Comparative study of hippocampal neuronal loss and in vivo binding of 5-HT1a receptors in the KA model of limbic epilepsy in the rat

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Abstract

A high density of 5-HT1a receptors is present in pyramidal hippocampal cells. Mapping of these receptors may be performed in vivo using the tracer no-carrier-added 4-18F-fluoro-N-2-(1-(2-methoxyphenyl)-1-piperazinyl)ethyl-N-2-pyridinyl-benzamide (MPPF). We tested the hypothesis of a relationship between MPPF binding and post-epileptic neuronal loss in the hippocampus. The model of limbic epilepsy induced by kainic acid (KA) in the rat was used. Rats were sacrificed at various times (1 h–240 days) after systemic injection of 10 mg/kg KA. Determination of MPPF binding in the brain was combined with a quantification of neuronal loss using DNA labeling with propidium iodide and confocal microscopy. Hippocampal MPPF binding varied according to time elapsed from KA injection. An initial decrease from day 1 to day 6 post injection was followed by a relative increase between day 6 and day 30. This effect was observed in rats which showed hippocampal neuronal loss but also in one rat which did not. In KA treated rats, statistically significant relationship between MPPF binding and neuronal count was found during the acute period (rats sacrificed 1 h–day 6 after KA injection) and the chronic phase (rats sacrificed beyond day 60 after KA injection). The late relative increase of MPPF binding suggests an epilepsy-induced increase of 5-HT1a receptors in the hippocampus. This effect needs to be further characterized before considering PET determination of hippocampal MPPF binding as a method of post-epileptic neuronal loss assessment. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

It is recognized that neuronal damage may occur in the brain of epileptic patients as a result of seizures. This deleterious effect of seizures has been studied for years on animal models of
epilepsy, including the perforant path stimulation model (Sloviter, 1994) and models of epilepsy induced by the injection of toxins like kainic acid (KA) (Ben-Ari, 1985) or lithium and pilocarpine (Sankar et al., 1998). These studies have shown that pyramidal cells of the hippocampus CA1 region are particularly vulnerable to seizure-induced injury and that loss of neurons occurs mainly after prolonged seizures (Auer and Siesjö, 1988). Neuronal loss, however, was also observed in the rat hippocampus after repeated brief seizures (Cavazos et al., 1994). There are strong arguments to support the hypothesis that these acute and chronic seizure-induced lesions also occur in human epilepsies. It is now largely accepted that prolonged febrile seizures are a cause of mesial temporal sclerosis (Cendes et al., 1993; Maher and McLachlan, 1995; Shinnar, 1998; VanLandingham et al., 1998; McNamara, 1999). Whether the repetition of seizures may cause progressive neuronal loss is a more difficult question to address in vivo in epileptic patients. Recent magnetic resonance imaging (MRI) studies based on volumetric hippocampal measurements or proton spectroscopic measurements suggest that neuronal loss correlates with duration and severity of the epileptic disorder (Kalviainen et al., 1998; Tasch et al., 1999). One longitudinal study has shown, however, that the appearance of hippocampal changes related to the repetition of seizures is an exceptional feature in adults with newly diagnosed partial epilepsy (Van Paesschen et al., 1998). In addition, MRI volumetric studies are complicated by possible hippocampal sclerosis with no detectable hippocampal atrophy (Jackson et al., 1994). This situation could be the consequence of gliosis, sprouting of the mossy fibers or neurogenesis of dentate granule cells induced by prolonged seizures (McNamara, 1992; Jackson et al., 1994). Positron emission tomography (PET) is another brain imaging technique that could be used to detect seizure-related damage. The PET tracer most commonly used to investigate epileptic patients is 18F-fluorodeoxyglucose. This tracer seems inappropriate to study seizure-induced brain damage because changes in cerebral glucose distribution frequently extends beyond the epileptogenic lesion (Sackella et al., 1990; Sperling et al., 1990; Henry et al., 1993; Van Bogaert et al., 2000) and may reverse after cure of epilepsy (Hajek et al., 1994). Tracers of receptor systems could be of interest, as shown in early PET studies of opiate receptors changes in temporal epilepsy (Frost et al., 1988). The central benzodiazepine receptor antagonist [11C]-flumazenil (FMZ) has been extensively used in epileptic patients. One study has shown a correlation between severity of the epileptic disorder and degree of FMZ binding reduction (Savic et al., 1996). However, FMZ binding is not correlated with neuronal density (Koep et al., 1997). Its reduction in the hippocampus CA1 region is larger than what could be attributed to neuronal loss (Hand et al., 1997). The study of the subtype 1a serotonin (5-HT) receptor could be a more useful technique to detect mesial temporal sclerosis. Indeed, these receptors are highly concentrated in the limbic regions and particularly on CA1 pyramidal cells (Zifa and Fillion, 1992). A new tracer of 5-HT1a receptors, the no-carrier-added 4-18F-fluoro-N-2-(1-(2methoxyphenyl)-1piperazinyl) ethyl-N-2-pyridinyl-benzamide (MPPF), has been recently synthesized (Le Bars et al., 1998) and validated for human studies (Plenevaux et al., 1999). Autoradiographic studies performed in the cat and in the rat have shown reversible and highly specific binding of MPPF for 5-HT1a receptors (Le Bars et al., 1998; Ginovart et al., 2000; Plenevaux et al., 2000). The high affinity of MPPF for hippocampal neurons led us to consider this tracer for the PET evaluation of post-epileptic brain damage, both at the acute phase and at the chronic phase after the injury. In order to test this potential tool, we used the rat model of KA intoxication. In this model, we compared in vivo binding of MPPF with a histological estimation of neuronal loss.

