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Amygdaloid kindling by repeated focal *N*-methyl-D-aspartate administration: comparison with electrical kindling

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Abstract

Limbic seizures were kindled by repeated, daily intra-amygdaloid microinjections of *N*-methyl-D-aspartate (NMDA; 2 nmol). The seizures, and accompanying afterdischarges, closely resembled those seen following electrical kindling of the amygdala. As with electrical kindling, co-administration of the competitive NMDA receptor antagonist DL-2-amino-7-phosphonoheptanoic acid (AP7; 70 nmol) prevented the development of seizure activity. NMDA-induced kindling was durable, lasting at least 1 month, and showed positive transfer to electrical kindling. Fully kindled seizures were inhibited by co-administration of the potent NMDA receptor antagonist DL-[*E*]-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849) with the agonist. These results strongly support a role for NMDA receptors in kindling epileptogenesis.

Keywords: Kindling; NMDA (*N*-methyl-D-aspartate); Seizure; Epilepsy; Excitatory amino acid; Amygdala

1. Introduction

The brain mechanisms underlying epileptogenesis are at present poorly defined. Kindling, an animal model of human complex partial and secondary generalised focal seizures, first described by Goddard and co-workers (1969), is a valuable model for the study of these processes.

The term kindling refers to the gradual development of epileptiform activity as a result of repeated, spaced stimulations of specific brain regions. Evidence is accumulating for an involvement of excitatory amino acids in kindling epileptogenesis. Thus, both tissue content and basal extracellular levels of glutamate (as shown by intracerebral microdialysis) are elevated in the amygdaloid complex following electrical kindling of this region (Kaura et al., 1995). Elevated extracellular concentrations of glutamate (and γ-aminobutyric acid) have also been reported in the hippocampus during early stages of amygdala kindling (Minamoto et al., 1992). Moreover, During and Spencer (1993), also using microdialysis, have reported a sustained increase in hippocampal extracellular glutamate, which preceded seizure onset, in six patients with refractory complex partial epilepsy. Competitive and non-competitive antagonists of the NMDA sub-type of excitatory amino acid receptors have been shown to inhibit the development of electrical kindling in rodents (Croucher et al., 1988; Gilbert, 1988; Vezzani et al., 1988). Additionally, full limbic seizures can be kindled by repeated intra-amygdaloid injections of the endogenous excitatory amino acids glutamate and aspartate, either alone or in combination (Mori and Wada, 1987; Croucher and Bradford, 1989), and this chemical kindling can also be inhibited by NMDA receptor antagonists (Croucher and Bradford, 1990a).

In the present study we demonstrate that seizures can be kindled by daily focal administration of the selective excitatory amino acid receptor agonist *N*-methyl-D-aspartate (NMDA) alone. These seizures closely resemble those seen with electrical kindling and strong positive transfer can be demonstrated between the two processes, suggesting that NMDA-induced and electrical kindling share common neurochemical mechanisms. As with electrical kindling, the competitive NMDA receptor antagonist DL-2-amino-7-phosphono-

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heptanoic acid (AP7) also inhibits the development of NMDA-kindled seizures confirming that NMDA receptors play a critical role in the kindling process. A preliminary account of some of this work has been published (Croucher et al., 1992b).

2. Materials and methods

2.1. Animals and surgery

Details of the experimental procedures are described elsewhere (Croucher and Bradford, 1989). Briefly, male Sprague-Dawley rats (280–320 g) were implanted with a guide cannula/bipolar electrode assembly into the right basolateral amygdala under halothane/nitrous oxide anaesthesia. The assembly was secured to the skull with two stainless steel anchor screws using cyanoacrylate cement and zinc powder. The chemical kindling procedure was begun at least 1 week after surgery.

2.2. Kindling with NMDA

N-Methyl-D-aspartate was focally administered, on a daily basis, by microinjection into the basolateral amygdala in a total volume of 0.5 μl of 50 mM phosphate buffer, pH 7.4. When required, DL-2-amino-7-phosphonoheptanoic acid (AP7) or DL-[E]-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849) was co-administered with the NMDA in the same final volume. Controls were given vehicle alone.

