LABORATORY INVESTIGATIONS

Chronopharmacological studies of ketamine in normal and NMDA ε1 receptor knockout mice†

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Background. The effectiveness and toxicity of many drugs depends on the dosing-time schedule, relative to the circadian rhythms of biochemical, physiological, and behavioural processes. Previous studies have found chronopharmacology of ketamine, which is a N-methyl-D-aspartate (NMDA) receptor antagonist. The in vivo contribution of the NMDA receptor ε1 subunit (NR2A) in this effect is unclear.

Methods. In the present study, daily variations in the hypnotic effect of ketamine were determined in wild-type mice and NMDA ε1 knockout (KO) mice.

Results. The effect of ketamine had a definite daily variation in wild-type mice. No significant difference in blood concentration was observed at different dosing times (10:00 and 22:00). In NMDA receptor ε1 KO mice, the hypnotic effect of ketamine was weaker than in wild-type mice and there was no dependence on the time of administration. Significant pharmacokinetic differences were not observed between wild-type and KO mice.

Conclusions. The enhanced hypnotic effect in the active phase of the circadian cycle is likely a result of changes with the time of day in the susceptibility of the central nervous system to ketamine. Knockout of the NMDA receptor ε1 subunit gene markedly reduced the effect of ketamine, and eliminated the time-dependent sensitivity to ketamine.

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Most organisms have biological rhythms synchronized to the natural light–dark cycle of approximately 24 h, known as circadian rhythms. Many drugs show differences in their efficacy or toxicity that depend on the time of their administration during the day.1 The study of this effect is known as chronopharmacology, focusing on temporal rhythmic variations in pharmacokinetics and pharmacodynamics. In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is an oscillator of locomotor activity and various physiologic phenomena.2,3 The recent isolation of the clock genes Per1, Per2, Per3, Tim and Cry from humans and mice has clarified the molecular mechanisms of the circadian clock.4–6 It has been shown that these genes are rhythmically expressed in both the SCN and also peripheral organs.7

Some i.v. anaesthetics are believed to act in an agent-specific fashion, by activating or inhibiting synaptic transmission or both.8 Ketamine is a widely used anaesthetic that exerts its depressant effect by reducing neuronal excitation via the N-methyl-D-aspartate (NMDA) receptor as a non-competitive antagonist and mediating sympathetic responses. The NMDA receptor channel is formed from at
least two families of subunits, the ε1–4 (NR2A–D) and ζ (NR1). The anaesthetic effect of ketamine is therefore believed to correlate with the circadian rhythms of neural activities; previous studies have demonstrated that the pharmacological response to ketamine shows daily variation.10 11 However, although in vitro studies showed ε1–4 subunits are involved in the effect of ketamine,12 the in vivo contribution of the NMDA receptor ε1 subunit to this chronopharmacological phenomenon is unclear.

In the present study we examined the chronopharmacological effect of ketamine and the resulting pharmacokinetics in wild-type mice. We then investigated the hypnotic effect of ketamine in NMDA receptor ε1 subunit knockout (KO) mice at different times of the day.

Materials and methods

Animals

We used 8–10-week-old male C57BL/6 (CLEA Japan Inc., Tokyo, Japan), wild-type (ε1 +/+ ) mice, and mice lacking the NMDA receptor ε1 subunit gene (ε1 −/− ) weighing 20–25 g. Both ε1 +/+ and ε1 −/− mice with highly homogenous genetic background have been described previously.13–15 Although impairment of hippocampal long-term potentiation and of spatial learning were observed in these KO mice, no abnormal motor function was observed under physiological conditions.13–15 These wild-type and KO mice were maintained in a pathogen-free room in a controlled environment (22 (2)°C room temperature, light/dark cycle (12 h each), light on at 07:00). During the experiments each mouse was housed in its own cage, and food and water were provided ad libitum. All experiments were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Measurement of daily variation of water intake in wild-type and NMDA receptor ε1 subunit KO mice

Water intake of experimental wild-type and NMDA receptor ε1 subunit KO mice was measured by high-performance liquid chromatography (HPLC) as described).19–21 Mice were killed at 22:00 and live samples were obtained.