2. Materials and methods

2.1. Preparation of animals

Forty-nine 2-month-old Male Wistar rats (body weight of about 200 g) were used in this study (Purchased from Iffa Credo, Brussels, Belgium). An intraperitoneal (i.p.) injection of either a KA
solution (44 rats) or a saline physiological solution (5 control rats) was performed. The rats were then housed in plastic cages and had free access to food and water. The KA solution was prepared by dissolving 125 mg KA (Sigma) in 25 ml of a 9‰ saline solution. The dose of KA was set at 10 mg/kg. This dose is known to produce status epilepticus in more than 90% of the animals with a low mortality rate (Schwob et al., 1980; Sperk et al., 1983; Ben-Ari, 1985; Balchen et al., 1993). This dose is also known to produce hippocampal damage that largely predominates in the CA1 region with relative sparing of the CA3 region (Sperk et al., 1983; Lason et al., 1988; Balchen et al., 1993). This pattern of lesion is quite similar to that observed in mesial temporal sclerosis in human.

2.2. MPPF injection

Premature death (8 out of the 44 rats injected with KA spontaneously died, and 3 others died during anesthetic procedure) and MPPF synthesis failures reduced the total number of rats studied to 34. A dose of about 1 mCi MPPF, synthesized as previously described (Le Bars et al., 1998), was intravenously injected at the following times after KA injection: 1–5 h (six rats), 24 h (one rat), 6 days (five rats), 13 days (three rats), 15 days (two rats), 30 days (five rats), 60 days (five rats) and 240 days (two rats). The five control rats were injected at various times. The injection was performed through a catheter introduced in a femoral vein under anesthesia with a solution of pentobarbital sodium 5% (0.2–0.4 ml i.p.). Animals were sacrificed by decapitation 30 min after the MPPF injection. This timing for ex vivo analysis has been determined by others in kinetics (Plenevaux et al., 2000).