Animals were observed for behavioural and electroencephalographic (EEG) changes for 30 min post-injection. Motor seizure activity was rated on a scale of 0–5 based on that of Racine (1972), as follows: 0 – no behavioural response; 1 – facial myoclonus and vibrissae twitching; 2 – jaw myoclonus and/or head bobbing; 3 – 2 plus unilateral forelimb myoclonus; 4 – 3 plus rearing with bilateral forelimb myoclonus; 5 – 4 plus repeated rearing and falling.

Following the development of the maximal (stage 5) seizure response, or after 30 microinjections, animals remained unstimulated for 1 month. After this period the effects of a further single dose of NMDA were determined. Subsequently, in animals which were fully kindled by repeated NMDA injection, the anticonvulsant activity of a single co-injected dose (1 nmol) of the competitive NMDA receptor antagonist CGP 37849 was tested.

Animals given NMDA and AP7 simultaneously received 20 daily microinjections and were then left unstimulated for 4 days to allow for drug clearance (Croucher and Bradford, 1989). NMDA alone was then administered daily, as above.

2.3. Electrical stimulation

Finally, both control and NMDA-kindled animals were stimulated electrically. The threshold current for evoking an electrical afterdischarge i.e. the afterdischarge threshold, was estimated in all animals using a method of ascending limits (Croucher and Bradford, 1989). The afterdischarge threshold, the duration of the first afterdischarge and the severity of the accompanying motor seizure (rated as above) were compared between buffer- and NMDA-treated animals.

2.4. Histology

On completion of the study, animals were anaesthetised and perfused transcardially with phosphate-buffered saline (200 mM, pH 7.4). The brains were removed and rapidly frozen in cooled isopentane. Serial 20 μm coronal sections were cut using a cryotome and stained with Toluidine Blue. Sections were examined by light microscopy for histological changes and to confirm the positioning of the injection cannula and electrode tips.

2.5. Drugs

NMDA and AP7 were purchased from Tocris Cookson (Bristol, UK). CGP 37849 was a kind gift from Dr. L. Maître, Ciba-Geigy Ltd., Basle, Switzerland.

3. Results

3.1. NMDA-induced kindling

In a preliminary study, increasing doses of NMDA were focally administered to a group of six animals on consecutive days and EEG and behavioural changes were carefully monitored. Vehicle alone and NMDA, 1 nmol, failed to evoke EEG or motor responses in any animal. NMDA, 2 nmol, evoked a mean seizure stage of 0.9 ± 0.5 (mean ± S.E.M., $n = 6$), typical of the response seen following the first suprathreshold stimulation of electrical kindling. This dose of NMDA was therefore selected for subsequent daily administration.

Repeated daily focal microinjection of NMDA, 2 nmol, evoked seizures of progressively increasing severity (Fig. 1). The developing seizure activity closely resembled that seen during electrical kindling. Thus, animals initially displayed facial and jaw myoclonus followed by unilateral, then bilateral forelimb jerking and myoclonus and ultimately rearing and falling. In addition, animals showed twisting of the head and upper trunk towards the tail, contralateral to the injected side. All animals progressed to generalised seizure activity following a mean (±S.E.M.) of 17.7 ±

