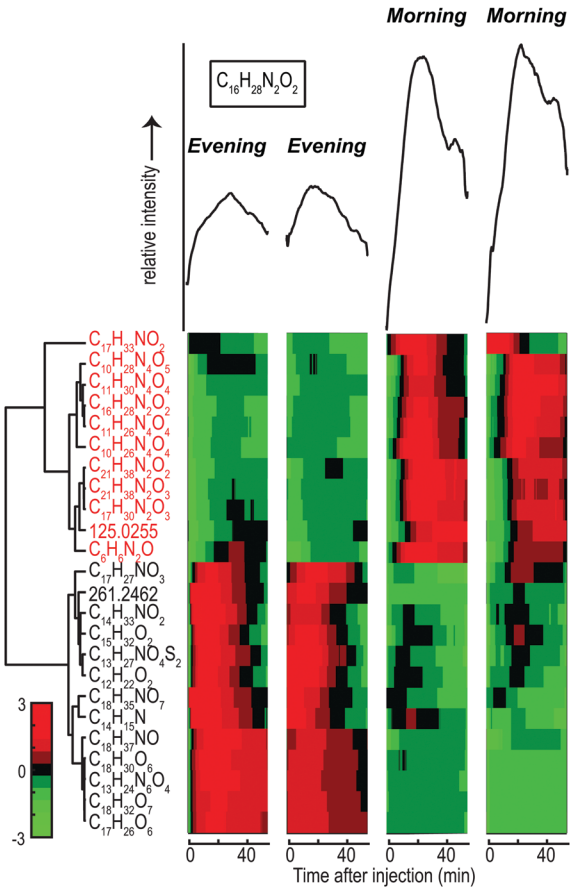

Fig. 3 The levels of other metabolites apart from those shown in Fig. 1 also showed a circadian behaviour. The heatmap provides an overview of 24 compounds found to have a distinct behaviour depending on the time-of-dose. On the top the actual (smoothed) time traces of one such compound with a molecular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

positive identification of these altered metabolites remains to be accomplished.

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, leading to 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 blood sampling is circumvented) and reduction (because multiple measurements can be taken from one animal).

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