Measurement of the effect of ketamine dosing at different times of the day

Ketamine (Sankyo Co., Ltd, Tokyo, Japan) diluted in saline was administered intraperitoneally. The volume injected was standardized to 0.2 ml·animal−1 in each experiment. The hypnotic effect was evaluated as ketamine-induced sleep times using the duration of loss of the righting reflex (LORR) after injection of 200 mg·kg−1 ketamine. Each mouse received a single dose of ketamine. LORR was defined as having occurred when the mouse failed to right itself for at least 10 s after being placed on its back. Recovery from the LORR was defined as having occurred when the mouse spontaneously righted itself.18 During anaesthesia the animals were kept warm on a plate heated to 38°C. To examine the daily variation of effect, ketamine was injected at 04:00, 10:00, 16:00, and 22:00 in wild-type mice (n=12 in each group). To analyse the effect of the dosing time in KO mice, ketamine was injected at 10:00 and 22:00 (n=12). The investigators were blinded to the experimental groups.

Measurement of blood plasma concentrations of ketamine and cytochrome P450 enzymes in liver

To compare the pharmacokinetics of drug administrations at 10:00 and 22:00, the blood concentration of ketamine was measured in wild-type mice. Mice were killed at each time point, and blood samples were obtained at the time of the i.p. injection and at 10, 20, 40, and 60 min after i.p. injection of ketamine 200 mg·kg−1 at 10:00 and 22:00 (n=8 in each group). The concentration of ketamine in plasma was determined by high-performance liquid chromatography.19 Since ketamine is metabolized by cytochrome P450s and then eliminated,19–21 we investigated the activity of total CYPs and isoenzymes (CYP2B6, CYP2C8/9, and CYP3A) in wild-type and KO mice to examine if the hepatic metabolism in KO mice was different from that of wild-type mice. Activity of total and isoenzymes of CYPs in the liver of wild-type and KO mice were measured by high-performance liquid chromatography (n=5 in each group as described).19–21 Mice were killed at 22:00 and live samples were obtained.

Statistical analysis

Statistical analyses were performed using t-test or analysis of variance (ANOVA). Laboratory data are expressed as the mean (SD). Single cosinor method was used to analyse the circadian rhythm.22–23 This method involves fitting to a curve of predefined period by the method of least squares.22–23 A value of P<0.05 was considered statistically significant. For analysis of dosing time-dependent effect of ketamine in wild-type and KO mice, a value of P<0.0125 was considered statistically significant by Bonferroni correction.

Results

Daily variation of water intake in wild-type and NMDA receptor ε1 subunit KO mice

To determine circadian locomotor activities of experimental wild-type and NMDA receptor ε1 subunit KO mice, water
intake was measured every 6 h. In both wild-type and KO mice, water intake showed similar circadian patterns (Fig. 1). Water consumption peaked between 19:00 and 07:00 in each dark phase, when the mice are active (mice are nocturnal), and was least between 07:00 and 19:00 in each light phase (Fig. 1), corresponding to the daily locomotor rhythm in both wild-type and KO mice.

Daily variations in hypnotic effect of ketamine in wild-type mice

The effect of ketamine dosing at different times of the day was determined by measuring the duration of LORR. In this experiment, ketamine was injected at 04:00, 10:00, 16:00, and 22:00. The hypnotic effect in wild-type mice differed according to the time of administration, and a significant circadian rhythm was observed using single cosinor analysis ($P<0.001$) (Fig. 2). The result of the fitted cosine function indicated that the longest sleep time was in the early active phase (22:00) and the shortest was in the early inactive phase (10:00). Comparison of the duration of LORR at 10:00 and 22:00 also showed a significant difference ($P=0.001$) (Fig. 2). The pattern of daily variation in the effect of ketamine was similar to that of the circadian locomotor rhythm shown in Figure 1.

