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ORIGINAL ARTICLE

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Involvement of δ-opioid receptors in pentylenetetrazol kindling development and kindling-related processes in rats

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Abstract The role of δ-opioid receptors on the development of kindling induced by the convulsant pentylenetetrazol (37.5 mg kg^{-1} i.p.) was investigated in rats. Besides the seizure development, the kindling induced enhancement of glutamate binding and the kindling-induced learning deficit were examined. A clear depression of kindling development by blocking of δ-opioid receptors by intracerebroventricular administration of naltrindole (10 nmol/5 μl) was found. In an acute convulsion test performed 8 days after kindling completion, animals pretreated with naltrindole during kindling induction showed lower seizure stages compared to saline-pretreated kindled animals. The kindling-induced increase in hippocampal glutamate binding was completely prevented by naltrindole, whereas the kindling-induced learning deficit was not influenced. The learning performance of control animals pretreated with naltrindole was very low. It was hypothesized that the various consequences of kindling induction could be influenced separately.
Summarizing the results, an involvement of the δ-opioid system in mechanisms underlying chemical kindling was clearly demonstrated. Interactions of endogeneous opioid systems with glutamatergic transmission were suggested.

Key words δ-Opioid receptors · Kindling · Learning deficit · Naltrindole · Pentylenetetrazol · Rat

Abbreviations *PTZ* pentylenetetrazol

Introduction

Opioid systems are involved in a number of important physiological functions. There has been considerable interest in the possible role of opioid receptors in the mediation of seizure activity. Epileptic properties of morphine have been known for a long time (Cogswell 1852) and it could be demonstrated that endogenous opiate agonists may play a role in modulating brain excitability and seizure activity (Urca et al. 1977; Frenk et al. 1978; Cain and Corcoran 1985; Hong et al. 1993; Simmons and Chavkin 1996). Conflicting results in the literature suggest that the route of administration, animal species and dosages are crucial factors for proconvulsant or anticonvulsant properties of opioids (Henriksen et al. 1978; Frenk 1983; Böhme et al. 1987; Cain et al. 1990). Because it has been demonstrated that opioids produce their effects through three major types of opioid receptor, i.e. m, κ and δ opioid receptors, the question of the role of opioid receptor subtypes in brain excitability and seizure activity arose. Stimulation of δ-opioid receptors by systemic (Comer et al. 1993; Pakarinen et al. 1995) or by hippocampal (DeSarro et al. 1992) application of δ-opioid receptor agonists produced convulsions in various species which could antagonized by specific δ-opioid receptor antagonists. An increased magnitude of the population spike in hippocampal slices by the δ-opioid agonist deltorphin was demonstrated in vitro (Sagratella and Scotti de Carolis 1993).

Recently, we investigated the role of the opioid system by the influence of an opioid receptor blockade on the development of kindling induced by the convulsant pentylenetetrazol (PTZ) (Becker et al. 1994a). Kindling represents an accepted model of human epilepsy and is characterized by progressive intensification of electroencephalographic and behavioural seizures evoked by initially subeffective chemical or electrical stimuli. This model offers unique opportunities to study pathophysiological mechanisms of epileptogenesis. Besides the convulsive component of epilepsy it is possible to investigate secondary alterations, in particular in the field of cognition. Previously, we reported dramatic learning impairment of active-avoidance acquisition in PTZ-kindled rats (Becker et al. 1992). Pretreatment with the opioid receptor antagonist naloxone before each PTZ injection during the kindling induction had a dissociative effect. Whereas no significant effect on

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seizure development was found, the learning impairment in kindled rats was ameliorated (Becker et al. 1994a).

The purpose of the present experiments was to examine the role of δ-opioid receptors on the development of the chemical kindling induced by the convulsant PTZ, investigating seizure development and the kindling-induced learning deficit. Moreover, kindling-induced enhancement of hippocampal glutamate binding sites was examined. The hippocampus of kindled rats mimics characteristic features of enhanced excitability such as increased glutamate binding (Becker et al. 1997; Schröder et al. 1993; Schröder and Becker 1996) and a specific kind of potentiation in the dentate gyrus (Rüthrich et al. 1996). The important role of an intact dentate gyrus for PTZ kindling development was supported by experiments using colchicine lesions (Grecksch et al. 1995).