2.3. Conditioning of the brain

The brain was removed from the skull and rapidly frozen on dry ice. Sagittal sections, 20-μm thick, were cut within the left hemisphere using a Leica CM 3000 microtome cryostat. Five lateral levels covering the entire hippocampus were defined for the study according to the rat brain Paxinos and Watson atlas (Paxinos and Watson, 1986) (level 1: lateral 4.20 mm, level 2: lateral 3.90 mm, level 3: lateral 3.18 mm, level 4: lateral 2.40 mm and level 5: lateral 1.40 mm). Two brain slices from each lateral level were mounted on a microscope slide for the study of MPPF binding. This study was thus performed on ten different slices. Two slices from level 4 were mounted on a microscope slide for neuronal count. This level was selected because all hippocampal regions were apparent on the same slice.

2.4. MPPF binding analysis

The 18F activity representing total binding of MPPF on the brain slices was revealed using the Phosphorimager technique (Phosphorimager SI, Molecular Dynamics). Microscope slides were exposed to the screen during 24 h. Digital images were obtained for each brain slice and were analyzed with the ImageQuaNT software (Molecular Dynamics). Several geometrical regions of interest (ROI) were drawn to sample the frontal cortex, the piriform cortex and the occipital cortex. Anatomical ROI were drawn to sample the entorhinal cortex and subfields CA1 and CA3 of the hippocampus. In addition, binding in the whole hippocampus was determined with ROI delineating contours of this structure on all slices where it was present. Fig. 1 illustrates ROI placement in a control rat. The average counts per pixel was determined for each ROI on each slice. For each brain region studied, a global average of these values was determined across the ten slides sampling the predefined levels of the hippocampus. Relative binding of MPPF in CA1, CA3, whole hippocampus and entorhinal cortex was expressed using a procedure of normalization to the mean values obtained in frontal, occipital and piriform cortices.

2.5. Neuronal loss determination

To obtain an indicator of KA induced neuronal death, loss of pyramidal cells was studied in restricted areas of the CA1 and CA3 regions of the hippocampus. Preserved pyramidal cells were counted using confocal microscopy after labeling
of their nuclei with propidium iodide. Brain slices were first fixed in 0.1 M phosphate-buffered 4% paraformaldehyde during 15 min and washed in 0.1 M Tris-buffered saline containing triton-X (TBS-TX) for 5 min. Then, they were incubated with a solution of 50 μg/ml propidium iodide (Aldrich-Chimie) and 500 μg/ml ribonuclease A type 1 (Sigma-Aldrich) buffered in Tris–HCl 0.05 M (pH 7.4) during 5 min at room temperature in the dark. After three rinses in TBS, sections were mounted with ‘Slow Fade Light™’ anti-fade mounting medium (Molecular Probes) in 50% glycerol and secured with nail polish before viewing on a confocal microscope (MRC 1024; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) fitted on an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat™ 63x/1.4 oil immersion objective (Zeiss). The 568 nm excitation beam of an Argon/Krypton laser and a 605/32 nm band-pass emission filter were used for selective detection of the red fluorochrome (propidium iodide). The resolution of our confocal system was measured according to the manufacturer’s instructions and was found to be better than 0.5 μm in the X–Y plane and better than 0.8 μm in the Z axis (depth). On brain slice, a single confocal section was acquired and geometrical ROI (square fields of 2500 μm²) were placed in areas of the CA1 and CA3 region of the hippocampus. This procedure was standardized in order to sample the same areas of these regions. Digital images were analyzed using the public domain NIH Image 1.61 program (National Institute of Health, USA). Neuronal death induced by toxic KA stimulation is morphologically characterized by a condensation of the chromatin (Sperk et al., 1983; Ben-Ari, 1985), which results in reduced dimension, irregular shape and hyperdensity of the nuclei. Morphological criteria of normal pyramidal cells were established in control rats based on dimension and density of the nucleus. We threshold all counting at a minimal nucleic size of 700 pixels on a binary image thresholded at 80% of gray scale maximum to apply these criteria. This procedure resulted in exclusion of glial cells and degenerative neurons.

