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ARTICLE

Changes and relationship of somatosensory cortical electrical activity and hind paw defensive reflex in rats under various anesthetics

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ABSTRACT The effect of four common anesthetics on the spontaneous and evoked activity of the somatosensory cortex and on hind paw withdrawal reflex was studied in rats in acute preparation. The aim was to determine to what extent the choice of anesthetic and the depth of anesthesia may interfere with the effects of agents tested in such a system. Electrical activity was recorded on the dura surface after opening the skull, from the primary projection area of the whiskers which were stimulated electrically. Defensive reflex was elicited by manually pinching the toes of the left hind paw. Anesthesia was initiated with chloral hydrate or ketamine-xylazine, and continued with urethane or thiopental and then urethane. In the spontaneous cortical activity, delta band power was in strong correlation with the depth of anesthesia. Anesthesia also had a clear effect on the amplitude, but not on the latency, of the somatosensory evoked potential. Under effect of chloral hydrate the shape of the evoked potential was different from that seen under ketamine-xylazine or urethane. The results showed that properly chosen electrophysiological parameters can reliably indicate the depth of anesthesia, and that choice of anesthetic and level of anesthesia may effectively interfere with the effects of tested substances
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KEY WORDS

evoked potential
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In spite of all development of *in vitro* and *in silico* methods in the last decades, *in vivo* animal experimentation remains a fundamental tool of pure and applied biomedical research. In performing animal experiments with invasive measures, however, appropriate anesthesia is necessary for both ethical and practical reasons. Anesthesia in animal experiments is regulated at national and international level, e.g. in Article 14 of Directive 2010/63/EU (European Union 2010).

The way of action of all anesthetics includes interference with the functioning of the central nervous system – but that means that the „control" state of the animals in a particular experiment, before giving any test substance, is to some extent non-natural. This is inevitable and has to be taken in consideration when evaluating and interpreting the results, but in order to be able to do that, the own effects of the anesthetics need to be described. In this work, four parenteral anesthetics, used commonly in work with animals – urethane, chloral hydrate, ketamine-xylazine mixture and thiopental – were investigated.

Urethane is (or, mainly, used to be) a preferred anesthetic in animal experimentation because of its long-lasting effect without major depression of vital functions (Maggi and Meli 1986). Some reports identified the increase of inhibition via $GABA_A$ and glycine receptors, and decrease of glutamatergic excitation, as its chief mechanism of action (Hara and Harris 2002) while Maggi and Meli (1986) and Sceniak and macIver (2005) found only minor effect on GABAergic inhibition. Its effect is long-lasting (24 hours or longer) making it suitable primarily for terminal anesthesia. Urethane is being phased out from laboratory use mainly because of its human toxicity (IARC 1974; Koblin 2002).

Chloral hydrate, also known as trichloro acetaldehyde, was used also as a human anesthetic but is regarded today as obsolete because of its toxicity and its narrow therapeutic range (Pershad et al. 1999); the narrow range is a problem also in animals (Murray et al. 2000). It is, in fact, a prodrug, metabolized to trichloro ethanol which acts as a $GABA_A$ agonist (Lu and Greco 2006). Narcosis induced by chloral hydrate lasts up to 2 hours, an advantageous length, but the substance causes atonic ileus if injected intraperitoneally in concentration over 5% (Davis et al. 1985).

Ketamine is frequently used in human medicine (in contrast to the above two agents) first of all in children and in deteriorated patients. It is also common in veterinary practice and in animal experimentation. Ketamine is primarily a noncompetitive antagonist of NMDA receptors, acting on a binding site within the open pore of the receptor-channel complex (Bergman 1999). At the dose causing complete anesthesia, it also acts as central muscarinergic as well as alpha- and beta-adrenergic agonist. An alternative hypothesis says that ket-