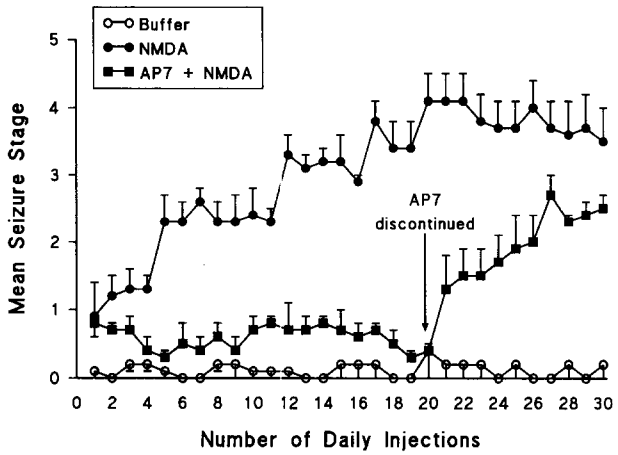

Fig. 1. The development of NMDA-induced kindling and its inhibition by a competitive NMDA receptor antagonist. Values shown are mean seizure responses (±S.E.M.; $n = 5$–6) following repeated daily intra-amygdaloid injections of NMDA, NMDA plus antagonist (AP7; 70 nmol) or vehicle (buffer) alone. Following 20 daily injections of AP7 plus NMDA, NMDA was administered alone for a further 10 days to assess the subsequent rate of development of kindling (see text).

1.5 NMDA injections. In four out of six animals stage 4 generalised seizure activity was reached but did not progress to stage 5 activity, even after 30 daily injections (Table 1).

Initial EEG responses comprised intermittent spiking gradually developing into full afterdischarges (and always accompanied by ipsilateral eye closure). These afterdischarges closely resembled, in both amplitude and frequency, those seen with electrical kindling as previously reported by this group (Croucher et al., 1988) and others (Goddard et al., 1969; Racine, 1972). However, in *contrast* to electrical kindling, where stimulation evokes a single seizure and afterdischarge, NMDA-treated animals showed repeated brief seizures with short afterdischarges (10–30 s). This intermittent seizure activity occasionally continued for up to 10–15 min. Animals were agitated and showed excessive grooming between seizure episodes. Buffer-injected animals behaved normally, only rarely showing signs of facial muscle twitching (Fig. 1).

Table 1
Parameters of limbic seizure kindling following repeated focal NMDA administration

Animal	No. of injections to maximal seizure	Maximal seizure stage	
		During NMDA kindling	1 month post-kindling
N1	17	4	4
N2	12	4	2
N3	20	5	5
N4	21	4	3
N5	21	4	3
N6	15	5	5
Mean ± S.E.M.	17.7 ± 1.5	4.3 ± 0.2	3.7 ± 0.5

Animals (N1–N6) were given daily focal intra-amygdaloid microinjections of NMDA, 2 nmol, as described in Materials and methods. The number of injections required to reach the maximal seizure stage (rated as described in the text), and the seizure stage reached, is shown for each animal. The seizure response evoked by a further single dose of NMDA, given 1 month after NMDA-induced kindling, is also shown. Control (buffer-injected) animals showed no kindled responses, even following 30 daily buffer injections (mean seizure stage 0.2 ± 0.2; $n = 5$)

3.2. Blockade of NMDA-induced kindling by AP7

Prior to NMDA plus AP7 administration in a further group of animals, the sensitivity of the animals to NMDA was confirmed with a dose of 2 nmol producing a mean seizure stage of 0.9 ± 0.4 (mean ± S.E.M., $n = 5$). This response did not differ significantly from the initial response seen in the NMDA-kindling group. During subsequent daily co-administration of AP7, 70 nmol, with NMDA, 2 nmol, the development of the seizure response was markedly suppressed with most animals showing only occasional facial myoclonus or transient chewing with jaw myoclonus (Fig. 1). No generalised seizure activity was seen in any animal. The NMDA/AP7-treated animals showed few other behavioural changes although some circling (two animals) and reduced exploratory activity was apparent. After 20 daily intra-amygdaloid injections, by which time most NMDA-treated animals had reached their maximal seizure stage (Table 1), the NMDA/AP7-treated group showed a mean seizure stage of only 0.4 ± 0.1, i.e. no significant kindling had occurred ($P > 0.05$ compared with control group; Student's t-test for independent groups) (Fig. 1). These animals subsequently responded to NMDA, 2 nmol, alone with a mean seizure stage of 1.3 ± 0.5 which was not significantly different to the initial NMDA-evoked response in these animals (see above). With further daily focal NMDA administration, kindling developed at a rate similar to that seen in the NMDA-kindled group (cf. injections 1–10 of the NMDA-treated group; Fig. 1).