Plasma blood concentration of ketamine at different dosing times in wild-type mice

To evaluate whether the observed circadian rhythm was caused by temporal rhythmic variations in pharmacokinetics, we examined plasma concentration of ketamine at different dosing times. Figure 3 shows curves of the mean ketamine concentration vs time in the two different dosing-time groups. No significant difference was observed between the two groups. The data therefore suggest that the pharmacokinetics of ketamine do not underlie the circadian variation in the effect of the anaesthetic.

Inhibition of dosing time-dependent effect of ketamine in NMDA receptor $\varepsilon_1$ subunit KO mice

We compared the hypnotic effects of ketamine in wild-type mice and NMDA receptor $\varepsilon_1$ subunit KO mice at 10:00 and 22:00 dosing times, as cosinor analysis showed ketamine was most effective in wild-type mice when administered at
22:00, and least effective at 10:00. The mean sleep time was shorter in KO mice than in wild-type mice at both dosing times (Fig. 4). However, although the mean duration of LORR was significantly shorter in the NMDA receptor ε1 subunit KO mice than in wild-type mice at 22:00 ($P<0.001$), no significant difference was observed at 10:00 (Fig. 4). Additionally, while significant dosing time dependency was observed in wild-type mice ($P=0.001$) a significant difference in the hypnotic effect was not found between the two injection times in KO mice. Error bar, SD ($n=12$ in each group).

**CYP activity in the liver of wild-type and KO mice**

Since ketamine is metabolized by cytochrome P450s and eliminated in the liver,19 21 we investigated whether the significant differences found in anaesthetic effects at 22:00 between wild-type and NMDA receptor ε1 subunit KO mice showed any dependence on hepatic metabolism. The apparent levels of total CYPs and isoenzymes (CYP2B6, CYP2C8/9, and CYP3A) activity were not different between the wild-type mice and the NMDA receptor ε1 subunit KO mice (Table 1). We also determined the blood concentration of ketamine 10 and 60 min after i.p. injection of ketamine 200 mg·kg$^{-1}$ in KO mice at 22:00 ($n=4$) and confirmed that pharmacokinetic difference was not observed between wild-type and KO mice (data not shown). These findings suggested that the differing anaesthetic effects were a result of pharmacodynamic differences, rather than pharmacokinetics.

**Table 1** Cytochrome P450 (CYPs) enzyme activity in wild-type and NMDA receptor ε1 subunit KO mice at 22:00. Values are mean (SD)

<table>
<thead>
<tr>
<th></th>
<th>Total CYPs</th>
<th>CYP2B6</th>
<th>CYP2C8/9</th>
<th>CYP3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pmol·mg$^{-1}$)</td>
<td>(pmol·mg$^{-1}$·min$^{-1}$)</td>
<td>(pmol·mg$^{-1}$·min$^{-1}$)</td>
<td>(pmol·mg$^{-1}$·min$^{-1}$)</td>
<td></td>
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<tr>
<td>0.38 (0.10)</td>
<td>96.7 (45.2)</td>
<td>19.6 (7.5)</td>
<td>0.65 (0.14)</td>
<td></td>
</tr>
<tr>
<td>0.29 (0.09)</td>
<td>122.0 (11.1)</td>
<td>23.1 (10.4)</td>
<td>0.84 (0.14)</td>
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**Discussion**

Chronopharmacological assessment of many agents has found recently that drug efficacy depends on the time of administration.10 11 24±27 Circadian effects in anaesthesia have been discussed for decades and a chronopharmacological effect of ketamine has been reported.10 11 28 29 However, little is known about the contribution of the NMDA receptor to the chronopharmacology of ketamine. In the present study, we have found that there is a marked dosing time-dependent effect of ketamine in wild-type, but not in NMDA receptor ε1 subunit KO mice.