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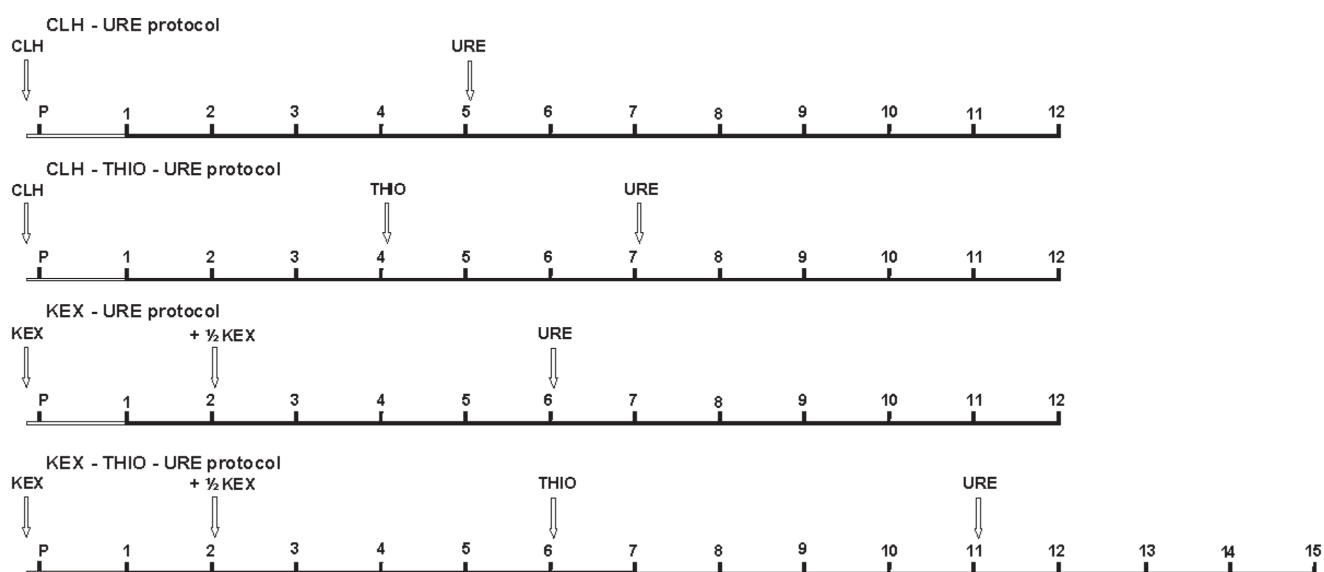

Figure 1. Timelines of the four protocols (*CLH – URE*, *CLH – THIO – URE, KEX – URE, KEX – THIO – URE)* used in the experiments. The time passed between injecting the first anesthetic (*CLH* or *KEX*) and preparing the scull for recording (P) was ca. 10 min; between P and the first recording session (1), 30 min were left; and between two subsequent recordings, 20 min.

amine blocks the hyperpolarization-activated neuronal cation channels, making cortical neurons tend to sleep-like rhythmic activity (Carr et al. 2007). For use in lab animals, ketamine is often combined with xylazine, an adrenergic agonist.

Pentobarbital was once a frequently used short-acting barbiturate for lab animals. Now, however, it is a restricted drug according to Schedule III of the UN Convention of Psychotropic Substances (United Nations 1971). Thiopental is a viable alternative with similar pharmacological properties (and its main metabolite is pentobarbital itself: Raj et al. 2011). Being a barbiturate, it acts on a distinct site of the $GABA_A$ receptor complex to enhance the efficacy of GABAergic inhibition (Twyman et al. 1989). Beyond short action, it is also important to remember that lower doses of thiopental induce narcosis without analgesia.

In the experiments presented here, two or three of the above mentioned anesthetics were given to rats one after another (see Fig. 1), and the changes in spontaneous and stimulus-evoked cortical electrical activity, and in peripheral defensive reflex (hind paw withdrawal on toe pinching, as a measure of the anesthetic action) were investigated in parallel.

Materials and Methods

Adult male Wistar rats (280-350 g body weight) were used, obtained from the breeding centre of the university. The animals were housed in an air conditioned animal room, maintained at 22ºC, with 12-hour light/dark cycle (light on at 06:00) and free access to tap water and standard rodent chow.

The effect of the anesthetics was tested using the doses given in Table 1; the corresponding protocols are presented in Figure 1. The doses were based on the literature sources given in Table 1 and on previous own experience; and the time span of observing the effect of one anesthetic (*i.e.* number of recording sessions, see below) on the typical length of action of each drug seen previously in comparable experiments. The maximal length of one experiment (15 x 20 min, that is, 5 hours) was in line with the experience (Pecze et al. 2005) that after anesthesia of such length the rats' general state starts to deteriorate. In each protocol, 8 rats were finally evaluated.

The rats were prepared for electrophysiological recording under anesthesia by the first agent (*CLH* or *KEX*). The animal's head was fixed in a holding frame, and the left hemisphere was exposed by opening the bony skull. Lidocaine (10% spray; EGIS, Hungary) was applied on the wounds, and the exposed dura was protected by a thin layer of petroleum jelly. The rat was put aside for a 30 minutes recovery, covered in a warm cloth, and was then transferred to the recording setup. Normal body temperature was maintained here by

Table 1. Doses of the anesthetics applied.