3.3. Durability of NMDA-induced kindling

One month after NMDA-induced kindling, all animals responded to a single injection of NMDA with generalised seizure activity (Table 1). Animals which had previously attained stage 5 activity responded immediately with a further fully kindled seizure. Those previously reaching only stage 4 activity showed lower stage generalised seizure activity. The mean seizure response of the group after 1 month did not differ significantly from that observed immediately following kindling (3.7 ± 0.5 compared with 4.3 ± 0.2, respectively; $P > 0.05$).

Table 2
Positive transfer of NMDA-induced kindling to electrical kindling of the rat amygdala

Animal	ADT (μA)	Response to initial electrical stimulation	
		ADD (s)	Seizure stage
Controls			
C1	125	15	1
C2	50	17	2
C3	175	19	2
C4	175	14	1
C5	100	17	1
Mean ± S.E.M.	125.0 ± 23.7	16.4 ± 0.9	1.4 ± 0.2
NMDA-kindled			
N1	175	29	3
N2	325	38	3
N3	325	90	5
N4	300	39	3
N5	450	35	3
N6	225	82	5
Mean ± S.E.M.	300.0 ± 38.7 [b]	52.2 ± 10.8 [a]	3.7 ± 0.4 [b]

After NMDA-induced kindling or daily vehicle (buffer) administration, animals were stimulated electrically, through bipolar intra-amygdaloid electrodes, and individual afterdischarge thresholds (ADTs) were estimated (see Materials and methods). The mean afterdischarge threshold (± S.E.M.), together with the mean afterdischarge duration (ADD) and mean seizure stage in response to the first suprathreshold stimulation, are shown for each group of animals. Significance of differences between the control and test groups were assessed using Student's *t*-test for independent groups or Wilcoxon's two-sample rank test (seizure ratings): [a] $P < 0.01$; [b] $P < 0.005$.

3.4. Blockade of NMDA-kindled seizures by CGP 37849

In the two animals showing stage 5 seizures following kindling with NMDA, co-administration of CGP 37849, 1 nmol, with NMDA, 2 nmol, inhibited the generalised seizures. Only brief periods of jaw myoclonus with occasional head bobbing were observed (stage 1–2 seizure activity). No marked behavioural abnormalities were apparent following intra-amygdaloid CGP 37849 plus NMDA. Maximal seizure responses to NMDA alone returned within 2–6 days.

3.5. Positive transfer to electrical kindling

Following NMDA kindling all buffer- and NMDA-treated animals were stimulated electrically and the motor and EEG responses were compared (Table 2). Fully NMDA-kindled animals responded with a maximal stage 5 seizure and a fully developed afterdischarge, the remaining animals responding with a submaximal generalised seizure consistent with their level of chemical kindling (mean seizure stage 3.7 ± 0.4; mean afterdischarge duration 52.2 ± 10.8 s). Buffer-treated controls responded with minimal seizure activity (mean seizure score 1.4 ± 0.2) and brief afterdischarges (mean duration 16.4 ± 0.9 s). Interestingly, NMDA-kindled animals showed a significantly higher mean afterdischarge threshold than the control group (Table 2).

3.6. Histology

Histological analysis confirmed the positioning of the injection cannula and electrode tips in the basolateral amygdala. There was no evidence of neuronal loss or glial cell proliferation in any of the NMDA- or NMDA/AP7-treated animals.

4. Discussion

The present results demonstrate that limbic seizures can be kindled by repeated daily focal microinjection of the selective excitatory amino acid receptor agonist NMDA. This NMDA-induced kindling shows many of the characteristics of both glutamate-induced (Croucher and Bradford, 1989) and electrical kindling (Goddard et al., 1969). Thus, behaviourally the developing seizures closely resemble those seen following repeated, daily intra-amygdaloid glutamate (1.5 μmol) injections or electrical stimulations, although during focal microinjections of NMDA more severe and longer lasting seizure activity is apparent (see Results) than is observed during either glutamate-induced or electrical kindling. This difference is most likely due to the absence of a high-affinity uptake system for NMDA in the brain (Skerritt and Johnston, 1981) resulting in the extracellular persistence of the agonist. Both microinjected glutamate and glutamate released by electrical stimulation, on the other hand, would be rapidly inactivated by the selective, high affinity neuronal and glial uptake systems for this amino acid (Balcar and Johnston, 1972; Davies and Johnston, 1976). This is also consistent with the much higher doses of glutamate (1.5 μmol) compared with NMDA (2 nmol) required to induce kindling, despite the higher affinity of glutamate for NMDA receptors (Watkins and Olverman, 1987).

