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An *in vivo* [^{18}F]MK-9470 microPET study of type 1 cannabinoid receptor binding in Wistar rats after chronic administration of valproate and levetiracetam☆

Karolien Goffin [a,*], Guy Bormans [b], Cindy Casteels [a], Barbara Bosier [c], Didier M. Lambert [c], Igor D. Grachev [d], Wim Van Paesschen [e], Koen Van Laere [a]

[a] Division of Nuclear Medicine and MOSAIC, University Hospital Leuven and Katholieke Universiteit Leuven, Leuven, Belgium
[b] Laboratory of Radiopharmacy and MOSAIC, Katholieke Universiteit Leuven, Leuven, Belgium
[c] Unité de Chimie Pharmaceutique et de Radiopharmacie, Université Catholique de Louvain, Brussel, Belgium
[d] Imaging Department, Merck Research Laboratories, West Point, PA 19486, USA
[e] Department of Neurology, University Hospital Leuven, Leuven, Belgium

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ABSTRACT

There is substantial evidence that the endocannabinoid system and in particular the type 1 cannabinoid receptor (CB1R) is involved in epilepsy. We evaluated the *in vivo* effect of chronic administration of the anti-epileptic drugs valproate (VPA) and levetiracetam (LEV) on rat brain CB1 receptors using the positron emission tomography (PET) tracer [^{18}F]MK-9470.

Six Wistar rats were treated with VPA (200 mg/kg) or LEV (50 mg/kg) IP daily for 2 weeks. Dynamic imaging after intravenous injection of 18 MBq [^{18}F]MK-9470 was performed on a FOCUS 220 microPET at baseline and after chronic treatment. Six animals were used as controls and were injected with saline, using the same protocol. Parametric images based on standardized uptake values (SUV) were generated and were spatially normalized to Paxinos space. These CB1R images were analyzed using a predefined volume of interest (VOI)-based analysis. Differences in SUV values between chronic and baseline scans in each condition (saline, VPA and LEV treatment) were calculated in each VOI. Direct binding affinity of the drugs at CB1R was assessed by competitive binding assay in Chinese hamster ovarian cells expressing human CB1R.

Chronic injections of saline did not produce significant changes in global [^{18}F]MK-9470 binding ($p = 0.43$), nor in tracer binding in individual VOIs. We found a significant increase in global cerebral [^{18}F]MK-9470 binding after chronic VPA administration compared to sham treated animals (+32.5%, $p < 0.001$), as well as in tracer binding in all individual VOIs. After chronic administration of LEV, there was no significant change in global cerebral CB1R binding (+6.9%, $p = 0.81$), nor in tracer binding in individual VOIs. As VPA does not exhibit high affinity for CB1R (displacement of [^{3}H]-SR141716A 1.3 ± 14.0%), such upregulation is most likely caused by an indirect effect on the endocannabinoid system.

This increase in CB1R tracer binding and possibly signaling may represent a supplementary and new mechanism of VPA, but not LEV, since activation of CB1Rs has been shown to decrease excitability and excitotoxicity on-demand.

1. Introduction

Since the discovery of specific membrane receptors of marijuana's primary psychoactive constituent Δ^9-tetrahydrocannabinol in the early 1990s, an endogenous signaling system, known as the endocannabinoid system (ECS), has been discovered. Two types of G-protein coupled cannabinoid receptor have been identified so far. Type 1 cannabinoid receptors (CB1R) are expressed in high concentrations in the central nervous system, where they are not only present on neurons, but also in lower concentrations on microglia, astrocytes and oligodendrocytes (Ramírez et al., 2005). CB1 receptors, located mainly on the presynaptic nerve terminal, modulate neuronal excitability through suppression of the release of other neurotransmitters, for example glutamate, γ-aminobutyric acid (GABA) and dopamine (Wilson and Nicoll, 2002). Type 2 cannabinoid receptors (CB2R) appear mainly in immune cells and play

☆ The authors state that all efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

* Corresponding author. Division of Nuclear Medicine, E901, University Hospital Leuven Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Tel.: +32 16 343 715; fax: +32 16 343 759.
E-mail address: karolien.goffin@uz.kuleuven.be (K. Goffin).