Anesthetic	Dose applied in this work (mg/kg b.w., ip.)	Reference for dose
Urethane (*URE*)	1000	Mook (2006)
Chloral hydrate (*CLH*)	400	Field et al. (1993)
Ketamine+xylazine (*KEX*)	100+8	Farkas et al (1999)
Thiopental (*THIO*)	30	Wixson (1994)

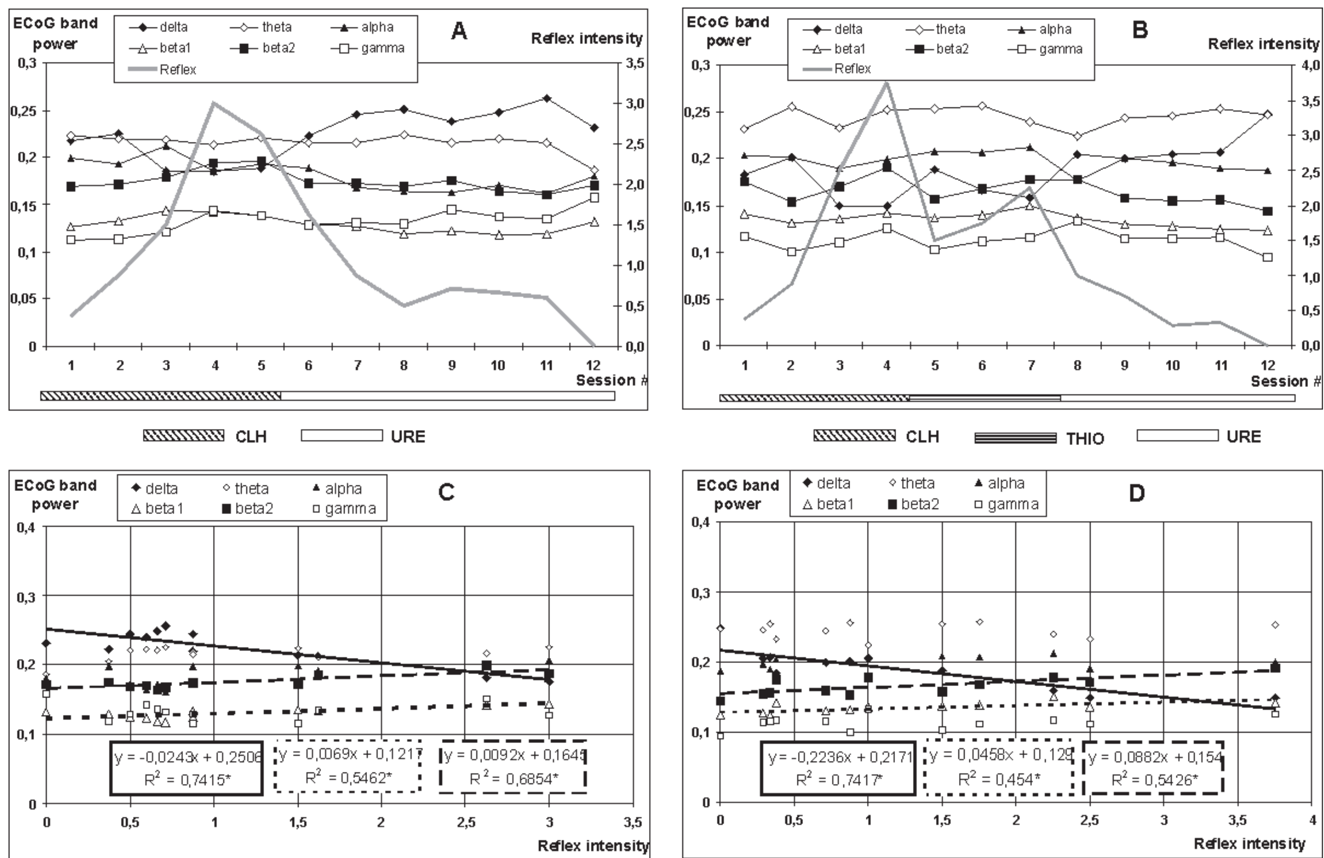

Figure 2. Top: time course of ECoG band power (left ordinate) and hind foot withdrawal reflex intensity (right ordinate) in rats treated using the *CLH – URE* (A) and *CLH – THIO – URE* (B) protocols (for the protocols, see Fig. 1). Recording sessions followed in 20 min intervals. The presented values are means, error ranges are omitted for clarity. Bottom: the corresponding correlation diagrams of reflex intensity and ECoG band powers (*CLH – URE* protocol, C*; CLH – THIO – URE* protocol, D). For substantial correlations, the trend line and its equation is given. Corresponding trend lines and equation boxes have the same line style (delta, solid; beta1, dotted; beta2, dashed), significant correlation (F>0.05 from Fisher's test included in "linear fit") is marked with * in the equation box and the data point symbols are enlarged.