2.6. Statistical analysis

Differences in CA1 MPPF binding between rats grouped by time of sacrifice after KA injection was assessed by one-way ANOVA with post-hoc Scheffé test. The relationship between MPPF binding and cell count was studied by linear regression analysis. To isolate early and chronic effects of KA injection, regression analyses were specifically conducted on the early phase (until day 6) and on the late phase (from day 60) post-injection. Significance level was set at 0.05 for all comparisons.

3. Results

3.1. Time course and localization of neuronal loss

Neuronal counts per field in control rats were 27 ± 3.5 and 14 ± 2 in CA1 and CA3 regions,
respectively. Maximal neuronal loss was observed in the CA1 region of rats treated with KA. Fig. 2 illustrates the morphological changes and the method of cell counting in geometrical ROI put in CA1 region of a control rat and of a KA treated rat, respectively. Fig. 3 illustrates neuronal count in control rats and in KA treated rats according to the time elapsed from KA injection to decapitation. We considered statistically significant neuronal loss under a neuronal count of 20 (mean in control rats minus 2 standard deviations). Neuronal loss was not observed in the 7 rats sacrificed in the 24 h time window after KA injection. Among the 22 rats sacrificed from day 6 after KA injection, statistically significant neuronal loss occurred in CA1 regions in 17 rats and was already maximal on day 6. Statistically significant neuronal loss was also observed in the CA3 region in 6 rats.

3.2. Time course of MPPF binding

In control rats, MPPF binding relative to the cortex were 2.9 ± 0.8, 3.6 ± 0.6 and 3.5 ± 0.4 in CA1, CA3 and the whole hippocampus, respectively. Fig. 4 illustrates binding quantification in CA1 region in control rats and KA treated rats according to time from KA injection. In this figure, KA treated rats are divided in two groups according to the presence ($n = 17$) or absence ($n = 12$) of significant neuronal loss in CA1 region. Visual analysis of this curve showed rapid decrease of binding with a nadir at day 6, followed by a progressive increase toward relatively stable values from day 60. This effect was particularly patent for rats showing neuronal loss. However, decreased binding was also observed in one rat sacrificed 15 days after KA injection which did
not develop significant neuronal loss in the CA1 region. Statistical analysis using one-way ANOVA showed that means of CA1 MPPF binding were significantly different ($P = 0.0018$) at the different times of sacrifice after KA injection. Post-hoc Scheffé’s test confirmed statistically significant nadir of CA1 binding at day 6 after KA injection ($P < 0.001$ for day 6 versus control and day 6 versus four different time points: 1–5 h, day 1, day 60 and day 240). MPPF binding in CA3, in the whole hippocampus and in the entorhinal cortex showed a similar evolution (one way ANOVA: $P = 0.0003$ for CA3 region, $P < 0.0001$ for the whole hippocampus and $P = 0.0015$ for entorhinal cortex).

3.3. Correlation between MPPF binding and neuronal loss

A statistically significant relationship between CA1 MPPF binding and CA1 cell count was found in the pooled set of control and KA treated rats ($R^2 = 0.3886$, $P < 0.0001$, see Fig. 5A). Among the KA treated rats, regression plots, illustrated in Fig. 5B and C, also showed statistically significant correlation between MPPF binding and neuronal loss in CA1 region during the early period (rats sacrificed 1 h–6 days after KA injection, $R^2 = 0.7268$, $P = 0.0004$) and the late phase (rats sacrificed beyond 60 days after KA injection, $R^2 = 0.6989$, $P = 0.0191$).

