Effect of kainic acid injected into raphe dorsal nucleus on sleep stages in cats

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Abstract. Early (up to 5 h) and late (up to 65 days) effects of kainic acid (KA) injected into raphe dorsal nucleus (NRD) at two doses, 4 and 12 nmol, on waking-sleep stages in cats were studied. Slow-wave sleep (SWS-1 and SWS-2) and rapid eye movement sleep (REM) were strongly reduced or even completely suppressed, while waking stage, especially quiet waking stage (W-2), was significantly increased during the first 5 h after the injection. The effect was much more pronounced after injection of KA at a dose of 12 nmol. These changes in sleep stages gradually subsided and starting from the first-third day up to the 65th day after injection of KA, sleep was completely normal. The effects were interpreted as reflecting the action of KA first as a strong excitant - when a suppression of sleep was established, and then as a toxic substance with respect to NRD cells - when sleep restored to normal. These results suggest that raphe dorsal nucleus is not essential for triggering or maintenance of sleep.

Key words: raphe dorsal nucleus, kainic acid, waking-sleep stages, cat
INTRODUCTION

The role of 5-hydroxytryptamine (serotonin, 5-HT) and serotonergic midbrain raphe nuclei in controlling vigilance is still unclear. There are many reports showing that inactivation of the 5-HT system either by intraventricular injection of 5,6-dihydroxytryptamine (Froment et al. 1974) or by systemic administration of the potent tryptophan-hydroxylase inhibitor parachlorophenylalanine (Mouret et al. 1967) leads to suppression of sleep in rats and cats. According to the serotonergic theory of regulation of waking-sleep cycle, serotonin is important especially for slow-wave sleep (SWS) (Koella 1969; Jouvet 1969, 1972). Many authors have shown that massive electrolytic lesions of the midbrain raphe nuclei in cats, i.e. raphe dorsal (NRD) and raphe central superior (RCs) produce awakening or insomnia and increased locomotion (Jouvet et al. 1966; Jouvet 1969; Juwanicz 1980). When only RCs is destroyed the effects are the same (Srebro and Lorens 1975, Mouret and Coindet 1980), but when NRD is inactivated by cooling, hypnogenic effects are observed (Cespuglio et al. 1979). From these experiments it has been concluded that the raphe dorsal nucleus functions are closer to those of the ascending activating reticular system than to those of the hypnogenic bulbar structures. However, in view of the nonspecific nature of the electrolytlic lesion which destroys not only local cell bodies but also fibres of passage, the contribution of the various nuclei of the raphe system to the observed changes in sleep is difficult to evaluate.

One of the methods employed for studying the role of a given structure in a given function is the widely used method of destroying the cells of this structure by means of the excitotoxic substance kainic acid (KA) (Olney et al. 1974). In our earlier investigations on immobilized (Moyanova et al. 1986) and freely-moving cats (Moyanova and Riche 1991) we have shown that paroxysmal EEG activity appears at the site of injection of KA-raphe dorsal nucleus, as well as in many other brain structures soon after the injection of KA. The seizure activity was recorded on the day of injection and rarely on the following day (Moyanova and Riche 1991). The present study was undertaken to examine the changes in waking-sleep stages after injection of kainic acid into the raphe dorsal nucleus in freely-moving cats.

METHODS

Animals and surgery

Ten male cats weighing 3.0-3.5 kg were used. Sodium pentobarbital anaesthesia (50 mg/kg i.p.) was used to implant standard electrodes for recording sleep-waking states. Geniculate and occipital components of pontogeniculooptical (PGO) waves were recorded. The electrode for recording from lateral geniculate body (LGB) was concentric - nichrome insulated wire inserted into a sleeve of a stainless steel tubing. Screws were fixed over the occipital (posterior lateral) cortex and over the somatosensory (posterior sigmoid) cortex. Additional screws were fixed over the frontal sinus and the occipital protuberance which served as indifferent and ground electrodes, respectively. Flexible nichrome insulated wires implanted into the orbital muscles and into the neck dorsolateral recti muscles of the two sides were used for recording the electrooculogram (EOG) and the electromyogram (EMG). A guide cannula (21-gauge hypodermic needle) was inserted caudally at 30° vs. the vertical plane to reach the raphe dorsal nucleus. The stereotaxic coordinates for the nucleus were P0.2, L0.0, H-0.5 (Berman 1968). The guide cannula insulated with epoxylite had a nichrome insulated wire soldered to its upper edge and served as a recording electrode. A mandril was inserted into the cannula. All electrodes were soldered to a 15-way p.c.b. socket (Radio Spares Ltd.) and the socket itself was fixed to the skull by means of a dental cement.