the support plate thermostated to 36.5°C. A ball-tipped (tip diameter ca. 0.6 mm) silver wire electrode was placed on the primary somatosensory (SS) projection area of the whiskers ("barrel field": Waite 2004); there, punctum maximum of the evoked potentials (EPs) was found by moving the electrode as necessary. The indifferent electrode was a stainless steel clip placed on the cut skin surface. In a recording session, first the spontaneous activity (electrocorticogram, ECoG) was taken for 6 minutes. Then, sensory cortical EPs were recorded by stimulating the contralateral whisker pad with square electric pulses (3-4 V; 0.05 ms; 1 Hz), given in one train of 50 stimuli through a pair of hook-shaped steel electrodes inserted ca. in the middle of the uppermost row of whiskers. This recording session was repeated every 20 minutes. All cortical activity was amplified (10^4 x, low- and high-pass filters set to 1.6 and 1000 Hz), digitized (at 4096 Hz) and stored on PC.

From the ECoG records, the relative spectral power of the frequency bands (delta, 0.5-4 Hz; theta, 4-7 Hz; alpha, 8-13 Hz; beta1, 13-20 Hz; beta2, 20-30 Hz; gamma, 30-50 Hz: Kandel and Schwartz 1985) was determined automatically by the software used by means of FFT analysis. The recorded EPs were averaged. On the averaged curve, the latency and amplitude data of specific points were measured manually. Latency was measured between stimulus artifact (point"0") and the points numbered 1 to 6, and amplitude between opposed peaks of the main waves (see Fig. 4). The complete electrophysiological recording and analysis was done by means of the Neurosys 1.11 software (Experimetria, Budapest, Hungary).

The depth of anesthesia was determined on a semiquantitative scale based on the defensive reflex response elicited by a strong pinch to the toes of the hind paw (as described in Zandieh et al. 2003). The pinch was applied once before and after each recording session (this was infrequent enough to avoid habituation) and the grade observed, or the mean of the two grades if it was different before and after, was assigned to the session in question. No or minimal response was grade 0; weak response (one faint movement), grade 1; moderate re-

