Central activation of the trigeminovascular pathway in the cat is inhibited by dihydroergotamine
A c-Fos and electrophysiological study

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Summary
Recent studies have delineated a clear role for the trigeminal innervation of pain-sensitive intracranial structures in the pathophysiology of migraine. The development of new compounds for the treatment of the acute attack of migraine has led to a greater understanding of serotonin (5-hydroxytryptamine; 5HT) receptor diversity. The ergot alkaloids have been used in the treatment of acute attacks of migraine for many years and parenteral administration of dihydroergotamine (DHE) can be a useful treatment strategy. In this study, the question of a possible central site of action of DHE is considered using both anatomical and physiological approaches. The c-Fos method has been used to map functional activation of central neurons in response to stimulation of the superior sagittal sinus (SSS) in the cat. This structure has been used as it refers pain to the ophthalmic division of the trigeminal nerve in humans, and in cats induces changes in neuropeptides and cranial blood flow similar to those seen in migraine. In addition, the temporal aspects of the effect of DHE have been studied by making extracellular recordings from cells in the most caudal aspect of the trigeminal nuclear complex. Stimulation of the SSS results in Fos expression in the superficial laminae of the trigeminal nucleus caudalis and in the dorsal horn of C1 and C2. This activation is blocked by a clinically relevant dose of DHE. Similarly, cells can be recorded in this region that respond to SSS stimulation. This linked cellular activity can be inhibited by the same intravenous dose of DHE. Together, these studies show that DHE can inhibit activity in central trigeminal neurons. Since the sinus and its nerve supply are directly stimulated, the peripheral nerve/vessel innervation is bypassed and this inhibition cannot have happened at any other site. These data imply that drugs acting at the central trigeminal neurons may have a role in the treatment of acute attacks of migraine.

Keywords: headache; migraine; acute treatment; trigeminal nucleus; ergotamine

Abbreviations: DHE = dihydroergotamine; SSS = superior sagittal sinus; 5HT = 5-hydroxytryptamine = serotonin

Introduction
Recent advances in the understanding of the pathophysiology of migraine have included more complete descriptions of the pathways that are involved in head pain. It is clear that migraine involves activation of the pain-sensitive innervation from the trigeminal nerve and that the second order neurons reside in the most caudal part of the trigeminal nucleus caudalis and in the dorsal horn of the first and second cervical segments of the spinal cord (Goadsby and Zagami, 1991; Kaube et al., 1993a). Drugs that are effective in migraine may, therefore, have an action upon these neurons in addition to effects at more peripheral sites (Moskowitz, 1992). The therapy of migraine has, until recent times, focused upon drugs used to treat acute attacks, that were thought to have a vascular role, such as the ergots (Graham and Wolff, 1966), or therapy for prevention of attacks, that has been considered to interact with inhibitory serotonergic mechanisms, such as methysergide or pizotifen (Peroutka, 1988). The latter group includes drugs that may act upon 5HT2 receptors (Hoyer et al., 1986). The synthesis of the novel 5HT1D-like agonist sumatriptan (Humphrey et al., 1991) and its successful use in the treatment of the acute attack of migraine (Ferrari, 1991; Goadsby et al., 1991b) has again highlighted serotonergic involvement in the syndrome. Dihydroergotamine is also widely used in clinical practice.
for the treatment of migraine (Goadsby, 1994) and particularly in the management of persistent severe headache (Raskin, 1986). In a similar way to sumatriptan it acts at the 5HT1D receptor (Peroutka, 1988) and its potential site of action is therefore of interest. It has been suggested that the action of anti-migraine compounds may be at the peripheral trigeminovascular synapse to block neurogenically mediated plasma extravasation (Moskowitz and Cutrer, 1993). We have proposed that, in addition, a peripheral action to it may be possible for anti-migraine drugs to act within the CNS on the central ramifications of the trigeminal system (Goadsby et al., 1991a).

There is good evidence in vitro that DHE binds with very high affinity to both α-adrenoceptors and to 5HT1D-like receptors in the rat brain (Hamblin et al., 1987). The question of whether peripherally administered DHE or ergotamine enters the CNS in humans in vivo is controversial. It has been reported that intravenous DHE (Kanto et al., 1981), and orally or rectally (Hovdal et al., 1982) administered ergotamine cannot be detected in the CSF, although Ala-Hurula et al. (1979) could detect small quantities of ergotamine in the CSF after a 2 mg oral dose. Methodological considerations, such as assay detection limits, have been cited to explain the differences seen in these studies of the CSF (Eadie, 1983).