Materials and methods

For all procedures followed, ethical approval was sought prior to the experiments according to the requirements of the National Act on the Use of Experimental Animals (Germany).

Animals. Experiments were carried out with male Wistar rats [Shoe:Wist(Shoe), Tierzucht Schönwalde] aged 7 weeks at the beginning of the experiments. The animals were kept under controlled laboratory conditions (light regime of 12 h light/12 h dark, light on at 06.00 a.m.), temperature 20±2 °C, air humidity 55–60%. They had free access to commercial rat pellets (Altromin 1326) and tap water. The rats were housed in groups of five per cage (Macrolon IV).

Implantation of microcannulas. Seven-week-old rats were chronically implanted with microcannulas under deep pentobarbital anaesthesia (40 mg kg^{-1} i.p.) into the right lateral ventricle (coordinates AP=0.25 mm; lateral=1.6 mm, deep 3.5 mm, according to Skinner 1971).

All experiments were started after a recovery period of 7 days after surgery.

Acute pentylenetetrazol convulsion test. Naive rats were injected with 45 mg kg^{-1} (=326 μmol kg^{-1}) i.p. PTZ. To investigate the acute effect of the δ-opioid antagonist on PTZ convulsions 1 or 10 nmol naltrindole in a volume of 5 μl were infused over a period of 30 s intracerebroventricularly 10 min before the injection of PTZ. Control animals received 5 μl of sterile 0.9% saline. The slow injection of 5 μl physiological saline was tolerated by rats without any reaction and alteration of neural activity like electroencephalographic parameters and evoked responses, respectively. The convulsive behaviour was observed for 20 min after PTZ injection and the resultant seizures were classified as follows:

1. Seizure stage 0: no response.
2. Seizure stage 1: ear and facial twitching.
3. Seizure stage 2: convulsive waves through the body, without rearing.
4. Seizure stage 3: myoclonic jerks, upright position.
5. Seizure stage 4: clonic-tonic convulsions, turn-over into side position.
6. Seizure stage 5: generalized clonic-tonic convulsions, loss of postural control.

PTZ kindling. The kindling induction was started using naive 8-week-old rats at least 7 days after implantation of intracerebroventricular microcannulas. For kindling initially subconvulsive doses of PTZ (37.5 mg kg^{-1}=271 μmol kg^{-1}=ED_{16} for induction of clonic-tonic seizures) were injected i.p. once every 48 h. Kindling control animals received the same number of injections of 0.9% saline i.p.. Naltrindole (10 nmol/5 μl) or saline were intraventricularly injected 10 min prior to each PTZ or saline application. Intracerebroventricular administration was chosen to avoid peripheral side effects. After each PTZ injection the animals were observed for 20 min and seizures were classified using the scale for seizures described above.

Four groups of animals were used:

1. Kindling group=saline/pentylenetetrazol (Sal/PTZ).
2. Kindling under naltrindole pretreatment=naltrindole/pentylenetetrazol (Nal/PTZ).
3. Kindling control group=saline/saline (Sal/Sal).
4. Kindling control group under naltrindole pretreatment=naltrindole/saline (Nal/Sal).

The kindling procedure was finished after 13 kindling injections.

Learning experiment. Twenty-four hours after the last kindling injection the learning experiment was started in order to study the rats' learning performance in a shuttle box.

The automatic shuttlebox (0.25 m × 0.25 m × 0.6 m) was divided into two identical compartments separated by a 5-cm hurdle. The conditioned stimuli were light (40-W bulbs located on the central ceiling of each compartment) and a sound produced by a buzzer. The unconditioned stimulus was an electric stimulation of 0.4–0.8 mA depending on the individual sensitiveness of the animal, delivered through stainless steel rods covering the floor. The conditioned stimuli-unconditioned stimulus interval was 4 s. The stimuli were switched off when the rat had changed to the goal compartment. One trial was limited to 20 s if the animal failed to react within this period. The mean intertrial interval was 30 s. Each session consisted of 20 trials and was repeated on four consecutive days. Sessions were performed between 08.00 a.m. and 12.00 p.m. during the light part of the 12:12 h cycle at about the same time. Prior to the first session, the rats were allowed to explore the box for 5 min, and the following days 1 min was provided.