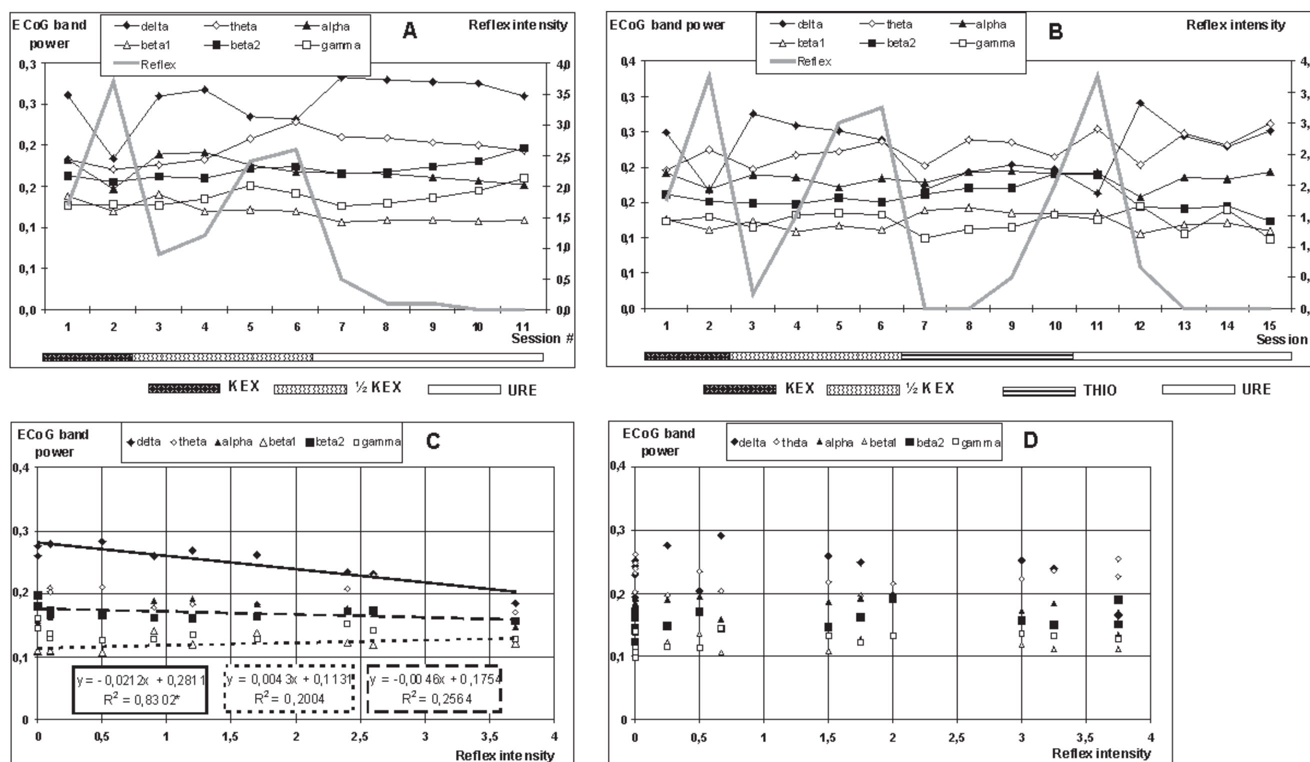

Figure 3. Top: time course of ECoG band power and hind paw withdrawal reflex intensity in rats treated using the *KEX – URE* (A) and *KEX – THIO – URE* (B) protocols. Bottom: the corresponding correlation diagrams of reflex intensity and ECoG band powers (*KEX – URE* protocol, *C; KEX – THIO – URE* protocol, D). The same display as in Figure 2.

sponse (one explicit movement), 2; vivid response (one strong movement or repeated weaker ones), 3; and strong response (repeated powerful movements), 4. On reaching grade 4, the next anesthetic in the protocol was administered forthwith.

For evaluation, all data – response grade (reflex intensity), ECoG band powers, EP latency and amplitude values – were averaged for whole groups (n=8) session by session; and were plotted against recording session number (*i.e.*, against time, see Fig. 1). Amplitude, and to a lesser extent, latency, of the EP was variable among the rats within a group, so that these values were first normalized to the mean of the values measured during the effect of the first anesthetic (that is, for the mean of sessions 1 to 5 in the *CLH – URE* protocol, of sessions 1 to 4 in the *CLH – THIO – URE* protocol, and of sessions 1 and 2 in both protocols starting with *KEX*). These plots then showed the time course of depth of anesthesia (response intensity) and the above mentioned electrophysiological parameters while the effect of the anesthetics was developing and fading out. Relationships suggested by the time courses were tested by means of correlation plots and R^2 values, calculated with the “linear fit” function of MS Excel. This function uses the least squares method to fit a straight line to the measurement data, and examines the strength of relationship with Fisher’s F test. The value of R^2 (determina-

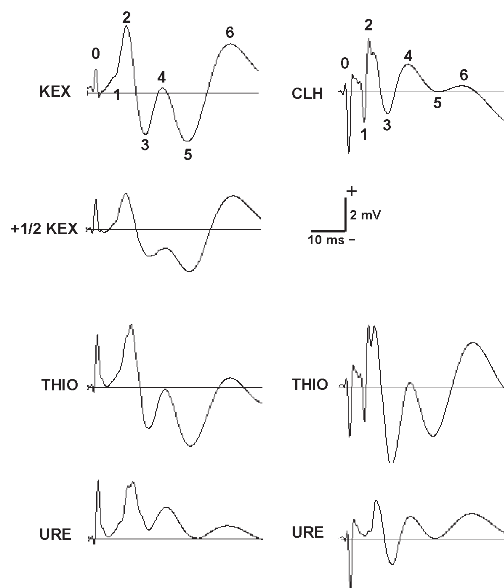

Figure 4. Typical examples of the somatosensory cortical evoked potential recorded according to the *KEX – THIO – URE* (left) and *CLH – THIO – URE* (right) protocols. Latency of the specific points (marked with numbering) was measured from the stimulus artifact (point 0); main wave peak-to-peak amplitudes were measured between points 2-3, and 5-6. Positive deflection is upwards.