Recording procedure

After 2-3 weeks recovery period during which all necessary post-operative precautions were made, the animal was placed for 2-3 days in a recording box. During this period of animal adaptation the cable was attached to the socket by means of a 15-way plug. The cable was connected to a rotating connector mounted to the box ceiling which permitted free movements of the animal. EEG, EOG and EMG recordings were made using Reega Duplex EEG machine 5 h/day (12 at noon - 5 p.m.) from the freely moving animals. The cats were placed in the recording box the night before each recording session. The control 5 h records were made 1-2 days before kainic acid injection into the raphe dorsal nucleus. Another control record with injection
of the KA vehicle (phosphate buffer solution - PBS) was made a week before the KA injection.

**Injection procedure and histology**

The injection of kainic acid (Sigma) into NRD was performed at 11.50 a.m. The mandril was removed from the guide cannula and an injection cannula (0.5 mm o.d., 0.3 mm i.d.) was inserted so that it protruded 0.5 mm from the first. A 1.0 µl Hamilton microsyringe was used and connected to the injection cannula by a length of polyethylene tubing. KA was dissolved immediately before use in a phosphate buffer solution (PBS) and pH of the KA solution was adjust at 7.4. In 5 animals the volume of KA solution injected into NRD was 0.9 µl containing 0.9 µg or 4 nmol of KA. In other 5 animals the volume of KA solution was 0.24 µl containing 2.6 µg or 12 nmol of KA.

Sixty-six days after KA injection the animals were deeply anaesthetized with thiopental (Spofa) and electrocoagulation was carried out (DC current 1.7 mA for 15 s) through the implanted electrodes. Then the brain was perfused with 0.9% saline followed by 10% formalin solution. Brain was stored in 4% formalin for at least 7 days. Frozen coronal 40 µm sections were cut on a carbon dioxide cryostat to verify the localization of the lesion.

**Sleep stages analysis**

The sleep stages analysis was carried out according to Ursin and Sterman (1981) and Trulson and Trulson (1983) as follows: *Waking-1* (*W-1*) - active waking with low voltage fast cortical EEG activity, gross bodily movements, frequent (> 20/min) and large (> 400 µV) eye movements potentials, high amplitude tonic EMG and phasic EMG bursts; *Waking-2* (*W-2*) - quiet waking and/or drowsiness without gross bodily movements, with 4-8 Hz synchronized EEG activity over posterolateral cortex for 50% or more of the scoring epoch, infrequent (< 10/min) or no large eye movement potentials (> 400 µV), with small eye movement potentials (< 40 µV) and tonic EMG activity; *Slow-wave sleep-1* (*SWS-1*) with 11-16 Hz sleep spindles over sigmoid cortex and less than 50% 1-4 Hz slow waves, 50 µV or more over posterolateral cortex; *Slow-wave sleep-2* (*SWS-2*) with sleep spindles and more than 50% 1-4 Hz slow waves, 50 µV or more over posterolateral cortex; *REM sleep* with low voltage EEG activity, no tonic EMG activity, with bursts of rapid eye movements (REM) and PGO waves.

The duration of each stage was expressed in minutes and hypnograms (total number - 100) were thus constructed for each animal and each experimental 5 h session. The duration of each sleep stage was determined every hour after KA injection and for the entire 5 h session on the day of injection (0 day) and on the 1st, 3rd, 6th, 15th, 30th and 65th day, as well as in the control records (before KA and after PBS).

**Statistical analysis**

Early effects of KA (up to the 5 h after the injection) were evaluated by means of two-way analysis of variance (ANOVA). Treatment (PBS, KA-4 nmol and KA-12 nmol) was chosen as independent factor and hour sessions (1,2,3,4,5 h) as repeated measures. Separate ANOVAs were performed for each sleep stage. If significant differences were detected, further post-hoc analyses (Newman-Keuls test and evaluation of the simple main effects by means of F-ratio (Winer 1971)) were made by determining which of the KA-treated groups (KA4 and KA12) differed from the animals injected with PBS. Since the effect of PBS was tested only on the day of its injection into NRD, in the studies investigating late effects of KA, numerical entries in ANOVA and in post-hoc analysis represented the differences between the amounts of the corresponding waking-sleep stages after and before the KA injection. KA treatment (KA4 and KA12) was chosen as independent factor in a two-way ANOVA, and day sessions (0,1,3,6,15,30 and 65 day) as repeated measures. The post-hoc analysis was as in the study of early effects. Additional parameters studied included the number of REM and SWS (NREM) sleep episodes and the latency between the time of injection (or the beginning of the record) and the first appearance of NREM or REM sleep episode. These parameters were compared with those of control records from the same cats and signed-ranks Wilcoxon test was used for statistical analysis of differences.