We have shown that DHE binds to receptors in the trigeminal nucleus caudalis and in the dorsal horn of the first and second cervical segments of the spinal cord using both in vitro and ex vivo methods in the cat (Goadsby and Gundlach, 1991). Intravenous administration of DHE has not been studied in models of central trigeminal activation, yet it is a widely employed route in clinical practice. In this study we have combined the functional/anatomical c-Fos method with extracellular electrophysiology to correlate more precisely the spatial relationships between the anatomical location of trigeminally activated cells and the possible sites of action of DHE.

Methods

Eleven cats were anaesthetized initially with 1.5% halothane and α-chloralose (60 mg/kg, intraperitoneally; Sigma, St Louis, USA) and prepared for physiological monitoring. The femoral artery and vein were cannulated in order to measure blood pressure and heart rate and provide access for drug and fluid administration, respectively. Cardiovascular parameters and pupillary reaction to noxious pinching of the forepaw were used to determine the need for supplementary anaesthesia. The animals were endotracheally intubated, ventilated with 40% oxygen and paralysed after the completion of the surgical procedures with repeated doses of gallamine triethiodide (6 mg/kg intravenously; May and Baker, UK). Body temperature and end-expiratory CO2 were monitored and maintained within physiological limits. The animals were mounted in a stereotactic frame and a circular midline craniotomy (2 cm in diameter) was performed for access to the SSS. The adjacent dura and falx were dissected parallel to the sinus over 10–15 mm. To prevent dehydration and for electrical insulation against the cortex, a paraffin bath was built with a dam of dental acrylic around the craniotomy and in addition a small polyethylene sheet inserted under the vessel. Fluid (4% glucose with 0.18% saline or normal saline) was administered intravenously at a rate of 3–5 ml/kg/h, while gallamine (6 mg/kg intravenously) and α-chloralose (10–15 mg/kg intravenously) were administered every 2 h. Blood pressure and heart rate were stable and within physiological range for all animals throughout the whole experiment. Arterial blood gas parameters were monitored intermittently as a guide to the end-expiratory CO2 output.

Fos study

Following completion of surgery, the animal was maintained for the following 24 h. After this resting phase, the SSS was suspended over a pair of platinum hook electrodes. We have shown that this would, of itself, not provoke Fos activation (Kaube et al., 1993a). The SSS was stimulated with a Grass S88 stimulator driving a stimulus isolation unit (SIU5, Grass Instruments, Quincy, Mass., USA; 150V, 250 μs duration) at a rate of 0.3/s for 1 h. After completion of the period of stimulation 1 h was allowed to elapse prior to perfusion.

Perfusion

Cats were perfused transcardially with 1–1.5 l of 0.9% saline (containing 1000 IU of heparin and 0.5ml of 1% sodium nitrite) this was followed by 2 l of 4% paraformaldehyde in phosphate buffer (pH 7.4) and finally by 500–600 ml of 30% sucrose solution in phosphate buffer. The brain and cervical spinal cord were removed and stored in 50% sucrose with azide. Coronal sections (40 μm) of the caudal medulla and upper cervical spinal cord were cut on a freezing microtome and every fifth section was collected for processing. Sections were cut from a block beginning at the level of the obex through to C3 segment of the cervical cord.