The number of escapes (reaction time >4 s–20 s) and conditioned reactions (reaction time <4 s) in each training session were recorded for further evaluation.

Challenge test. All rats from the four different treatment groups received a challenge dose of 30 mg kg^{-1} (=217 μmol kg^{-1}) PTZ i.p. without any pre-treatment 7 days after the final kindling injection. This dose is in general subconvulsive in unkindled animals. After PTZ injection convulsion behaviour of the animals was observed for 20 min and classified in the same way as during kindling induction. This experimental schedule allows us to distinguish an influence on kindling development from an acute anticonvulsive effect.

Glutamate binding assay. Twenty-four-hours after having received the challenge dose of PTZ, six animals were randomly selected from the four groups used in the kindling experiment for neurochemical studies. The animals were sacrificed by decapitation and the brains were quickly removed. The hippocampi were dissected for measuring glutamate binding. Crude membrane fractions were prepared by the modified method of Zukin et al. (1974) and the [^{3}H]-L-glutamate binding was determined using the method described by Schröder et al. (1993). Specific binding was calculated by subtracting non-specific binding (defined as that seen in the presence of 50 nM [^{3}H]-L-glutamate plus 100 μM unlabelled L-glutamate) from total binding obtained with [^{3}H]-L-glutamate alone. The data were determined as femtomoles bound radioligand per milligram protein. The labelled L-glutamate concentration used in the binding assay was 50 nM.

Statistical evaluation. Concerning seizure stages in the acute PTZ convulsion test and challenge test analysis of variance was used. To evaluate the development of seizures in the course of kindling development and the learning performance of the animals in the shuttle box experiment, the repeated measures model was applied several times to test hierarchical hypotheses.

The basis of statistical decision was a significance level of 0.05. The calculations were carried out by means of SPSS/PC+ software (procedure ANOVA and MANOVA).

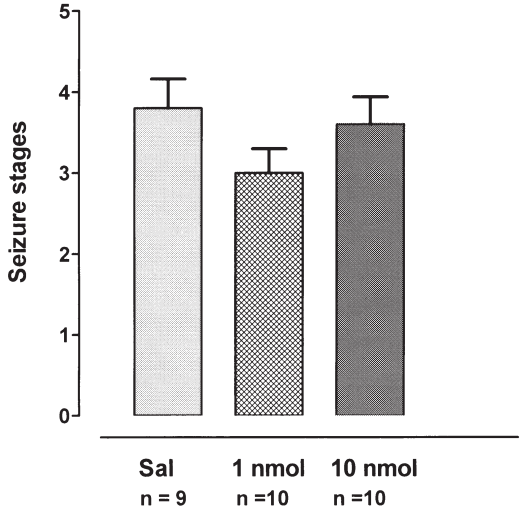

Fig. 1 Acute pentylenetetrazol (PTZ) convulsion test. Convulsive behaviour of naive rats induced by an injection of 45 mg kg^{-1} PTZ after pretreatment with saline (Sal) or different doses of naltrindole (Nal). *Ordinate*: seizure stages (means±SEM). Analysis of variance (ANOVA), one-way. $F(2,26)=1.496$; $P=0.29$

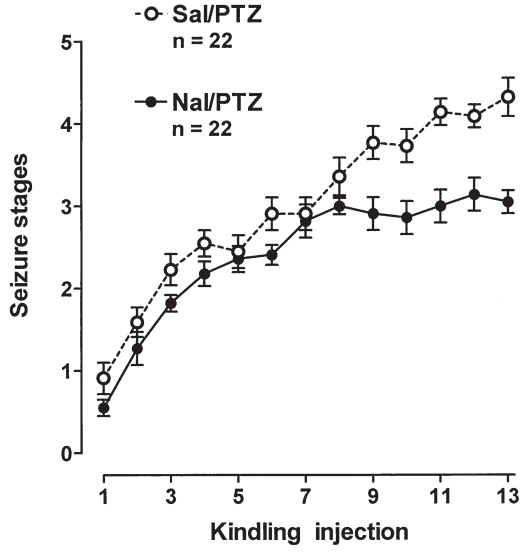

Fig. 2 Kindling development in rats pretreated i.c.v. with 10 nmol naltrindole or saline 10 min before each PTZ kindling injection of 37.5 mg kg^{-1}. *Ordinate*: seizure stages (means±SEM). *Abscissa*: 13 kindling stimulations. Repeated measures Sal/PTZ versus Nal/PTZ $n_1=22$; $n_2=22$; $F(42,1)=15.18$; $P<0.001$