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an important role in inflammation (Klein et al., 2003). The discovery of cannabinoid receptors was followed by the demonstration of endogenous cannabinoid receptor agonists, of which anandamide and 2-arachidonoyl glycerol (2-AG) are of primary importance (Di Marzo et al., 1998).

Several lines of evidence point towards a link between the endocannabinoid system and mechanisms involved in generation and cessation of epileptic seizures. The endocannabinoid system would provide an 'on-demand' protection against acute excitotoxicity in neurons of the central nervous system (CNS) and would contribute to a signaling system that protects neurons against the consequences of abnormal discharge activity (Marsicano et al., 2003). In humans, cannabis may act as an anticonvulsant in the treatment of partial and secondarily generalized seizures (Gross et al., 2004), although prospective and randomized studies are lacking. In cell cultures, endocannabinoids block status epilepticus in hippocampal neurons (Deshpande et al., 2007). In the mouse maximal electroshock-induced seizure model, administration of exogenous as well as endogenous cannabinoids is protective against seizures (Wallace et al., 2001, 2002). Cannabinoids also affect the seizure frequency and duration in the pilocarpine model of temporal lobe epilepsy in rats and 2-AG and CB1R levels are increased in these epileptic animals (Wallace et al., 2003).

Levetiracetam (LEV) and valproate (VPA) are frequently used antiepileptic drugs (Glauser et al., 2006). VPA has been used clinically for many years, but its mode of action as an anticonvulsant drug remains unclear. Several mechanisms have been suggested: VPA potentiates the inhibitory postsynaptic GABA responses in the central nervous system and may act through antagonism of *N*-methyl-D-aspartate (NMDA) receptor-mediated excitation and via blockade of voltage-dependent Na^+-channels (Löscher, 2002). Furthermore, it has been shown that levonantradol, a cannabinoid derivative, and arachidonyl-2′-chloroethylamide, a highly selective cannabinoid CB1R agonist, potentiate the anticonvulsant effects of VPA in animal models of epilepsy (Ehlers et al., 1981; Luszczki et al., 2006), but the mechanism of this interaction is unclear.

LEV, on the other hand, is a newer potent drug with a broad antiepileptic potential and a good tolerability (Dooley and Plosker, 2000). Its mechanisms of action are unrelated to those of classical antiepileptic drugs: LEV does not affect the activity of voltage-dependent Na^+-channels or T-type voltage-gated Ca^{2+}-channels (Zona et al., 2001). However, it is well established that the drug binds to a specific protein binding site, recently identified as the synaptic vesicle protein subtype SV_{2A} (Lynch et al., 2004). Although a role in the homeostasis of synaptic vesicle constituents such as ATP or calcium has been suggested for SV_{2A} (Janz et al., 1999), the functional consequences of the binding of LEV to this protein remain to be elucidated. Furthermore, LEV could act by reducing high voltage-activated Ca^{2+}-channels (Lukyanetz et al., 2002). It decreases $GABA_A$-receptor sensitivity to Zn^{2+} and β-carbolines (Rigo et al., 2002) and may reduce glutamate-induced excitotoxicity and enhance GABAergic inhibition by interacting with glutamate- and GABA-transporters (Ueda et al., 2007). So far, no interactions have been described between LEV and the ECS.

In this study, we investigated the effect of VPA and LEV on the binding of the CB1R *in vivo* in the brain of healthy rats using the recently developed high-affinity, highly selective radiolabeled inverse agonist, [^{18}F]MK-9470 (Burns et al., 2007).

2. Methods

Eighteen female Wistar rats weighing 230–290 g were group-housed under controlled conditions of temperature, humidity and 12-h light/dark cycle. Food and water were available *ad libitum*. All research protocols were approved by the local university's Animal Ethics Committee and were carried out in accordance with the European Communities Council Directive (86/609/EEC).

The radioligand [^{18}F]MK-9470 (*N*-[2-(3-cyano-phenyl)-3-(4-(2-[^{18}F]fluorethoxy)phenyl)-1-methylpropyl]-2-(5-methyl-2-pyridyloxy)-2-methyl proponamide) is a high-specificity, high-affinity radioligand at the CB1R (inverse agonist), and was developed at Merck Research Laboratories (MRL, West Point, USA). The precursor was obtained from MRL and labeling was performed by alkylation of the precursor with 2-[^{18}F]fluoroethylbromide. Tracer preparation and characteristics are described elsewhere (Burns et al., 2007).