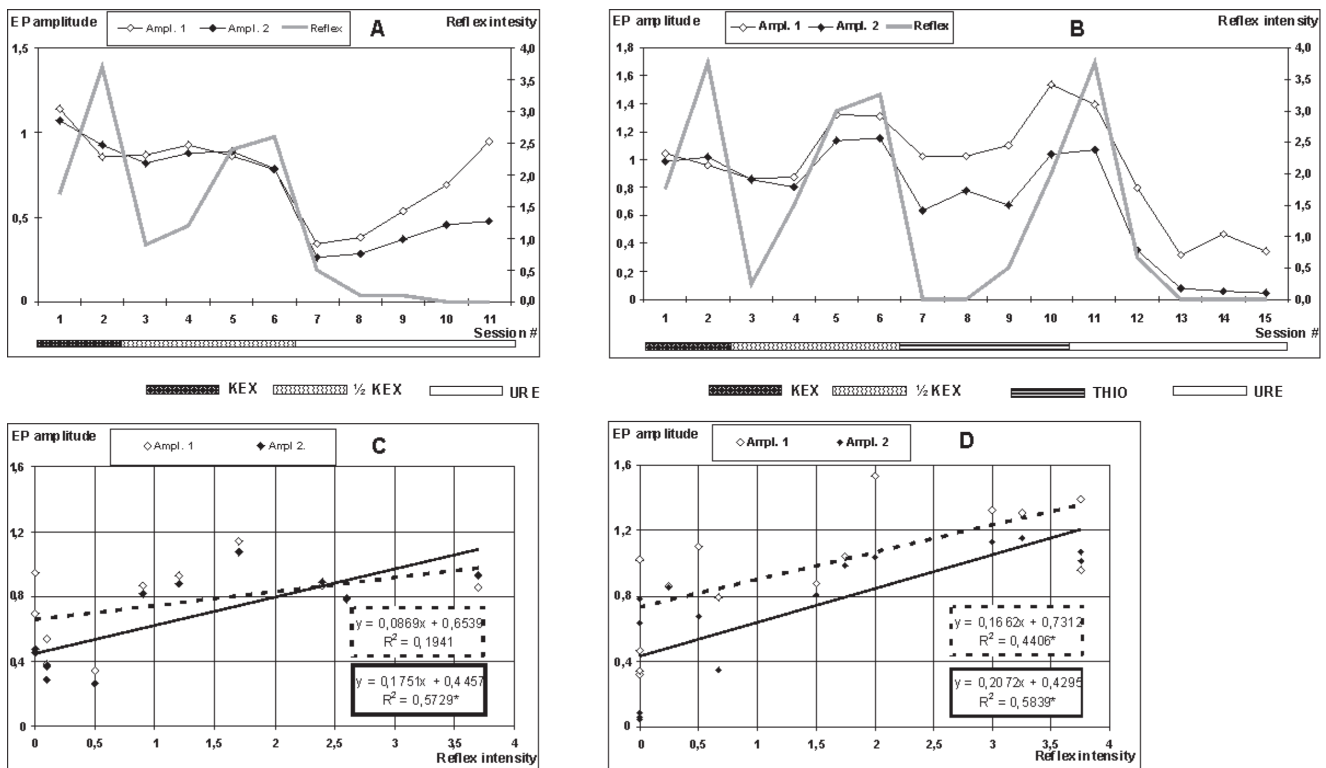

Figure 5. Top: time course of the EP main wave amplitudes (left ordinate) and hind paw withdrawal reflex intensity (right ordinate) in rats treated using the *KEX – URE* (A) and *KEX – THIO – URE* (B) protocols. Recording sessions followed in 20 min intervals. The presented values are means, error ranges are omitted for clarity. Bottom: the corresponding correlation diagrams of reflex intensity and EP amplitude (displayed as in Fig. 2: amplitude 1, light symbols and dotted line; amplitude 2, dark symbols and solid line), significant correlation (F>0.05 from Fisher's test included in "linear fit") is marked with * in the equation box.

tion coefficient) shows to what extent the variability of one (here: electrophysiological) parameter is explained by the other one (here: depth of anesthesia).

In the course of the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed.

Results

Effects of the anesthetics on the electrcorticogram

CLH – URE, *CLH – THIO – URE* protocols: As seen in Fig. 2A, reflex intensity gradually increased – that is, the depth of anesthesia achieved by *CLH* gradually decreased – in the first ca. 1.5 hours of these experiments. In parallel with that, ECoG delta activity decreased while beta and gamma activity increased. On administering *URE*, deep anesthesia was reached again after ca. 40 minutes and remained stable, and the shift in the ECoG band powers was reversed. Reflex intensity was quite strongly and significantly correlated with ECoG delta, beta1 and beta2 activity (Fig. 2C). In the *CLH – THIO – URE* protocol (Fig. 2B, D) the same effects were observed but the short action of *THIO* was also obvious.

KEX – URE, *KEX – THIO – URE* protocols: The action of *KEX* was short, so that reflex intensity became high (ca. 3.5) already at the 2nd recording session and a booster dose of *KEX* (1/2 of the original dose) had to be injected. The effect of this booster is clearly reflected in Fig. 3A. ECoG delta activity showed strong, significant correlation to reflex intensity, but for beta1 and beta2, R^2 values were lower (in contrast to the protocols with *CLH:* Fig. 3C). In the *KEX – THIO – URE* protocol, the time course of reflex intensity was similar to that seen with *CLH – THIO – URE*, but ECoG changes were less clear (Fig. 3B). Correlations were poor for the whole course of this protocol (hence, no trend lines are given in Fig. 3D)