**RESULTS**

**Early effects**

No significant changes were detected in the waking-sleep stages between control records (without any treatment) and records with injection of PBS.

Two-way ANOVA with repeated measures on time (hours after KA or PBS) revealed that the effect of
Late effects of kainic acid injected into NRD on waking-sleep stages (summary of analysis of variance)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>W-1</th>
<th>W-2</th>
<th>SWS-1</th>
<th>SWS-2</th>
<th>REM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F(2,17)</td>
<td>1.09</td>
<td>8.87</td>
<td>8.76</td>
<td>9.93</td>
<td>7.24</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F(4,68)</td>
<td>3.09</td>
<td>2.26</td>
<td>2.80</td>
<td>0.84</td>
<td>2.18</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Treatm. X Hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F(8,68)</td>
<td>1.20</td>
<td>0.40</td>
<td>0.93</td>
<td>0.44</td>
<td>0.59</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, no significance

treatment (PBS, KA - 4 nmol, KA - 12 nmol) on all stages of waking-sleep cycle (except for the active waking) was significant (Table 1). No significant interaction effect (treatment x hours) was found indicating that the amount of the waking-sleep changes did not depend on the time after the injection (at least to the 5th hour). The post-hoc analysis (Newman-Keuls test) for total amounts of waking-sleep stages per 5-h recording time and the evaluation of the simple main effects of the KA treatment at each hour of recording (by means of F-ratio) showed that W-2, SWS-1, SWS-2 and REM were dramatically changed all the time up to the 5th hour, but only after KA at a dose of 12 nmol (Fig. 1). Quiet waking stage (W-2) was significantly increased, while light sleep (SWS-1), deep sleep (SWS-2) and REM sleep were suppressed as compared with the total duration of these stages in control records with PBS. There was a significant difference between the effects of KA at a dose of 4 nmol and 12 nmol for W-2, SWS-1 and SWS-2 and between the effect of KA at a dose of 12 nmol and the effect of PBS for all stages (except for W-1) but the effect of KA at a dose of 4 nmol differed from the effect of PBS only for the REM sleep.

Late effects

Late effects of KA injected into NRD were tested up to the 65th day. ANOVA revealed a significant effect only

Fig. 1. The early effects on the waking-sleep cycles of KA injected into NRD in cats. KA was administered in two doses: 4 nmol (black circles) and 12 nmol (triangles) compared with the PBS effect (squares). On the ordinate – mean durations (in min ± SEM) per 1-hour recording epoch spent in active waking (W-1), quiet waking (W-2), light slow-wave (SWS-1), deep slow-wave (SWS-2) and rapid eye movement (REM) sleep. Newman-Keuls test made on total amount of waking-sleep stages per 5-h recording time revealed: for W-1 – KA12/PBS (NS), KA4/PBS (NS) and KA12/KA4 (P < 0.05); for W-2 – KA12/PBS (P < 0.01), KA4/PBS (NS) and KA12/KA4 (P < 0.05); for SWS-1 – KA12/PBS (P < 0.01), KA4/PBS (NS) and KA12/KA4 (P < 0.05); for SWS-2 – KA12/PBS (P < 0.01), KA4/PBS (NS) and KA12/KA4 (P < 0.01); and for REM – KA12/PBS (P < 0.01), KA4/PBS (P < 0.05) and KA12/KA4 (NS). *P < 0.05, **P < 0.01 and ***P < 0.001 refer to the analysis of the simple main effects (F-ratio) of the KA treatment at every hour of recording. Asterisks show significance of the differences between pairs of treatments indicated on the right side of the graphs.
TABLE II

Late effects of kainic acid injected into NRD on waking–sleep stages

(source of analysis of variance)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>W-1</th>
<th>W-2</th>
<th>SWS-1</th>
<th>SWS-2</th>
<th>REM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F(1,8) )</td>
<td>3.02</td>
<td>0.13</td>
<td>0.51</td>
<td>0.54</td>
<td>0.00</td>
</tr>
<tr>
<td>( P )</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F(6,48) )</td>
<td>0.15</td>
<td>8.16</td>
<td>4.27</td>
<td>6.18</td>
<td>2.96</td>
</tr>
<tr>
<td>( P )</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Treatm. X Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F(6,48) )</td>
<td>0.23</td>
<td>0.77</td>
<td>0.55</td>
<td>0.96</td>
<td>0.40</td>
</tr>
<tr>
<td>( P )</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