c-Fos procedure

Free-floating sections were incubated at 4°C for 3–7 days in a commercially available rabbit, polyclonal antibody to Fos protein (‘Ab-2’, Oncogene Science Ltd, USA) in a 1:1000 dilution with 1% phosphate buffered horse serum, containing 0.1% bovine serum albumin and 0.2% Triton-X100. Fos-like immunoreactivity (hereafter simply called Fos) was visualized using standard avidin–biotin peroxidase immunohistochemical techniques. Following the primary incubation, sections were washed in 0.1 M phosphate buffer (pH 7.4) for 30 min and then incubated in a biotinylated goat anti-rabbit IgG (1:200 dilution) (Vector Labs, USA) for a minimum of 2 h at room temperature on a rotating table. Following the second incubation, the sections were washed again in...
0.1 M phosphate buffer (pH 7.4) for 30 min. The sections were then incubated for 2.5 h in a 1:1000 dilution of ExtrAvidin–peroxidase (Sigma), sections were again washed in 0.1 M phosphate buffer (pH 7.4) for 30 min. The sections were then incubated in 20 ml of 0.1 M phosphate buffer containing 0.05% dianaminobenzidine (Sigma), 0.005% of 4% ammonium chloride, 0.005% of 20% (+)–glucose and 0.02% of a 1% solution of nickel ammonium sulphate for 20 min. The sections were then placed in a fresh identical 20 ml solution and 20 μl of glucose oxidase (Sigma) was added to initiate the chromogenic reaction. The reaction was allowed to proceed until Fos positive nuclei could be clearly seen under the microscope. The dianaminobenzidine reaction product was visible as a black precipitate due to the presence of the nickel ammonium sulphate. Following this reaction the sections were washed two or three times in 0.1 M phosphate buffer (pH 7.4) and they were mounted on gelatinized slides.

Fos-positive cells were distinguished by their black nickel enhanced nuclei from the background. Using the procedure adopted by Hammond et al. (1992), cells were only considered positive if the black precipitate of the dianaminobenzidine reaction within the cell nucleus was distinguishable from the background throughout a range of magnifications between ×20 and ×4 (Hammond et al., 1992). Fos-positive cells were plotted onto sections of the caudal medulla and upper cervical spinal cord modified from the atlases of Berman (1968) and Rexed (1954). Control incubations in the presence of the antigen were not carried out in this series of experiments. However, the omission of the primary antibody in other experiments performed in this laboratory have failed to produce positive staining. Furthermore, preabsorption of the antibody with Fos protein has demonstrated its specificity in other studies.

Distributions of cells were quantified for each individual animal by taking 10 sections at random, from each of the levels (SpV, CI, C2 and C3) and plotting the label from a single side onto one of the schematic sections described above. The plotting was performed by one person who, although they had knowledge of the experimental design, was not aware of the experimental group to which each animal belonged.

**Electrophysiology**

To activate trigeminal primary afferents, the SSS was stimulated with a Grass S88 stimulator with stimulus isolation unit (150 V, 250 μs, 0.3 Hz; SIUSA). Tungsten-in-glass microelectrodes (tip length/diameter: 50/15 μm, impedance: <200 kΩ) were lowered into the dorsolateral spinal cord 4–5 mm caudal to the mid-point of the C2–rootslet between 400 and 800 μm below the surface with a hydraulic micropositioner (Kopf, Model 650, USA). Electrical responses were amplified (NeuroLog, total system gain 20 000–30 000) and low-pass filtered (NeuroLog, high cut-off frequency 5.5 kHz) to prevent aliasing. The signal from the amplifier was passed to the analog input of an A/D converter (LabMaster, Ohio, USA) in an IBM-compatible microcomputer (80386/80387 based) for on-line analysis of single units responses by custom written program (Microsoft C). Single unit activity was analysed after digital online high pass filtering (cut-off frequency 500 Hz) and passing a digital window discriminator to create a post-stimulus histogram over 25 or 100 recordings (sweep length 50 ms) to identify linked responses. Baseline recordings with 100 averages each were repeated at least three times to ensure that single unit responses to SSS stimulation were reproducible over time.

**Drugs**

Dihydroergotamine (Sandoz) was administered in a dose of 15 μg/kg intravenously 30 min prior to stimulation for the Fos studies. For the electrophysiological studies three control post-stimulus histograms were obtained before DHE was administered.

**Statistics**

Physiological data are reported as mean±SD, whereas Fos data are reported as the median response with the interquartile range. Although the Fos technique provides quantitation it is likely to result in ordinal rather than interval data (Siegel, 1956) and therefore is better analysed by non-parametric methods. To compare the two groups a Mann–Whitney U test has been employed (Siegel, 1956). The electrophysiological data were compared using a Student's t test. All comparisons were considered significant at the P < 0.05 level.

**Results**

Eleven cats were studied in all with a mean weight of 2.4±0.3 kg (mean±SD). The physiological data for the animals reported are presented in the Table 1.