Imaging was performed in baseline condition and after chronic administration of VPA or LEV ($n = 6$ for each drug) or saline ($n = 6$). Chronic treatment consisted of one daily intraperitoneal (IP) injection of VPA (200 mg/kg; Depakine®, Sanofi-Aventis, Diegem, Belgium), LEV (50 mg/kg; Keppra®, UCB Pharma, Braine-l'Alleud, Belgium) or saline (1 ml) during 2 weeks. The last dose of the chronic treatment was injected 1 h before the microPET scan. The doses of VPA and LEV administered were selected from typical doses used in literature (Bosetti et al., 2005; Dedeurwaerdere et al., 2005).

All injections were given between 10 am and noon, all microPET (positron emission tomography) experiments were carried out between 1 and 5 pm. Prior to microPET imaging, rats were fasted for at least 6 h. MicroPET imaging was performed using a FOCUS 220 tomograph (Siemens/Concorde Microsystems, Knoxville, USA), which has a transaxial resolution of 1.35 mm full-width at half-maximum. Data were acquired in list mode in a $128 \times 128 \times 95$ matrix with a pixel width of 0.475 mm and a slice thickness of 0.796 mm. Pentobarbital anesthesia was used (50 mg/kg, IP; Nembutal®, Ceva Sante Animale, Brussels, Belgium) for all conditions. All rats were breathing spontaneously throughout the experiment. A dynamic acquisition (60 min) was started immediately after IV injection of 18 MBq [^{18}F]MK-9470. Static sinograms of the last 20 min of each scan period were reconstructed using filtered backprojection.

Baseline and chronic images were coregistered and spatially normalized to a predefined CB1R template oriented in Paxinos space as previously described (Casteels et al., 2006). Parametric images based on standardized uptake values (SUV) (activity concentration (MBq/ml) × body mass (g)/injected dose (MBq)) of the last 20 min of each scan period were generated as a measure of CB1R binding (Burns et al., 2007). On these images, a standardized volume-of-interest (VOI) map was placed (Casteels et al., 2006) and average SUV values within each VOI were determined (PMOD, version 2.65; PMOD Inc.). For statistical analyses, differences in SUV values between chronic and baseline scans in each condition (saline, valproate or levetiracetam treatment) were calculated in each VOI: $\text{difference}_{SUV} = (SUV_{chronic} - SUV_{baseline}) \times 2/(SUV_{chronic} + SUV_{baseline})$. To determine whether the difference_{SUV} values of sham treated animals were different from 0, a single sample *t*-test was performed. For the comparison of VPA or LEV to sham treated animals, a one-way analysis of variance (ANOVA) followed by 2-sided Dunnett post hoc test was performed on the difference values in each VOI (Statistica, version 6.1; Statsoft Inc., Tulsa, OK, USA).

Direct binding affinity of VPA and LEV at CB1R was assessed using a competitive binding assay as previously described (Govaerts et al., 2004). Experiments were conducted on membrane preparation from human CB1R transfected Chinese hamster ovarian cells (CHO-CB1) incubated with 1 nM [^{3}H]-SR141716A (Amersham, Roosendaal, The Netherlands) and appropriate concentration of competition ligand. Non-specific binding was determined in the presence of 10 μM of the potent cannabinoid agonist HU 210 (Tocris Cookson, Bristol, UK).

3. Results

Chronic injections of saline did not produce significant changes in global [^{18}F]MK-9470 binding (difference_{SUV} $+4.2 \pm 11.9\%$ for overall rat brain, mean ± SD; $p = 0.43$ in single sample *t*-test to 0; Fig. 1), nor in tracer binding in individual VOIs (Table 1). The intersubject variability of global CB1R binding ranged from 5 to 15%. Variability in SUV values of different VOIs within one animal was small in baseline and chronic conditions (5–8%).

