Heat injury-induced drop of the noxious heat threshold measured with an increasing-temperature water bath: A novel rat thermal hyperalgesia model

Kata Bőleskei, Dóra Horváth, János Szolcsányi*, Gábor Pethő

Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Pécs, Szegedi u. 12., H-7624 Pécs, Hungary

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Abstract

Conventional thermoneciceptive tests are based on measurement of the latency of nocifensive reactions evoked by constant, suprathreshold heat stimuli. In the present study, a novel, increasing-temperature water bath was developed for determination of the noxious heat threshold temperature of lightly restrained conscious rats. One of the hindpaws of a rat was immersed into the water bath whose temperature was increased from 30 °C at a rate of 24 °C/min until the animal withdrew its hindpaw from the water. The corresponding bath temperature was considered as behavioural noxious heat threshold. The heat threshold of untreated rats was 43.5±0.4 °C (n=10) and was reproducible upon repeated measurements at intervals of 10 min for 60 min. Thermal hyperalgesia was induced by mild heat injury (51 °C water for 20 s) which led to a 7–8 °C decrease of the noxious heat threshold. Thermal hyperalgesia was detected at least for 60 min after heat injury. Morphine, diclofenac, ibuprofen and paracetamol administered intraperitoneally 20 min after heat injury dose-dependently inhibited the drop of heat threshold with minimum effective doses of 0.3, 0.3, 10 and 30 mg/kg, and ED50 values of 0.5, 3, 18 and 100 mg/kg, respectively. Thermal hyperalgesia was also decreased by intraplantar treatment with morphine (10 μg), diclofenac (10 μg) or ibuprofen (100 μg). In conclusion, the mild heat injury-induced drop of the noxious heat threshold measured with the increasing-temperature water bath is a novel thermal hyperalgesia model highly sensitive to both opioid and non-opioid analgesics upon systemic or local administration.

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1. Introduction

Conventional methods for the study of thermoneciception in conscious animals (hot plate, tail flick, plantar test) measure the latency of nocifensive (pain-avoiding) behavioural reactions evoked by heat stimuli of suprathreshold intensity (for review see Le Bars et al., 2001; Siddall and Munglani, 2003). A disadvantage of these methods is that latencies may vary upon repeated measurements due to habituation or sensitization (for review see Le Bars et al., 2001). In electrophysiological studies, however, threshold temperatures for the activation of nerve fibers or ion channels are routinely determined. Furthermore, in human psychophysical experiments noxious heat sensitivity is also typically assessed by determination of the heat pain threshold, i.e. the lowest temperature that evokes pain. Threshold determination is a widely employed principle in methods for assessing mechanoneciception such as the Randall–Selitto test. However, measurement of the noxious heat threshold has not been introduced as a routine approach in animal studies (Le Bars et al., 2001; Siddall and Munglani, 2003), most probably owing to technical difficulties.

The first published implementation of noxious heat threshold measurement dates back to about 20 years ago when Szolcsányi (1985, 1987) determined the approximate noxious heat threshold of rats by immersing one of their hindpaws into a thermostatic water bath whose temperature was increased stepwise by 1 °C at a time until the animals withdrew their paws. This approach was suitable to reveal the heat-desensitizing action of high capsaicin doses, a phenomenon for which contradictory data had been obtained with latency measurements (Szolcsányi, 1976; Ohá et al., 1979; Hayes and Tyers, 1980; Gamse, 1982; Bittner and Lahmann, 1984). The exact heat threshold could be determined by the development of the
increasing-temperature hot plate which emerged as a reliable tool for the detection of the antinociceptive effect of morphine and certain cyclooxygenase inhibitors as well (Hunskaar et al., 1986; Álmási et al., 2003). We have developed a heat allodynia test based on the massive drop of heat threshold induced by intraplantar injection of resiniferatoxin, a potent agonist of the noxious heat-gated transient receptor potential vanilloid 1 (TRPV1) receptor and measured with an increasing-temperature hot plate (Álmási et al., 2003). This model proved to be highly sensitive to both opioid and cyclooxygenase inhibitor analgesics (Álmási et al., 2003).