**Fos studies**

Stimulation of the SSS results in expression of Fos-like immunoreactivity in restricted regions of the caudal medulla and high cervical spinal cord as has been previously reported from our group (Kaube et al., 1993a). The data here extend the number of animals that we have studied (n = 3) at a rate of stimulation of 0.3 Hz. The regions expressing Fos in the trigeminal system are the ventrolateral portion of laminae I and IIo of the trigeminal nucleus caudalis and C3 dorsal horn grey matter and the medial aspect of laminae I and IIo of the C2 dorsal horn grey matter. In addition to these cells Fos was also detected in the caudal portion of the medial nucleus of the solitary tract and in the nucleus retroambigualis at the level of the trigeminal nucleus caudalis and in laminae X in the high cervical spinal cord. The quantitative data for these regions are presented in Table 2.
Table 1  Physiological data*

<table>
<thead>
<tr>
<th></th>
<th>Weight (kg)</th>
<th>Blood pressure (mmHg)</th>
<th>pH</th>
<th>pCO₂</th>
<th>pO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Fos studies (n = 6)</td>
<td>2.5±0.3</td>
<td>110±8</td>
<td>7.31±0.05</td>
<td>37±6</td>
<td>211±47</td>
</tr>
<tr>
<td>Electrophysiological studies</td>
<td>2.3±0.3</td>
<td>101±7</td>
<td>7.33±0.04</td>
<td>36±5</td>
<td>221±23</td>
</tr>
</tbody>
</table>

*Mean±SD for n = 11 animals.

Table 2  Fos expression following SSS stimulation is reduced after treatment with DHE*

<table>
<thead>
<tr>
<th>Region</th>
<th>Control stimulation</th>
<th>Dihydroergotamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Caudal medulla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminae I/IIo</td>
<td>76</td>
<td>50-95</td>
</tr>
<tr>
<td>cNTS</td>
<td>37</td>
<td>35-48</td>
</tr>
<tr>
<td>C₁</td>
<td>88</td>
<td>72-105</td>
</tr>
<tr>
<td>Lamina X</td>
<td>25</td>
<td>17-39</td>
</tr>
<tr>
<td>C₂</td>
<td>92</td>
<td>71-95</td>
</tr>
<tr>
<td>Lamina X</td>
<td>18</td>
<td>14-26</td>
</tr>
</tbody>
</table>

*Median with interquartile range for n = 6 cats; \(^{c}c\)NTS, caudal extension of the medial nucleus of the solitary tract; ^*P < 0.05.

DHE
Treatment of three animals with DHE resulted in marked changes in Fos expression in the regions studied (Fig. 1). The number of Fos-positive cells in laminae I and IIo of the trigeminal nucleus caudalis fell from the control level of 81 (76-114) to 9 (7-10; P < 0.05) following DHE (Fig. 2). Similarly, Fos expression in laminae I and IIo of the C₁ (P < 0.05) and C₂ (P < 0.05) cervical spinal cord were markedly reduced by DHE administration (Table 2).

Electrophysiology
Cells were recorded at the C₂ level of the cervical spinal cord with a baseline probability of firing of 0.73±0.2 and a latency for the fastest component of the histogram of 8.3±1.1 ms (n = 5; Fig. 3). The probability of firing was reproducible across three consecutive averages each consisting of 100 stimulations followed by 50 ms sweeps. The data for each control period and the post-treatment period are shown in Fig. 4. The variability between each of the control periods was 12% in total. About 30 min after the intravenous administration of DHE, the probability of firing began to reduce reaching a minimum at 45 min (0.21±0.04). This represented a significant reduction from the control level (P < 0.01) with no change in the latency of the units that remained. In no animal did the responses return to baseline after 3 h observation.

Discussion
We have examined the activation of central trigeminal neurons using two approaches both of which have shown that intravenous administration of DHE can inhibit trigeminal neurons. Fos activation provides a good overall picture of the neuronal activation during stimulation of a pain-sensitive intracranial structure. The data show that there are robust increases of Fos in trigeminal neurons after stimulation of the SSS which are markedly reduced by pretreatment with DHE. These data suggest that the effect involves a large proportion of the caudal trigeminal neurons and is robust in the sense that the data are mirrored by what is seen observing cell firing. The electrophysiological data supports the Fos data in that cells studied had a marked reduction in probability of firing after DHE and demonstrated that this effect took ~30 min to reach its maximum.