4. Discussion

This is the first study of 5-HT1a receptors distribution in an animal model of epilepsy. It makes use of the PET tracer MPPF to assess in vivo binding on 5-HT1a receptors in the brains of rats sacrificed at various times after systemic in-
jection of KA (10 mg/kg). Ex vivo binding experiments have been combined to a morphological approach using DNA labeling with propidium iodide and confocal microscopy in order to correlate 5-HT1a receptors distribution with a marker of neuronal loss. Neuronal loss largely predominates in the CA1 region of the hippocampus in most rats sacrificed more than 24 h after KA injection. The time course and the topography of neuronal loss are comparable to data reported in other studies using the same model of temporal lobe epilepsy. Indeed, if the systemic injection of this relatively low dose of KA induces status epilepticus in nearly all rats, neuronal loss is not observed in all of them and it predominates in CA1 region (Schwob et al., 1980; Sperk et al., 1983; Ben-Ari, 1985; Balchen et al., 1993). Neuronal loss in this region is probably a glutamate-mediated mechanism secondary to intense KA induced excitation of CA3 pyramidal neurons projecting to CA1 through the Schaffer collaterals (Lason et al., 1988). We found highest MPPF binding in limbic brain areas of control rats. These data are consistent with studies of 5-HT1a receptors density using MPPF or other tracers (Zifa and Fillion, 1992; Barnes and Sharp, 1999). Within the hippocampus, a high density of 5-HT1a receptors has been previously reported in CA1 region and in dentate gyrus (Zifa and Fillion, 1992). In the present study, quite similar binding of MPPF is found in CA1 and CA3 regions; this is attributed to the possible influence of dentate gyrus binding on CA3 quantification due to the close anatomical relationships between these structures and the relatively low spatial resolution of the Phosphorimager system.

MPPF binding study shows a rapid decrease of binding with a nadir at day 6, followed by a progressive increase toward relatively stable val-
ues from day 60. There is a statistically significant relationship between CA1 MPPF binding and CA1 cell count in the pooled set of control and KA treated rats. This correlation is already present in the early period after KA injection. At this stage, reduced MPPF binding is probably largely related to neuronal loss but another hypothesis to consider is the occupation of 5-HT1a binding sites by endogenous 5-HT, massively released as a consequence of status epilepticus. This latter hypothesis appears unlikely because (1) hippocampal release of 5-HT was not found in a previous study using the same model of epilepsy (Arias et al., 1990), and (2) release of neurotransmitters occurs mainly in the first 72 h after KA injection (Sperk et al., 1986). However, we cannot exclude that the time-course of MPPF binding in the hippocampus may have been partially influenced by seizure-induced 5-HT release since another study has shown sustained local release of 5-HT up to 21 days following an intrastriatal KA injection (Nakazato and Akiyama, 1997). The existence of one rat showing MPPF decrease at day 15 without significant neuronal loss leads us to consider that decreased MPPF binding could also follow depressed expression of 5-HT1a receptors as part of a stress reaction induced by status epilepticus. This hypothesis is supported by in situ hybridization studies showing decreased expression of various neurotransmitter receptors genes, including glutamate, GABA and muscarinic acetylcholine receptor genes, after KA induced status epilepticus (Friedman et al., 1994; Mingo et al., 1997; Sperk et al., 1998). This potential cellular survival mechanism should be better characterized in the prospect of MPPF use in human. Indeed, this PET tracer could be proposed for the detection of hippocampus post-epileptic neuronal loss in the early phase after status epilepticus.
Fig. 3. Time course of neuronal loss in CA1 region. Neuronal count is expressed in terms of morphologically normal nuclei per square field of 2500 μm². KA treated rats showing significant neuronal loss (cell count < 20) are represented with filled symbols (n = 17). Empty symbols represent KA treated rats without significant neuronal loss (n = 12). The line drawn on this figure represents the threshold value (of 20) under which significant neuronal loss was considered.