of factor “Day” (Table II). The Newman-Keuls test showed that this difference was due only to the changes occurring on the day of KA injection (0 day) when compared to the pre-KA day (Fig. 2). Further evaluation of the simple main effects (by means of F-ratio) revealed that there was no significant difference in the amounts of waking-sleep stages between the days of recording when KA was applied at a dose of 4 nmol. However, when KA was injected at a dose of 12 nmol, the corresponding values of \( F(6,48) \) were as follows: for W-1 - 0.36 (NS), for W-2 - 5.32 (P < 0.001), for SWS-1 - 3.52 (P < 0.01), for SWS-2 - 3.79 (P < 0.01) and for REM - 2.31 (P < 0.05). In other words, KA at a dose of 12 nmol significantly decreased the duration of SWS-1, SWS-2 and REM and increased the duration of W-2 on the day of its injection when compared with their duration measured before the KA injection.

Figure 3 shows that the occurrence of NREM and REM sleep episodes was decreased on the day of the KA injection (0 day) and on the following day (1st day), but only on the 0 day the decrease was statistically significant. On this day, NREM and REM sleep begin much later than in the control record or did not appear at all during the 5 h recording. This is particularly noticeable in the animals injected with KA at a dose of 12 nmol (in 4 cats out of 5).
Fig. 3. Time course of KA-induced late changes (KA, 4 nmol – open bars; 12 nmol – hatchet bars) in the mean occurrence of NREM (SWS-1 and SWS-2) and REM sleep episodes per 5-h recording time in the day preceding KA (C), 10 min after injection of KA (0 day) and on the 1st, 3rd, 6th, 15th, 30th and 65th day. The two bottom histograms show, mean latency between the beginning of the recording and the first appearance of NREM and REM sleep (delay NREM, delay REM). Data represent the mean ± SEM. Vertical arrows indicate that these values are actually much greater because a delay of 300 min was considered when NREM or REM did not appear in the course of 5-h recording epoch (in 4 out of 5 cats of the KA12-group on the 0 day). *P < 0.05 – KA12/C and †P < 0.05 – KA12/KA4 (Wilcoxon signed-ranks test).

DISCUSSION

The present results showed that KA injected into the raphe dorsal nucleus at a dose of 12 nmol markedly suppressed SWS and REM sleep in freely moving cats during 5-h recording after the KA injection and to a lesser extent (statistically insignificant) on the following day. The effect of KA at a dose of 4 nmol was statistically insignificant. The post-injection suppression of REM sleep may have been secondary to the SWS reduction, because REM sleep normally follows sustained periods of SWS-2. This was supported by the finding that the REM stages were normal when cats exhibited restored SWS-2 periods at later post-injection time. The early changes in waking-sleep stages gradually restored to the pre-KA injection values and starting from first to third day up to the 65th day after KA injection the sleep profile of injected cats was completely normal.

About 70% of the cells of the raphe dorsal nucleus in cats are serotonergic (Wiklund et al. 1981) and this structure together with the raphe central superior nucleus are the main source of serotonergic innervation of the forebrain. The numerous investigations on the effects of various pharmacological manipulations of the brain 5-HT activity on vigilance have led so far to contradictory results. The serotonergic theory of sleep is based on earlier pharmacological data (Mouret et al. 1967; Jouvet 1972; Froment et al. 1974) which are not consistent with the more recent data relating to the 5-HT release and the firing rate of the 5-HT neurones during wakefulness and during sleep (McGinty and Harper 1976; Trulson and Jacobs 1979; Puizillout et al. 1979; Cespuglio et al. 1981). Thus, it has been demonstrated in cats that the transition from wakefulness to sleep is accompanied by a decrease in the firing rate of the 5-HTergic cells of the raphe dorsal nucleus, the lowest rate being during paradoxical (REM) sleep. This decrease of the functional activity of the serotonergic cells during sleep is shown to correlate well with a decrease in the 5-HT release from the forebrain serotonergic terminals during sleep as compared with the 5-HT release in the waking phase (Puizillout et al. 1979; Cespuglio et al. 1983). It might be hypothesized therefore that manipulations or treatments which increase the activity of the 5-HT cells and whereby the release of serotonin from the terminals would decrease the duration of sleep at the expense of the waking phase. Thus, stimulation, either electrical or pharmacological of the NRD cells might be expected to inhibit sleep by increasing serotonergic activity in the brain to a level that is incompatible with sleep. Nevertheless the data about the effects of electrical stimulation of the raphe dorsal nucleus on sleep are contradictory. Low-frequency train of stimuli (0.2-10 Hz) applied to NRD exerts sedative effects (Gumulka
et al. 1971) or provokes only week arousal reaction (Jacobs et al. 1973). Activating effect has been attributed to high-frequency (20-100 Hz) stimulation of NRD (Gumulka et al. 1971; Garcia-Ramoz 1978). We failed to find data about effects of pharmacological stimulation of the NRD cells on waking-sleep stages in any species of animals. There is some evidence of stimulatory effect of KA injected into NRD on the locomotion (Przewlocka et al. 1986).