These studies rely, in the first instance, on the validity of the model of SSS stimulation for their interpretation and significance. It is clear in humans that mechanical or electrical stimulation of the SSS in vivo is painful (Ray and Wolff, 1940). Furthermore, accidental stimulation of the trigeminal ganglion during thermocoagulation for trigeminal neuralgia, when anaesthesia is not adequate, is extremely painful and well remembered by the few patients who experience the pain. Moreover, pain from sagittal sinus stimulation is referred to the ophthalmic division of the trigeminal nerve by patients. There is good evidence in migraine that there is activation of the trigeminovascular system since the neuropeptide marker for that innervation, calcitonin gene-related peptide, is released during migraine (Goadsby et al., 1990). In the cat, SSS stimulation leads to similar changes in neuropeptides (Zagami et al., 1990). The sinus model, therefore, acts as an excellent method by which to examine aspects of the trigeminovascular system that cannot be studied in humans. The method of stimulation used in these studies...
DHE inhibits trigeminal nucleus activity

Electrical stimulated SSS

Electrical stimulation pre-treated with DHE

Fig. 1 Individual Fos-positive cells (closed circles) from 10 40-μm sections of the caudal medulla (SpV), first (C1), second (C2) and third (C3) cervical spinal cord segments of the cat. Data are presented for the control animals in which the superior sagittal sinus (SSS) was stimulated and for animals also stimulated but treated with dihydroergotamine (DHE). The plots show a marked reduction in Fos-positive cells after treatment with intravenous dihydroergotamine. INT = intermediate grey matter; I/IIo = laminae I and IIo of the dorsal horn; cNTS = medial nucleus of the solitary tract; VH = ventral horn; X = lamina X; Gr = gracile nucleus; Cu = cuneate nucleus; LCN = lateral cervical nucleus; i = ophthalmic, ii = maxillary and iii = mandibular divisions of trigeminal nucleus.

is very important. By stimulation of the sinus with a hook electrode, the trigeminal nerves innervating the sinus are directly activated. This eliminates the nerve/vessel interface and in so doing the peripheral 5HT1D receptor that mediates plasma extravasation (Moskowitz and Cutrer, 1993). The remaining site for the action of any drug, and in this study DHE, must be within the trigeminal nucleus at the second order synapse. The only intervening structures are the axons of passage and the cells bodies of the trigeminal ganglion both of which are not known to have functional 5HT1D receptors. The next available site for the drug is the trigeminal nucleus which is the structure examined by both the Fos and electrical studies.

Given that the model as it has been applied essentially examines central trigeminal behaviour, at least for the measurements that have been made, the data suggest that DHE acts in the brain. There is good evidence in vitro that DHE binds with very high affinity to both α-adrenoceptors and to 5HT1-like receptors in the rat brain (Hamblin et al., 1987). The question of whether peripherally administered DHE or ergotamine enters the CNS in humans in vivo is not as clear. Neither intravenous DHE (Kanto et al., 1981) nor orally or rectally (Hovdal et al., 1982), administered ergotamine can be detected in the CSF although Ala-Hurula et al. (1979) could detect small quantities of ergotamine in the CSF after a 2 mg oral dose. Methodological consider-
Electrical stimulation of the superior sagittal sinus: effect of DHE

Fig. 2 Histograms demonstrating the effect of DHE on various regions of the caudal medulla (SpV) and first (C₁), second (C₂) and third (C₃) cervical spinal cord segments studied using the Fos method. The median number of cells found in each area along with the interquartile ranges for the entire cohort of animals studied is plotted. I/IIo = laminae I and IIo of the dorsal horn; III/IV = laminae III and IV of the dorsal horn; cNTS = medial nucleus of the solitary tract; INT = intermediate grey matter; NRA = nucleus retroambigualis; RF = reticular formation; VH = ventral horn; X = lamina X.