NMDA-kindled seizures appear to develop more slowly than either glutamate- or electrically kindled seizures, requiring a mean of approximately 18 injections (present study) compared with 11 glutamate injections (Croucher and Bradford, 1989) or 12 electrical stimulations (Goddard et al., 1969; Racine, 1972). Additionally, although *all* NMDA-kindled animals showed generalised seizure activity, many of them did not achieve the maximum seizure stage (stage 5) within the present experimental time course. This may have been a direct result of the dose of agonist selected. Alternatively, it may have been due to the selective agonist action of NMDA, compared with the mixed agonist

action of electrically released (Kaura et al., 1995) or exogenous glutamate, or due to blockade of part of the NMDA receptor population by Mg^{2+}. Thus, in addition to stimulating NMDA receptor populations, a mixed agonist also causes depolarisation of post-synaptic neurones by activation of non-NMDA ionotropic and metabotropic receptors. This in itself may enhance the kindling process. In addition, post-synaptic neuronal depolarisation reduces the level of NMDA receptor-linked ion channel blockade by extracellular Mg^{2+} ions (Mayer et al., 1984; Nowak et al., 1984) thereby enhancing the NMDA receptor-mediated response. However, Rainnie et al. (1991) have suggested that NMDA receptors are active in the basolateral amygdala under normal physiological conditions. Furthermore, the present demonstration of NMDA-induced kindling indicates that at least a substantial proportion of NMDA receptors in this region is directly responsive to their selective agonist (NMDA) and is *not* blocked by Mg^{2+}. It has also been reported that NMDA added to hippocampal slices causes a substantial increase in Ca^{2+} uptake, which is blocked by NMDA receptor antagonists (Crowder et al., 1987). Thus, here too, NMDA receptors show marked response to their selective agonist and are clearly not Mg^{2+}-blocked. A further relevant mechanism may therefore involve mixed agonist activation of *presynaptic* excitatory amino acid receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or metabotropic sub-types. Stimulation of these receptor populations has recently been shown to enhance the neuronal release of excitatory amino acids (Herrero et al., 1992; Barnes et al., 1994; Patel et al., 1994; Patel and Croucher, 1995). Such an action by endogenously released or microinjected glutamate, not achieved by NMDA, is likely to facilitate the kindling process.

Co-administration of AP7 (70 nmol) with NMDA almost entirely blocked the kindling process (Fig. 1). A similar effect of AP7 has previously been reported against glutamate-induced amygdala kindling (Croucher and Bradford, 1990a). However, the blockade by AP7 was less effective following glutamate microinjections probably because other, non-NMDA and metabotropic glutamate receptors remained active. Centrally or systemically administered competitive, or non-competitive, NMDA receptor antagonists have also been shown to inhibit the development of electrical kindling (Croucher et al., 1988; Croucher and Bradford, 1990b; Gilbert, 1988; Vezzani et al., 1988).

When NMDA alone was administered following completion of NMDA/AP7 treatment, animals displayed only minimal seizure activity (Fig. 1). This was an important observation as it indicated that AP7 prevented the *development* of the epileptic focus and did not merely mask the expression of the kindling process. A comparable action has been shown for NMDA receptor antagonists against electrical kindling (Croucher and Bradford, 1990b).

One month after NMDA-induced kindling, animals responded to a further single dose of the agonist with generalised seizure activity. This clearly demonstrates the persistence of kindling due to multiple focal injections of NMDA. This is also a feature in common with glutamate and electrical kindling, and further suggests that NMDA receptors play a critical role in the generation of the kindled state in each of these conditions.