Heat injury to the skin is widely employed for inducing thermal hyperalgesia and leads to a decrease of heat pain threshold and enhanced pain ratings to suprathreshold stimuli in the injured area denoted as primary thermal hyperalgesia (Hardy et al., 1950; Coderre and Melzack, 1987). Primary thermal hyperalgesia develops mainly as a consequence of sensitization of the peripheral terminals of cutaneous polymodal nociceptors (Meyer and Campbell, 1981; LaMotte et al., 1982).

The aim of the present study was to validate a newly developed increasing-temperature water bath allowing an exact determination of the behavioural noxious heat threshold. As a part of this process, a heat hyperalgesia model based on the mild heat injury-induced drop of heat threshold was implemented and its sensitivity to various analgesics was assessed.

2. Materials and methods

2.1. Animals

Female Wistar rats (Charles River Hungary Ltd, Budapest, Hungary) weighing 150–200 g were used in the experiments. The animals were kept in the Animal House of the University of Pécs in a pathogen-free, temperature-controlled room providing a 12 hour light–dark cycle. Animals were brought to the air-conditioned laboratory the day before and were provided with food and water ad libitum. Throughout all the experiments the same assistant handled all the animals and they were habituated to the measurement’s conditions prior to the experiment by performing two threshold measurements whose results were not included in the final analysis. The observer was blind to the drug treatment of animals.

2.2. The increasing-temperature water bath

An increasing-temperature water bath was developed in cooperation with Experimetria Ltd. (Budapest, Hungary). The equipment is suitable for the determination of the behavioural noxious heat threshold of rats defined as the lowest temperature at which the animal withdraws its hindpaw immersed into the water bath. The equipment consists of a tap water-filled water container and a controlling unit (Fig. 1A). The cylindrical plastic container (120 mm inner diameter, 140 mm height) is equipped with a built-in heating unit in its bottom that provides a homogenous and fast increase in the water temperature. The controlling unit serves for setting different starting temperatures (30 or 40 °C) and heating rates (6, 12 or 24 °C/min) and has a display continuously showing the actual bath temperature measured by a thermocouple at the middle position 35 mm below the water level. Heating can be interrupted by a foot switch and the corresponding bath temperature remains on the display to be recorded. After each measurement, the water bath is cooled back to the starting temperature by pumping cold water into the container controlled by a feedback mechanism while the excess water is drained through a spillway.

The homogeneity of the temperature distribution in a given layer of the water bath was investigated as follows. Five thermocouples connected to a multi-channel thermocouple thermometer (Columbus Instruments, Columbus, Ohio, USA) were arranged along a circumference 30 mm from the center and immersed 35 mm deep into water. These parameters were selected to correspond to the position where the rat’s hindpaw can conveniently be immersed upon heat threshold determination. Temperatures were measured at every 4 s upon 6 repeated heating processes using a starting temperature of 30 °C and a heating rate of 24 °C/min. No significant difference was revealed among temperatures measured at the 5 sites at the same time point of the heating process as assessed by two-way repeated measures analysis of variance (ANOVA) followed by Newman–Keuls post hoc test.

2.3. Determination of the noxious heat threshold of rats

A starting temperature of 30 °C and a heating rate of 24 °C/min were employed and the cut-off temperature was set to 53 °C. Rats were lightly restrained and held in an upright position above the water bath allowing free movement of the hindlimbs (Fig. 1B). One of the hindpaws was immersed into the water and the heating process was started afterwards. At the moment when the animal withdrew its paw, heating was immediately stopped by the foot switch and the corresponding temperature was recorded as the noxious heat threshold of the examined paw. Two control threshold measurements separated by a 30 min interval were performed for the same paw of each animal and the mean of the two values was used for analysis.

2.4. Induction of thermal hyperalgesia by heat injury and assessment of the antihyperalgesic effect of analgesics

After control threshold measurements rats were anaesthetized with diethyl ether and one of the hindpaws was immersed...
in a constant, 51 °C hot water bath for 20 s. These parameters for the heat injury were empirically established in a series of preliminary measurements in order to evoke a substantial drop of heat threshold without spontaneous nocifensive behaviour. Following recovery from anaesthesia, heat threshold determinations were repeated 10 and 20 min after heat injury to confirm the development of hyperalgesia. Drugs were administered intraperitoneally (i.p., 0.3 ml/100 g) or intraplantarly (i.pl., 0.1 ml/paw) after the 20-min measurement, which was followed by repeated heat threshold measurements at 10 min intervals. The effect of each dose of drugs was examined by comparison to an actual solvent control, i.e. one half of the group was treated with the drug and the other half with its solvent.