Fig. 3 Post-stimulus time histogram demonstrating the effect of DHE on units linked to stimulation of the SSS that are monitored in the C₂ level of the spinal cord. Probability of firing is plotted on the ordinate while the abscissa contains the time scale (ms). The control plot demonstrates two units that are closely linked to SSS stimulation and the firing of the same units is shown after DHE injection demonstrating a marked reduction in the probability of firing.

Fig. 4 This figure illustrates the probability of firing for each of three consecutive averages plotted as the mean with SEM for all animals studied. The response of the cells is consistent with respect to time and markedly diminished by the administration of DHE.

DHE has been linked to sites of action in the trigeminal nucleus caudalis and in the dorsal horn of the first and second cervical segments of the spinal cord using both in vitro and ex vivo methods in the cat (Goadsby and Gundlach, 1991). The latter data showing that after intravenous administration of [³H]DHE radioactivity could be detected in the trigeminal nucleus suggesting that DHE is capable of accessing at least some central sites. It has been suggested that attempts to measure DHE binding in the brain

...ations, such as assay detection limits, have been suggested as a possible explanation for these differences (Eadie, 1983). Data from our group have shown that DHE binds to receptors in the trigeminal nucleus caudalis and in the dorsal horn of the first and second cervical segments of the spinal cord using both in vitro and ex vivo methods in the cat (Goadsby and Gundlach, 1991). The latter data showing that after intravenous administration of [³H]DHE radioactivity could be detected in the trigeminal nucleus suggesting that DHE is capable of accessing some central sites. It has been suggested that attempts to measure DHE binding in the brain using classical whole brain or whole brainstem methods may have failed due to lack of sensitivity. Taken together these data support a possible site of action for DHE in the central trigeminal neurons with sites available for drug action and accessible after intravenous administration. These data do not exclude an additional peripheral action in humans during headache but widen the scope of evaluations of the possible sites of action of anti-migraine drugs.

If DHE were to access trigeminal neurons what would be expected to happen? There are several pieces of data that...
would suggest that DHE should inhibit trigeminal neuronal activity if it were to access these neurons. Sumatriptan has an inhibitory action at peripheral trigeminal nerve terminals at their point of innervation of pain-sensitive structures, such as the dura mater (Buzzi et al., 1991). If the blood–brain barrier is intact sumatriptan does not affect trigeminal neuronal activity in vivo; however, if the blood–brain barrier is disrupted then trigeminal neuronal activity is markedly inhibited (Kaube et al., 1993b). It has also been shown that intravenous administration of ergotamine reduces trigeminal evoked potentials from the high cervical spinal cord that arise from stimulation of the SSS and that a closely related compound, ergometrine, when iontophoresed onto trigeminal cells will reduce their firing rate (Lambert et al., 1992). Ergotamine and DHE are closely related and certainly ergotamine is an effective treatment of migraine attacks (Raskin, 1988). It remains to be seen whether specific 5-HT$_{1D}$ antagonists can reverse the effects of DHE. The recent demonstration that a more lipophilic 5-HT$_{1D}$ receptor agonist 311C90 is capable of blocking a centrally mediated trigeminovascular reflex (Goadsby and Edvinsson, 1994) and the impressive efficacy of that compound in treating an acute attack of migraine (Dahlof et al., 1994) provides clinical evidence that central sites must be considered in any formulation of the problem of migraine. Indeed the demonstration that a centrally placed electrode in the periaqueductal grey (Raskin et al., 1987) and the finding of receptors binding DHE in this region (Goadsby and Gundlach, 1991) makes the likelihood of a central dysfunction even more reasonable in migraine.

In summary, intravenous administration of DHE in a dose similar to that used in the clinical setting results in inhibition of trigeminal neuronal activity. This effect can be seen for the entire caudal trigeminal nucleus complex and is quantitatively substantial. The effect is seen reasonably soon after injection and is prolonged for many hours. This inhibition is consistent with what is known about the pharmacology of DHE and with the known distribution of DHE receptors in the caudal medulla and upper cervical spinal cord of the cat but does not exclude an involvement of more rostral structures of the central pain control system such as the dorsal raphe nucleus. The effect seen in these studies cannot be due to a peripheral action of DHE since the model employed uses an activation that bypasses the peripheral receptor site. The data suggest that the central trigeminal neurons may be an alternate site for anti-migraine drugs and supports development of such compounds for clinical trial.

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