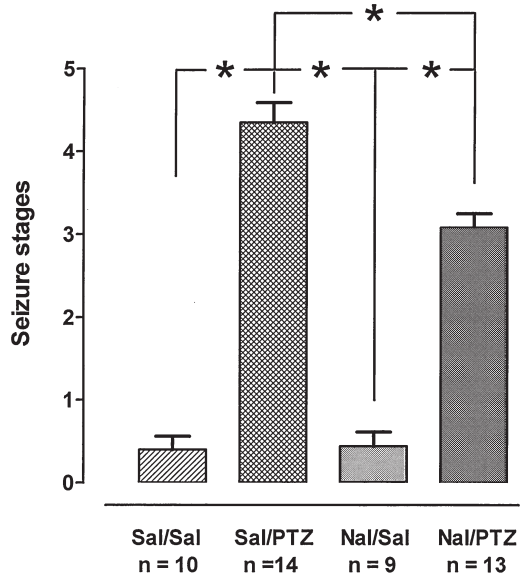

Fig. 3 Challenge test with kindled rats 7 days after the last kindling injection (without naltrindole or saline injection in the test). All rats received a dose of 30 mg kg^{-1} of PTZ. *Ordinate*: seizure stages (mean±SEM). Analysis of variance (ANOVA), one-way $F(3,42)=90.34$: $P<0.001$. Multiple-range tests: Student-Newman-Keuls test with significance level 0.05. Significances: Sal/Sal versus SalPTZ; Sal/PTZ versus Nal/PTZ; Nal/Sal versus Sal/PTZ

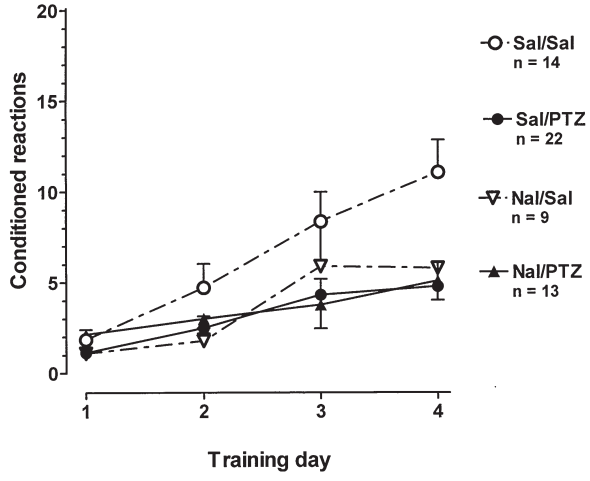

Fig. 4 Learning performance of kindled rats in the shuttle box. *Ordinate*: conditioned reactions (mean±SEM) were given for four training sessions. Repeated measures: Sal/Sal versus Sal/PTZ $n_1=14$; $n_2=22$; $F(34,1)=7.27$; $P=0.011$. Sal/PTZ versus Nal/PTZ $n_2=22$; $n_4=13$; $F(33,1)=0.10$; $P=0.755$. Sal/Sal versus Nal/PTZ $n_1=14$; $n_4=13$; $F(25,1)=4.59$; $P=0.42$

Results

Acute i.c.v. pretreatment of naive rats with naltrindole 10 min before injection of a convulsive dose of 45 mg/kg PTZ i.p. did not significantly influence the resultant convulsions (Fig. 1).