The minimum effective dose of drugs for the antihyperalgesic action was defined as the lowest dose applied causing a statistically significant inhibition of the heat injury-induced drop of heat threshold. The ED_{50} values determined by interpolation refer to the dose corresponding to the 50% reduction of the drop of heat threshold. The percentage inhibition of hyperalgesia was calculated using the sum of threshold drops measured 40, 50 and 60 min after heat injury according to the following formula: \[(\text{Drop}_{\text{solv}} - \text{Drop}_{\text{drug}}) / \text{Drop}_{\text{solv}}\] \times 100, where \text{Drop}_{\text{solv}} and \text{Drop}_{\text{drug}} refer to the average of the sum of threshold drops measured at the 40, 50 and 60 min time points in the solvent- and drug-treated animals, respectively.

### 2.5. Materials

Morphine hydrochloride (pharmaceutical grade, Ph. Hg. VII., University Pharmacy of Pécs), diclofenac sodium (Research Biochemicals International, Natick, MA, USA) and ibuprofen sodium (Sigma Chemical Co., MO, USA) were dissolved in saline. Paracetamol (acetaminophen, pharmaceutical grade, Ph. Hg. VII., University Pharmacy of Pécs) was dissolved in 1,2-propanediol and further diluted with saline resulting in a final solution containing 12.5% 1,2-propanediol.

### 2.6. Statistical analysis

One-way repeated measures analysis of variance (ANOVA) followed by Newman–Keuls post hoc test was used for comparison of thresholds determined upon repeated measurements in the same group of untreated rats and animals exposed to heat injury but not to solvent or drug treatment. Two-way repeated measures analysis of variance (ANOVA) followed by Newman–Keuls post hoc test was used for comparison of threshold drops of drug- and solvent-treated animals at various time points. A value of \( p < 0.05 \) was considered statistically significant.

### 2.7. Ethics

The experiments were carried out according to the Animals (Research Procedures) Act of 1998 (Hungary) and complied with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983). The studies were approved by the Ethics Committee on Animal Research of the University of Pécs.

### 3. Results

The behavioural noxious heat threshold of untreated rats measured with the increasing-temperature water bath was \( 43.5 ± 0.4 ^\circ C \) \((n=10)\). The threshold was reproducible upon repeated measurements at intervals of 10 min in the same group of animals (Fig. 2). No significant difference was found among thresholds of the same paw upon repeated measurements. There was no difference between the means of thresholds of different groups of animals \((n=8-12)\) measured on the same day (data not shown). No animals were excluded from the study due to lack of reproducibility of the noxious heat threshold or controverses in the evaluation of the nocifensive behaviour i.e. paw withdrawal.

After the heat injury, rats recovered from anaesthesia within minutes. There were no signs of spontaneous nocifensive behaviour in any of the animals. Upon heat threshold measurements

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following heat injury, a 7–8 °C drop of the threshold was observed, and this heat hyperalgesia was maintained at the same level for at least an hour (Fig. 3). The threshold of the injured paw returned to a near-control level within 4–5 h. The anaesthesia alone failed to have any after-effect on the heat threshold as indicated by the lack of statistically significant change in the noxious heat sensitivity of the uninjured paw at any time point (Fig. 3).