Changes in the cortical evoked potential

In the rats anesthetized using the *KEX – URE* and *KEX – THIO – URE* protocols, the general shape of the cortical EP (Fig. 4) was identical to that seen in earlier experiments (Pecze et al. 2005; Takács et al. 2009) performed under pure *URE* anesthesia. Latency and amplitude values, determined by means of the specific points defined in Methods and marked in Figure 4, showed that presence of various anesthetics and depth of anesthesia had much more weak and inconsistent

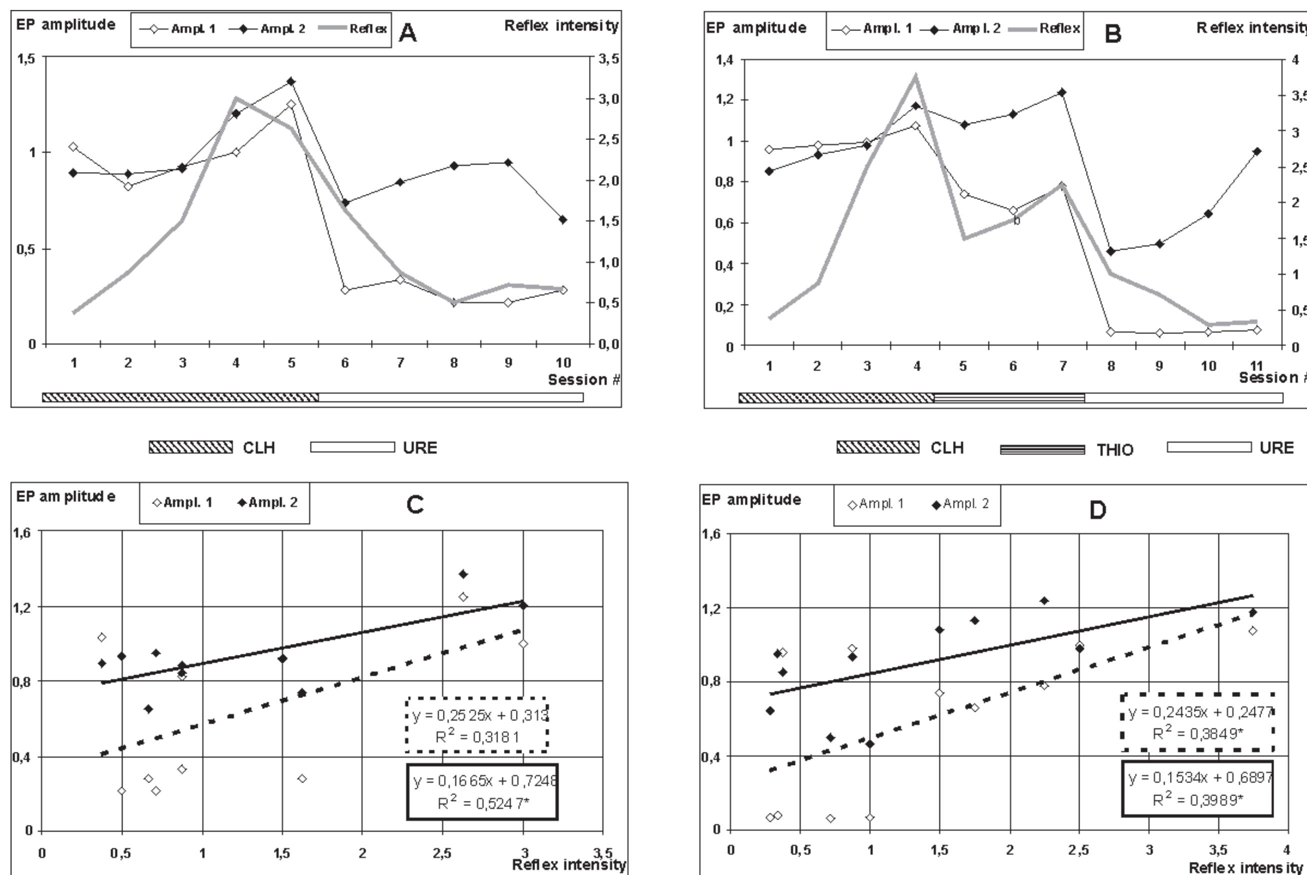

Figure 6. Top: time course of the EP main wave amplitudes (left ordinate) and hind paw withdrawal reflex intensity (right ordinate) in rats treated using the *CLH – URE* (A) and *CLH – THIO – URE* (B) protocols. Bottom: the corresponding correlation diagrams of reflex intensity and EP amplitude. The same display as in Fig. 5.

effect on the latencies of the EP than on its amplitude. The time course curves of Figure 5A and C also show that EP amplitude did not follow the changes of depth of anesthesia before and after the *KEX* repeat dose. Regarding the whole course of the protocols it can be stated all the same that the amplitude of the first wave (Y axis difference of points 2 and 3 in Figure 4) showed less strong correlation to reflex intensity (depth of anesthesia) than that of the later wave (difference of points 5 and 6).