Fully developed NMDA- and electrically kindled seizures also respond similarly to the potent, competitive NMDA receptor antagonist CGP 37849. Thus, a dose of the antagonist which has previously been shown to raise the generalised seizure threshold of electrically kindled seizures (Croucher et al., 1992a) considerably reduced the severity of seizures induced by NMDA in fully kindled animals in the present study. Also notable in the comparison of electrical and NMDA-induced kindling is the phenomenon of 'positive transfer' in which NMDA-kindled animals displayed equivalent seizure activity when stimulated by *either* focal NMDA injection *or* electrical pulses. This positive transfer strongly supports a common mechanism between the two kindling processes suggesting that the electrical stimulation releases an endogenous NMDA receptor agonist which is critical in establishing the kindled state. This is consistent with recently published work from our own group (Kaura et al., 1995) demonstrating an increase in release of endogenous glutamate from the amygdaloid complex in vivo following electrical kindling stimulations.

The increase in electrical afterdischarge threshold in NMDA-kindled compared to control animals (Table 2) was unexpected and the mechanism responsible for this change is unclear. It may, however, be related to the observed longer duration of seizure activity following NMDA injections compared with electrical kindling stimulations (see Results) which would be followed by a prolonged refractory period during which the kindled focus would be less responsive to seizure-inducing stimuli. Indeed, studies using rat cortical slices have recently shown that low-level NMDA receptor activation causes release of adenosine which appears to provide a purinergic inhibitory threshold against further NMDA-mediated transmission (Craig and White, 1992).

Considerable electrophysiological and biochemical evidence also suggests that increased NMDA receptor-mediated activity occurs during and following electrical kindling (Morrisett et al., 1989; Gean et al., 1989; Mody and Heinemann, 1987; Mody et al., 1988). Recent studies, however, have provided contradictory data concerning changes in NMDA receptor populations which may account for this enhanced activity. Thus, Wu et al. (1990) and Yeh et al. (1989) identified

increases in NMDA-sensitive binding sites and 3-([+]-2-carboxypiperazin-4-yl) (CPP), 1-thienylcyclohexylpiperidine (TCP) and glycine binding sites associated with the NMDA receptor complex following electrical kindling in rats. Conversely, Jones and Johnson (1989) found no changes in either NMDA receptor number or in the modulation of the receptor and its associated ion channel by Mg^{2+}, glycine or phenycyclidine (PCP) following electrical kindling. Similarly, Okazaki et al. (1989) and Vezzani et al. (1990) failed to observe any increase in NMDA-sensitive [^{3}H]glutamate binding sites during or after kindling. No similar studies have yet been reported following excitatory amino acid-induced kindling.

Changes 'downstream' from the NMDA receptor, e.g. Ca^{2+}-dependent enzyme-mediated processes responding to Ca^{2+} influx through NMDA receptor-linked cation channels, may also be important in the development of kindled seizures. Indeed, electrical kindling has been shown to be associated with altered intracellular Ca^{2+} homeostasis resulting from a progressive decrease in neuronal Ca^{2+}-binding protein (Baimbridge and Miller, 1984; Sonnenberg et al., 1991). In addition, a decrease in voltage-dependent Ca^{2+} fluxes has been reported in kindled compared to control hippocampus (Mody et al., 1990).

It may be that the afterdischarge induced by electrical stimulation, NMDA, glutamate or other agents is the trigger for epileptogenesis and the mechanism by which the afterdischarge induces these secondary epileptogenic processes may be common to all of these. However, neither electrical stimulation nor NMDA are endogenous agents which could cause the generation of the afterdischarge. Glutamate however *is* and has been shown to be released before (i.e. during electrical kindling) and simultaneously with the onset of seizures of various kinds (Dodd and Bradford, 1976; Ueda and Tsuru, 1994; Kaura et al., 1995). Extracellular glutamate at abnormally raised levels could therefore be the agent triggering many forms of epilepsy, including kindling.

Thus, although a unitary hypothesis remains to be formulated, it is clear that NMDA receptor-mediated mechanisms play a central role in kindling processes. Excitatory amino acid, and particularly NMDA-induced kindling provides a valuable new animal model for the further study of these mechanisms of epileptogenesis.

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