Morphine administered after the 20-min measurement dose-dependently reduced the heat injury-induced drop of heat threshold as compared to the solvent-treated groups (Fig. 4). Its minimum effective dose was 0.3 mg/kg i.p. and the 1 mg/kg dose abolished heat hyperalgesia as threshold values measured 50 and 60 min after heat injury did not differ significantly from control (Fig. 4). The non-selective cyclooxygenase inhibitors diclofenac and ibuprofen were also able to decrease the heat injury-induced threshold drop in a dose-dependent manner (minimum effective doses: 0.3 and 10 mg/kg i.p., respectively, Fig. 4). Paracetamol (acetaminophen) also inhibited the heat injury-induced thermal hyperalgesia (minimum effective dose: 30 mg/kg i.p., Fig. 4). The dose–response relationship for the antihyperalgesic effects of these drugs is shown in Fig. 5. In the case of paracetamol, the heat thresholds under the influence of the 30 and 100 mg/kg dose were similar (Fig. 4), but their respective solvent controls were different (data not shown) resulting in different percentage inhibition of hyperalgesia (Fig. 5).

We have previously developed another equipment for determination of the behavioural noxious heat threshold, an increasing-temperature hot plate (Almási et al., 2003). Although the high degree of reproducibility of the heat threshold upon repeated measurements at short intervals is a characteristic of both instruments, several differences exist between the increasing-temperature water bath and hot plate. (i) The animals are slightly restrained in the case of the increasing-temperature water bath (although only for 30–35 s owing to the high heating rate), unlike the increasing-temperature hot plate. (ii) In the increasing-temperature water bath the whole skin area of one hindpaw is exposed to the heat stimulus whereas on the increasing-temperature hot plate the plantar (volar) skin of four extremities is in contact with the heat source. (iii) The evoked nocifensive behaviour in the increasing-temperature water bath is paw withdrawal which is a spinal reflex (Le Bars et al., 2001) in contrast to the supraspinally controlled paw licking behaviour seen on the increasing-temperature hot plate.

Heat injury, which is an acute, natural and clinically relevant noxa, is widely employed for inducing thermal hyperalgesia (Hardy et al., 1950; Meyer and Campbell, 1981; LaMotte et al., 1982; Coderre and Melzac, 1987; Jun and Yaksh, 1998; Hamura et al., 2000; Johaneck and Simone, 2004; Wang et al., 2005), however, the antihyperalgesic effect of standard analgesics, especially cyclooxygenase inhibitors, has not been investigated in this model. The drop of heat threshold induced by mild heat injury in rats represents a novel thermal hyperalgesia model mimicking a first-degree burn injury to the skin. An important advantage of the model is that the mild heat injury does not lead to spontaneous nocifensive reaction (pain behaviour) which could otherwise confound subsequent threshold determinations. Furthermore, the measurement of noxious heat sensitivity and the induction of thermal injury corresponded to the same stimulus modality and affected exactly the same skin area. The reproducibility of heat threshold allowed assessment of the degree of hyperalgesia at every 10 min. The extent and time course of the hyperalgesia

Table 1
Minimum effective doses (MED) and ED_{50} values of morphine, diclofenac, ibuprofen and paracetamol determined for the inhibition of the heat injury-induced drop of heat threshold

<table>
<thead>
<tr>
<th>Drug</th>
<th>MED (mg/kg i.p.)</th>
<th>ED_{50} (mg/kg i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

The MED value for the antihyperalgesic action was defined as the lowest dose of the drug applied causing a statistically significant inhibition of the heat injury-induced drop of heat threshold. The ED_{50} values determined for the inhibition of the heat injury-induced drop of heat threshold. The percentage inhibition of hyperalgesia was calculated according to the following formula: \( \left( \frac{\text{Drop}_{\text{sol}} - \text{Drop}_{\text{dmg}}}{\text{Drop}_{\text{sol}}} \right) \times 100 \), where \( \text{Drop}_{\text{sol}} \) and \( \text{Drop}_{\text{dmg}} \) refer to the average of the sum of threshold drops measured at the 40, 50 and 60 min time points in the solvent- and dmg-treated animals, respectively.

5), i.e. a dose-dependent effect. The minimum effective doses and the calculated ED_{50} values for the antihyperalgesic action of morphine, diclofenac, ibuprofen and paracetamol are summarized in Table 1. In separate experiments it was shown that the highest doses of morphine, diclofenac, ibuprofen and paracetamol applied failed to alter the noxious heat threshold of animals whose hindpaw was not injured (data not shown).