Fig. 4. Time course of MPPF binding in CA1 region. KA treated rats with significant neuronal loss in CA1 region (n = 17) are represented with filled symbols and those without significant neuronal loss (n = 12) are represented with empty symbols. The time course of MPPF binding shows a three-stage evolution in the two groups: decrease binding after 24 h, slow increase from the 6th day to the 30th day, and stabilization from the 60th day. On this figure, a line is drawn at the 2.9 counts/pixels value corresponding to the mean value in control rats (standard deviation = 0.8).
The fact that neuronal loss cannot explain all variations observed in MPPF binding is further supported by the late increase of CA1 MPPF binding after KA injection. Such a phenomenon may reflect a regulatory enhancement of endogeneous serotonergic neurotransmission in response to epileptiform activity. There are indeed several lines of evidence coming both from human and animal studies, which indicate that endogenous 5-HT exerts an antiepileptic effect. This regula-

Fig. 5. A. Correlation between MPPF binding and cell counting. A statistically significant relationship between CA1 MPPF binding and CA1 cell count is found in the pooled set of control and KA treated rats ($R^2 = 0.3886, P < 0.0001$). B. Correlation between MPPF binding and cell counting for KA treated rats in the early period. A statistically significant correlation between MPPF binding and cell count CA1 region is found for rats sacrificed 1 h–6 days after KA injection ($R^2 = 0.7268, P = 0.0004$). C. Correlation between MPPF binding and cell counting for KA treated rats in the chronic phase. A statistically significant correlation between MPPF binding and cell count CA1 region is found for rats sacrificed beyond 60 days after KA injection ($R^2 = 0.6989, P = 0.0191$).
Fig. 5. (Continued)

A previous study has demonstrated that an increased uptake of \( \alpha^{11} \text{C}\)-methyl-L-tryptophan has been observed in humans with tuberous sclerosis, suggesting a seizure-related increase of 5-HT synthesis (Chugani et al., 1998). In addition, serotonergic hyperinnervation has been shown in materials of focal cortical dysplasia obtained from patients who underwent surgery for refractory epilepsy (Trottier et al., 1996). In rats, it has been shown that 5-HT increases the threshold for electroconvulsions (Loscher and Czuczwar, 1985). The role of the serotonergic system on the modulation of epileptic activity has been particularly studied in genetically epilepsy-prone rats. In this animal model of epilepsy, it has been shown that (1) the severity of epilepsy is inversely correlated with the brain concentration of 5-HT (Dailey et al., 1989), (2) this reduced concentration is due to a reduction in the number of serotonergic neurons (Statnick et al., 1996), and (3) fluoxetine, a selective 5-HT re-uptake blocker, has anticonvulsant properties (Yan et al., 1994). More recently, it has been shown that the antiepileptic action of fluoxetine is mediated by 5-HT1a receptors (Lu and Gean, 1998). An increased synthesis of postsynaptic 5-HT1a receptors in surviving hippocampal pyramidal cells is still to be demonstrated in our model of KA induced epilepsy. Indeed, it has been suggested that 5-HT1a receptors are also localized on terminals of serotonergic and nonserotonergic neurons and on glial cells (Zifa and Fillion, 1992). Overexpression of receptors in these structures, secondary to gliosis, sprouting of serotonergic axons due to early loss of post-synaptic 5-HT1a sites, or even neurogenesis of dentate granule cells, could therefore participate in the increase of hippocampal MPPF binding observed at this late phase of KA neurotoxicity.

In KA treated rats, we found a statistically significant relationship between MPPF binding and neuronal loss in CA1 region during the chronic phase after KA injection (rats sacrificed beyond day 60 after KA injection). Thus, during this period, rats which underwent neuronal loss showed a significant MPPF binding decrease compared to preserved rats. However, the possible seizure-induced increase of 5-HT1a receptors synthesis may have attenuated MPPF binding decrease in injured hippocampi and therefore underestimated extent of neuronal loss. The potential regulatory enhancement of endogenous serotonergic neurotransmission in response to epileptiform activity should be better characterized in order to establish the role of MPPF for the diagnosis of mesial temporal sclerosis.
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References


Savic, I., Svanborg, E., Thorell, J.O., 1996. Cortical benzodiazepine receptor changes are related to frequency of partial seizures: a positron emission tomography study. Epilepsia 37, 236–244.