As shown in Fig. 2, PTZ-injected animals (Sal/PTZ) showed increasing seizure severity during the course of development of kindling. The experimental group pretreated with naltrindole (Nal/PTZ) reacted at the beginning of kindling induction in a similar manner. But after reaching seizure stages of about 3 on kindling day 8, no further intensification of convulsion behaviour was evident, whereas the Sal/PTZ group showed constant seizure stages higher than 4, that means tonic-clonic seizures in the last part of kindling procedure.

The result of the challenge test, in which the animals were tested without new injection of naltrindole (Fig. 3), demonstrated lower seizure stage in the group pretreated with naltrindole (Nal/PTZ) during kindling induction com-

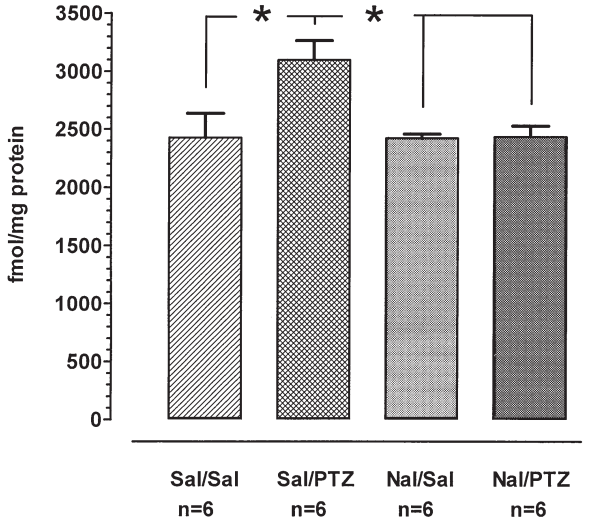

Fig. 5 Specific [^{3}H]-L-glutamate binding to synaptic membranes of the hippocampus of PTZ-kindled rats in comparison to kindling controls and pre-treatment during kindling induction with saline or naltrindole (mean±SEM). *U*-Tests: Sal/Sal versus Sal/PTZ $U(6,6)=4$. Nal/Sal versus Sal/PTZ $U(6,6)=2$. Sal/PTZ versus Nal/PTZ $U(6,6)=2$, $^*P<0.05$

pared with the control kindling group (Sal/PTZ). The behaviour of the kindling control group was not influenced by naltrindole (Sal/Sal and Nal/Sal).

The learning experiment in the shuttle box demonstrated a poor learning performance of kindled rats to acquire the conditioned reaction (Fig. 4). Rats kindled under naltrindole pretreatment (Nal/PTZ) did not differ from saline-pre-treated kindled rats (Sal/PTZ). Interestingly, the learning performance of naltrindole control rats (Nal/Sal) was markedly lowered compared to the control group (Sal/Sal).

Figure 5 demonstrates glutamate binding in the four groups of the kindling experiment. The binding was significantly enhanced in the control kindling group (Sal/PTZ) compared to kindling control animals (Sal/Sal). Pretreatment with naltrindole during kindling induction (Nal/PTZ) abolished this enhancement. The naltrindole treatment did not influence the binding rate in control animals (Nal/Sal).

Discussion

The results presented clearly demonstrate a depression of kindling development by blockade of opioid δ-receptors by intracerebroventricular administration of naltrindole (Fig. 2). Compared with the opioid receptor antagonist naloxone (Becker et al. 1994a) the effect of naltrindole on seizure development is much more pronounced. Administration of naloxone during the kindling procedure resulted in no major changes in seizure development, although a tendency to depress seizure development was repeatedly found in different experimental series using naloxone.

Interestingly, the first part of the kindling curve is identical with and without blockade of δ-opioid receptors. This corresponds to the failure of an anticonvulsive effect of naltrindole in an acute PTZ convulsion test in naive rats (Fig. 1). This is in agreement with results in the literature (Comer et al. 1993) that naltrindole produces a shift to the right in the potency of a δ receptor agonist to evoke convulsions, but has no effect on the dose-effect curve of PTZ. In a challenge test, it was proved that in the present experiment the kindling development is impaired by blockade of δ-opioid receptors. To this end all kindling groups were tested in a convulsion test without presence of naltrindole. The animals pretreated with naltrindole during kindling induction showed lower seizure stages compared to saline pretreated kindled animals. In control animals the pretreatment with naltrindole had no effect on the excitability in the acute convulsion test.