Intraplantar injection of morphine (10 µg), diclofenac (10 µg) and ibuprofen (100 µg) administered 20 min after heat injury all significantly decreased subsequent thermal hyperalgesia (Fig. 6).

4. Discussion

The recently developed increasing-temperature water bath proved to be a suitable equipment for the measurement of the noxious heat threshold in conscious rats. Remarkable features of the heat threshold are that within the same group the values of the individual animals vary only slightly and the average values calculated in different groups also show little variability. Furthermore, the heat threshold is highly reproducible in the same group of animals upon repeated measurements. This is a remarkable advantage as reflex latencies determined in the hot plate or tail flick tests may vary upon repeated determinations (Gamble and Milne, 1989; Plone et al., 1996). The excellent reproducibility of the heat threshold might be due to a stimulation just up to the threshold temperature inducing no lasting alterations in nociceptor responsiveness. In contrast, raising the temperature to the suprathreshold range can evoke sensitization to heat of nociceptive fibers and dorsal root ganglion cells (Fitzgerald and Lynn, 1977; Lyfenko et al., 2002). Furthermore, it is advantageous to compare the behavioural noxious heat threshold with results of the electrophysiological studies in which the threshold temperature for activation of nerve fibers or ion channels is typically determined. It is worth mentioning that also in human psychophysical studies the heat pain threshold but not latency is measured (Hardy et al., 1950; Meyer and Campbell, 1981; LaMotte et al., 1982; Sycha et al., 2003).

investigated in the present study correlate well with the results of earlier studies on heat injury in which threshold determinations were performed either in human psychophysical studies or electrophysiological experiments on cutaneous heat-sensitive nociceptors (Meyer and Campbell, 1981; LaMotte et al., 1982). The time course of hyperalgesia revealed is also comparable with those observed in behavioural rat models of heat injury in which reflex latency was measured to assess thermal hyperalgesia (Coderre and Melzack, 1987; Nozaki-Taguchi and Yaksh, 1998), although in contrast to the observations made by Coderre and Melzack (1987), no referred hyperalgesia developed in the contralateral paw, which can be explained by the mildness of the injury applied. It is worth mentioning that following heat injury, the variability of the decreased heat threshold around its mean was small, which is advantageous for testing analgesics.

The present model based on heat injury-induced drop of the noxious heat threshold displays a high sensitivity to both opioid (morphine) and nonopioid reference analgesics (diclofenac, ibuprofen and paracetamol) as shown by their dose-dependent inhibitory effect on thermal hyperalgesia at rather low dose ranges. It is also worth mentioning that morphine at the highest dose applied was even capable of abolishing heat hyperalgesia. Highly advantageous is the reproducibility of heat threshold upon repeated testings, since the time course of the antihyperalgesic effect of drugs can be followed in the same group of animals. The administered low systemic doses of the analgesics failed to alter the unconditioned heat threshold of animals not exposed to heat injury. Thus, the reduction of the heat injury-induced threshold drop by the drugs represents a true antihyperalgesic action. Since heat threshold was also determined after heat injury but before drug administration, development of hyperalgesia could be confirmed in each animal. The antihyperalgesic effect of each analgesic dose was assessed in comparison with an actual solvent control and the degree of hyperalgesia was found rather similar in animals treated afterwards with the solvent or the drug. Thus, drugs were administered after the development of hyperalgesia, mimicking the usual clinical practice when drug treatment follows the appearance of pain and contrasting with the typical design of animal experiments in which drug application is performed before induction of hyperalgesia. It should be emphasized that the minimum effective doses as well as the ED50 values for the antihyperalgesic effect of morphine, diclofenac, ibuprofen and paracetamol were very low, with the former values being within or very close to their usual dose ranges applied in the human clinical practice.

The reason why the dose–response curve for the antihyperalgesic action of diclofenac has a different slope compared to other non-steroidal anti-inflammatory drugs investigated is not clear. Pharmacokinetic differences among these drugs, e.g. in rate and extent of tissue accumulation, might be a reason for the different slopes. In addition, evidence exists that certain non-steroidal anti-inflammatory drugs may have cyclooxygenase-unrelated actions not shared by other members of the group. For example, activation of the nitric oxide–cGMP–K+ channel pathway was shown to be involved in the effect of diclofenac, but not indomethacin (Tonussi and Ferreira, 1994; Ortiz et al., 2003). These data indicate that various cyclooxygenase inhibitors are not pharmacodynamically interchangeable which might also account for the different dose–response curves.