When anesthesia was initiated using *CLH* (*CLH – URE* and *CLH – THIO – URE* protocols) the cortical EP had a different shape (Fig. 4); it was dominated by a very early sharp wave (sometimes present also under *KEX* or *URE* in rudimentary form) while the later parts were diminished. As the effect of *CLH* gradually subsided and that of *URE* set in, the shape of the EP was closer to that seen in the *KEX – URE* protocol but the unusually high and sharp early phase often remained. The parallelism between depth of anesthesia and EP amplitude was generally less strong than in case of *KEX* (Fig. 6).

Discussion

Spectral composition of the ECoG and amplitude of SS EP both showed a clear, monotonous, and mostly significant relationship to the intensity of the defensive reflex (withdrawal of hind paw on toe pinching). Such a relationship, and its potential use to monitor the depth of anesthesia, has repeatedly occurred in the literature. Field et al. (1993) tested *CLH* (300-450 mg/kg b.w.), *URE* (1.2-1.5 mg/kg b.w.) and pentobarbital (40 mg/kg b.w.) on rats, and found that the toe pinch response disappeared permanently under *URE*, but only for ca. 1 hour under *CLH*, and not at all under pentobarbital (experience from our lab also showed that *THIO* alone cannot produce surgical level anesthesia). The time course of the likelihood of response on toe pinch in rats treated with *URE* or *CLH* was similar to our curves of response intensity, indicating that both would be reliable measures of depth of anesthesia. In human volunteers receiving ketamine, subjective pain rating and amplitude of the cortical evoked response on a non-noxious galvanic stimulus changed in parallel (Kochs et al. 1996) and the authors mentioned the usability of evoked

response recording to assess the depth of anesthesia (our results, however, are at variance with this statement). According to other sources (Horn et al. 2009) only sophisticated joint analysis of EEG and auditory EPs of patients might provide a reliable indicator of level of anesthesia. The influence of anesthetic depth on the vibrissa–cortex transmission in rats, more exactly the shrinking receptive field of VPM thalamic neurons with deepening anesthesia (indicated by decreasing dominant ECoG frequency) is another clear illustration why and how the knowledge about the actual level of anesthesia in animal experimentation is a primary concern (Friedberg et al. 1999).

The artificial sleep in general anesthesia results from the imbalance between neuronal excitation and inhibition, induced by the drug applied. Most anesthetics are GABAergic agonists (*URE*: Hara and Harris 2002; *CLH*: Lu and Greco 2006). Suppression of glutamatergic excitation is also of importance, present in the action of *CLH* and *URE* (Kreuter et al. 2004) and being crucial in the action of *KEX* (Bergman 1999). Neurons of the thalamic reticular nucleus inhibit, by a GABAergic mechanism, thalamic pacemaker neurons, the slowed rhythm of which brakes specific afferentation to designated cortical areas via the relay nuclei. Sufficiently strong inhibition of the relay neurons can unmask the thalamic and cortical delta-oscillators, resulting in slow EEG typical for deep sleep (Otto 2008). The above mentioned GABA agonist effect of the anesthetics obviously enhances this intrinsic GABAergic inhibition while their glutamatergic antagonist effect reduces the activity of the ascending reticular activating system which acts not only directly on the cortex but also suppresses the inhibitory effect coming from the thalamic reticular nucleus (Otto 2008).

The qualitative difference between the shape of the SS EP recorded under *CLH* vs. under *URE* or *KEX* cannot be explained by the mentioned shift of excitation-inhibition balance. The very first deflections in the whole SS EP are, in all likelihood, of subcortical origin, arising from thalamic activation (Stienen et al. 2005) or from direct effect of the electric stimulus on the whisker pad muscles (Freeman and Sohmer 1996). Under effect of *CLH*, centripetal spread of excitation at and above the level of thalamus seems to be more strongly blocked than in case of *URE* or *KEX*, so that this fast, sharp wave is more pronounced in the cortical lead-off than with the other anesthetics.

The changes in the numerical parameters of the investigated cortical electrophysiological phenomena, resulting from the choice of the anesthetic and the actual level of anesthesia, were apparently large enough to interfere with the effects of test substances in neurotoxicological or neuropharmacological investigations, underlining the importance of correct choice and dosage of the anesthetic. The result also support that properly chosen electrophysiological parameters could reliably indicate the depth of anesthesia.

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