As demonstrated in earlier kindling experiments using pentylenetetrazol the glutamate binding to hippocampal membranes was increased after kindling completion. We had shown that a single generalized convulsion induced by PTZ did not result in any discernable change in glutamate binding after 24 h, whereas PTZ-kindling resulted in increased glutamate binding persisting up to 9 weeks after kindling completion. These results suggest long-lasting alterations in glutamatergic mechanisms in the hippocampus (Schröder et al. 1993, 1994; Becker et al. 1997). Blockade of δ-opioid receptors during kindling induction completely prevented this enhancement. Therefore a close correspondence exists between seizure development and kindling induced changes in glutamatergic neurotransmission. On the other hand, we have recently demonstrated that the DAMGO [Tyr-D-Ala-Gly-(Me)Phe-Gly-ol] and naltrindole binding to hippocampal membranes displayed no significant changes in kindled rats compared to controls. Also, the naltrindole binding was not altered by naltrindole pretreatment during kindling induction (Schröder et al. 1998). These results further support the assumption that specific changes in glutamatergic neurotransmission play an important role for the expression of PTZ kindled seizures. Interestingly, we have recently demonstrated that pretreatment with the competitive NMDA receptor antagonist CGP 43487 and the non-competitive NMDA receptor antagonist MK 801 depresses kindling development (Krug et al. 1998). δ-Opioid receptors seem to be involved in the processes leading to enhanced glutamate binding. A cross-talk between NMDA receptors and δ-opioid receptors was demonstrated recently (Cai et al. 1997).

Considering the fact that endogenous opioid peptides also play an important role in the modulation of learning and memory (McGaugh et al. 1993), we investigated the effect of δ-opioid receptor blockade on the kindling induced learning impairment. Although naltrindole depressed the seizure development during kindling, the learning deficit in the shuttle box experiment of kindled rats was not antagonized. In addition, the learning performance of control animals pretreated with naltrindole was very low. Although many exceptions can be found, the general action of μ- and δ-opioid receptor agonists is to impair learning and memory and of the antagonists is to

facilitate them and to alleviate amnesia (Olson et al. 1990, 1995; Izquierdo 1980; McGaugh 1990; Ilyutchenok and Dubrovina 1995). Specifically, δ-opioid receptor agonists have been demonstrated to produce amnesia (Schulteis et al. 1988; Pakarinen et al. 1995; Ukai et al. 1997) and selective δ-opioid antagonist reversed an induced learning impairment (Schulteis and Martinez 1990) and enhanced memory retrieval (Ilyutchenok and Dubrovina 1995). Our results demonstrating a low learning performance do not necessarily conflict with the literature, because the animals in the experiments presented were chronically injected over a period of 4 weeks.

On the other hand our results clearly indicate that a depression of kindling induced seizure development did not automatically prevent the kindling induced cognitive impairment and also normalization of glutamate binding alone was not sufficient to normalize learning performance. Similarly, we have recently found that seizure prevention by the benzodiazepine receptor agonist diazepam did not result in prevention against learning deficit in PTZ-kindled rats (Becker et al. 1994b). To the contrary, we could prevent the learning impairment in kindled rats by treatments during kindling development by gangliosides and the opioid receptor antagonist naloxone without an effect on seizure expression (Grecksch et al. 1991; Becker et al. 1994a). Regarding these results it could be suggested that development of seizures and impairment in learning abilities in kindled animals are different processes which develop independently of each other, probably founded on one common underlying mechanism. Also, the various consequences of kindling induction could be partly influenced separately.

Summarizing the results, an involvement of δ-opioid systems in the mechanisms underlying chemical kindling could be suggested. An interaction with glutamatergic systems seems likely, but with the present results it is not possible to characterize more precisely if this is a direct interaction with glutamate receptors or an indirect modulator effect including other parameters of neuronal transmission. For instance, there is experimental evidence that δ-opioid receptors interfere with parameters of GABA-ergic neurotransmission (Kalyuzhny and Wessendorf 1998; Piguet and North 1993), which might additionally contribute to anticonvulsive effectiveness of δ-opioid antagonists as demonstrated in our study.

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