Regarding the antihyperalgesic effects of diclofenac and ibuprofen, two considerations deserve attention. Since diclofenac and ibuprofen belong to two chemically different groups of cyclooxygenase inhibitors, their shared antihyperalgesic action is likely to indicate that our paradigm is suitable to measure the antinociceptive effects of other cyclooxygenase inhibitors as well. On the other hand, as these drugs were applied i.p. 20 min after the heat injury, inflammatory processes had already been initiated until the onset of drug effect. It cannot be excluded, however, that the antihyperalgesic effect of these drugs is partly due to actions unrelated to the anti-inflammatory effect, e.g. to centrally mediated antinociception. Indeed, upon intrathecal administration, several cyclooxygenase inhibitors including diclofenac and ibuprofen displayed antinociceptive actions pointing to pure antinociceptive effects (for review see Svensson and Yaksh, 2002). In accord with this, induction of cyclooxygenase-2 in the spinal cord was revealed in response to painful stimuli (for review see Svensson and Yaksh, 2002). In addition to inhibition of spinal cyclooxygenase-2, a further possible underlying mechanism of the central antinociceptive actions could be the inhibition of the recently revealed cyclooxygenase-3 occurring mainly in the central nervous system and inhibited by either diclofenac or ibuprofen (Chandraksharan et al., 2002).

In terms of the minimum effective dose and ED50 values, the present model displays a similar sensitivity to morphine, diclofenac and paracetamol as does our previously developed heat hyperalgesia/ailodynia model based on the massive drop of heat threshold induced by resiniferatoxin and measured with the increasing-temperature hot plate (Álmasi et al., 2003). In a human study employing determination of the heat pain threshold, the inhibitory effect of ibuprofen on the heat threshold drop induced by ultraviolet irradiation of the skin could be revealed (Sycha et al., 2003). The present antihyperalgesic ED50 values for both morphine and the three cyclooxygenase inhibitors are also similar to or lower than those found in other experimental paradigms of thermal hyperalgesia based on measurement of reflex latencies with the conventional tail flick and plantar tests (Gelgor et al., 1992; Bianchi and Panerai, 1996; Dirig et al., 1998; Berg et al., 2000; Hamura et al., 2000; Sluka, 2000).

The heat injury-induced thermal hyperalgesia was also inhibited by intraplantar administration of low doses of morphine as well as cyclooxygenase inhibitors diclofenac and ibuprofen. The intraplantarily applied dose was substantially lower than the respective systemic minimum effective antihyperalgesic dose for each drug, which clearly supports a local, peripheral site of action. In accord with this, a peripheral analgesic effect for both opioids and cyclooxygenase inhibitors has been revealed long ago (Ferreira et al., 1978; Levine and Taiwo, 1989; Stein et al., 1988; Stein, 1995) which is more prominent under inflammatory conditions. Our results are in line with those of a previous study investigating heat injury-
induced hyperalgesia which showed the antihyperalgesic effect of loperamide, a peripherally acting opioid (Nozaki-Taguchi and Yaksh, 2002). Opioids can act on peripheral opioid receptors found on nociceptors (Coggshall et al., 1997), whereas cyclooxygenase inhibitors suppress the formation of prostaglandins that sensitize nociceptive nerve endings to heat and mechanical stimuli. On the basis of the above data, the present model complies with the requisite to detect the peripheral antinociceptive effects of different compounds with high sensitivity.

In conclusion, the mild heat injury-induced drop of the noxious heat threshold of rats measured with the novel increasing-temperature water bath is a new thermal hyperalgesia model that is (i) based on highly reproducible heat threshold values; (ii) suitable for revealing the antihyperalgesic effect and its time course of very low doses of systemically and locally applied reference analgesics; (iii) a correlate of a human clinical pain state. For these reasons, this model seems to be a reliable and promising method for investigating the mechanisms of hyperalgesia and for preclinical testing of analgesics.

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