The observed initial effects of behavioural and EEG activation immediately after the injection of kainic acid into NRD (in some animals also on the following day) were likely due to the well known excitatory properties of KA demonstrated in many other brain structures (Hall et al. 1979). The injections of KA into hippocampus or amygdala lead to an appearance of local and distal paroxysmal EEG activity (Schwarcz et al. 1978; Ben-Ari et al. 1979). Kainic acid injected into the core of the midbrain induces immediate and strong activation of the cortical EEG which lasts for 12-24 h (Kitsikis and Steriade 1981). Initial desynchronization followed by a dose- and time-dependent appearance of paroxysmal EEG activity in NRD and in many other brain structures has been shown in our earlier studies on the effects of KA injections into NRD in cats under acute conditions (Moyanova et al. 1986) and recently in freely-moving cats (Moyanova and Riche 1991). The paroxysmal EEG activity in NRD is probably a manifestation of an excitation by KA of the NRD cells as a result of which an increase in 5-HT release might be expected to occur at the terminals. This has recently been shown in rats (Przewlocka et al. 1986). There is no doubt that not only 5-HT but also other neurotransmitters might be released as a result of KA injection into NRD. In addition to serotonin, neurons of raphe dorsal nucleus contain catecholamines (Ochi and Shimizy 1978; Descarries et al. 1986), enkephalin (Uhl et al. 1979), neurotensin (Uhl and Snyder 1981), cholecystokinin (Van der Kooy et al. 1981), and GABA (Gamrani et al. 1979). After the initial strong excitation by KA, though it is difficult to say exactly when, the cells of NRD probably begin to undergo chromatolytic changes and to degenerate as a result of the toxic effect or KA. The GABAergic intrinsic neurones of NRD are the first ones to be damaged by KA (McGeer et al. 1979). They are also known to be the most vulnerable to the toxic action of KA injected into the hippocampus (Fischer and Alger 1984). The destruction of the GABAergic neurones would lead to a disinhibition of the main cells of NRD projecting to the forebrain and thus to an even greater increase of their functional activity. In other words, it might suggest that the decrease of the sleep duration and even the total sleep suppression at the expense of the increase of the waking phase for a few hours after the KA injection in all animals studied (not only in those exhibiting seizure activity) is due either to direct excitation of the main projecting neurones of NRD or to damage of the GABAergic interneurones, or most probably to both of them. It is well known that a certain time is necessary for restoration of the 5-HT pools, especially after an intense release of the neurotransmitter. By this time, also the main cells of NRD (including catecholaminergic and enkephalinergic) might undergo chromatolytic changes which lead to disturbances of their functional activity. This stage of the above-described hypothetical series of events, did not lead, however, to furthering of the initial sleep-suppressing effect of KA, but instead, to a restoration of the normal organization of sleep beginning from 1-3 days after the KA injection. The observed late effects of KA injections into NRD (complete restoration of sleep) did not resemble those induced by electrolytic lesion of this nucleus in cats (Petitjean 1981): a decrease of SWS-2 and an increase of SWS-1 up to the 8th day after the lesion, or in rats (Mouret and Coindet 1980): a decrease of SWS up to the 22nd day. These discrepancies might be due to the significant damage caused by the electrolytic lesion to axons of neurones lying outside NRD and actually involved in the control of sleep. In our recent study (Moyanova and Riche 1991), in addition to the chromatolytic changes in the NRD cells, we observed degenerative alterations in many other brain structures in cats injected with KA into NRD. It remains to be explained how behaviour and sleep organization return to normal in spite of such widespread damage in the brain. Thanks to functional plasticity of the brain, functional recovery from destruction of many areas of the brain has been shown to be possible (Irle 1987).

In conclusion, we would like to suggest that the cells of raphe dorsal nucleus are not essential for triggering off or maintenance of sleep because the initial suppression of sleep after KA injection into this nucleus seems to correlate with an increased activity of the raphe cells while the degeneration of these cells occurring at a later stage has no influence on sleep.

REFERENCES

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