In wild-type mice, ketamine administered during the active phase-induced longer sleep, which we take as a surrogate measure of the hypnotic effect of ketamine, than in the inactive phase. The pattern of daily variation in the chronopharmacological effect of ketamine is similar to that of circadian locomotor activity, corresponding to water intake. Because changes in drug effectiveness based on pharmacokinetics depend on the time of administration,30 31 it is possible that pharmacokinetic rhythms contribute to variations in the response to drugs. We accordingly investigated blood plasma concentrations of ketamine at the time of administration and at 10, 20, 40, and 60 min after administration in both the active and inactive phases in wild-type mice. There was no significant dosing time-dependent difference in plasma pharmacokinetics between the phases. However, sleep time differed significantly between the two wild-type groups, and most mice given an injection at 10:00 were already awake 60 min later. These findings suggest that the enhanced effect of ketamine in the active phase is more likely a result of the enhanced susceptibility or sensitivity of the central nervous system rather than the result of differences in pharmacokinetics in wild-type mice.

It has been suggested that the anaesthetic effects of ketamine are mediated through block of NMDA receptor channels, which are a subtype of glutamate receptors.32 33 NMDA receptors play an important role in excitatory neurotransmission.9 They are ligand-gated cation channels with high Ca$^{2+}$ permeability, and are a combination of a ζ (NR1) subunit and any one of the ε1–4 (NR2A–D) subunits.9 Analysis of recombinant receptors expressed in *Xenopus oocytes* indicates that ketamine blocks the four ε/ζ channels to a similar extent.12 To determine the contribution of the NMDA receptor ε1 subunit to the hypnotic effect of ketamine in *vivo*, we examined ketamine-induced sleep
times using LORR and compared the hypnotic effect of ketamine in wild-type mice and in mutant mice lacking the NMDA receptor ε1 subunit. In these KO mice, no abnormal development or abnormal motor function has been reported under normal conditions.\(^{13} 15\text{--}17\) Our results showed that the effect of ketamine on the NMDA receptor ε1 subunit KO mice was significantly weaker at the time of day that ketamine was most effective in wild-type mice. Because the levels of CYPs and the blood concentration of ketamine after i.p. injection were not different from those in wild-type mice at 22:00, the difference in hypnotic effectiveness could be a result of sensitivity differences of the CNS resulting from disruption of the NMDA receptor ε1 subunit gene, rather than pharmacokinetic differences. Consequently, the data suggest that the hypnotic effect of ketamine involves an NMDA receptor channel, comprising the ε1 subunit, and NMDA receptor ε1 subunit contributes to the dosing time-dependent effect of ketamine.

As NMDA receptor antagonist inhibited the light-induced phase shift of locomotor activity rhythm, it might be possible that NMDA receptors mediate photic entrainment of the biological clock in the SCN.\(^{14\text{--}37}\) It has been shown that glutamate also increases the amplitude of the circadian of the biological clock in the SCN.\(^{34\text{--}37}\) It has been shown that glutamate also increases the amplitude of the circadian clock-associated gene products Per1 and Per2; either or both of these may be critical for photic phase shifts.\(^{17\text{--}38\text{--}39}\) Thus, although the similar circadian patterns of water consumption in NMDA receptor ε1 subunit KO mice and wild-type mice suggest that abrogation of the dosing time-dependent hypnotic effect of ketamine in KO mice was caused by the NMDA receptor ε1 subunit related hypnotic pathway rather than a circadian pathway, anaesthetic drugs such as ketamine or knocking out the NMDA receptor ε1 subunit might modify the circadian oscillation of the clock genes to change their expression in the SCN in a dosing time-dependent fashion.

To conclude, we have shown that the hypnotic action of ketamine has a marked dosing time-dependent effect. This might be the result of daily variations in anaesthetic sensitivity in the CNS rather than pharmacokinetic variation. Our findings also indicate that the NMDA receptor ε1 subunit is involved in the anaesthetic effect of ketamine, and plays a major role in the induction of the dosing time dependency.

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