Fig. 2 shows the mean CB1R images of the baseline and chronic condition after VPA and LEV administration. Variability in SUV values between the different animals and in SUV values of different VOIs within one animal was small in both experimental settings (6–10% and 5–9%, respectively). We found a significant increase in global cerebral [^{18}F]MK-9470 binding after chronic VPA administration (difference_{SUV} $+32.5 \pm 7.4\%$ for overall rat brain, mean ± SD; $p < 0.001$; Fig. 1). VOI-based assessment showed that this increase in [^{18}F]MK-9470 binding was also present in all studied brain regions (Table 1). When comparing LEV to sham treated animals, no significant changes in tracer binding were found in the overall rat brain (difference_{SUV} $+6.9 \pm 5.7\%$ for overall rat brain, mean ± SD; $p = 0.81$; Fig. 1), nor in individual VOIs (Table 1).

The *in vitro* direct binding affinity of VPA and LEV at human CB1R was negligible ($IC_{50} > 100$ μM; $5.4 \pm 1.9\%$ (mean ± SD) and

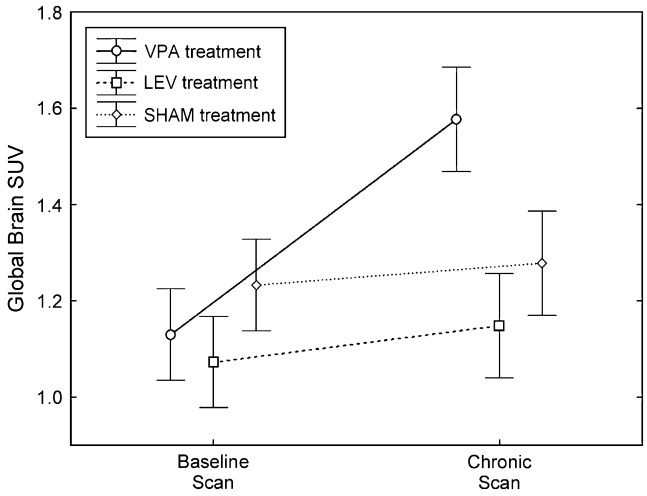

Fig. 1. Means with error plot of the global brain standard-uptake values (SUV) versus condition for VPA, LEV and sham treatment.

22.4 ± 12.6% displacement at 10 μM and 100 μM VPA respectively – 1.3 ± 14.0% and 18.2 ± 14.2% displacement at 10 μM and 100 μM LEV respectively).

4. Discussion

Antiepileptic drugs are widely used clinically although their mechanisms of action are not always well understood. There is evidence pointing towards a link between the cannabinoid system and mechanisms involved in epilepsy. Here we show that VPA, but not LEV increases the overall binding of the CB1R in the rat brain *in vivo* after chronic administration as measured by microPET imaging using the highly selective, high-affinity radioligand [^{18}F]MK-9470.

Table 1
Mean difference_{SUV} values in different VOIs studied for sham, VPA and LEV treated animals and corresponding *p* values of single sample *t*-test comparing difference_{SUV} values of sham treated animals to 0 and of ANOVA comparing difference_{SUV} values of VPA or LEV to those of sham treated animals

	Sham		Valproate		Levetiracetam	
	Mean difference_{SUV}	*p* value	Mean difference_{SUV}	*p* value	Mean difference_{SUV}	*p* value
Cortex	+4.2%	0.41	+30.2%	<0.001	+6.8%	0.82
Frontal cortex	+3.3%	0.52	+29.5%	<0.001	+5.7%	0.84
Sensorimotor cortex	+4.6%	0.37	+31.4%	<0.001	+7.4%	0.77
Lateral temporal cortex	+3.6%	0.44	+31.5%	<0.001	+9.8%	0.37
Medial temporal cortex	+4.1%	0.47	+33.0%	<0.001	+7.2%	0.78
Hippocampus	+3.4%	0.50	+34.7%	<0.001	+7.2%	0.68
Striatum	+4.5%	0.43	+34.6%	<0.001	+6.9%	0.87
Substantia nigra	+3.2%	0.63	+35.4%	<0.001	+6.5%	0.81
Thalamus	+3.0%	0.56	+37.3%	<0.001	+8.4%	0.51
Lateral globus pallidus	+4.5%	0.47	+33.6%	<0.001	+6.5%	0.91
Medial globus pallidus	+0.3%	0.96	+34.7%	<0.001	+2.8%	0.85
Nucleus accumbens	+4.6%	0.46	+30.6%	<0.001	+6.2%	0.94
Hypothalamus	+0.9%	0.86	+38.3%	<0.001	+7.7%	0.33
Cerebellum	+4.9%	0.38	+34.5%	<0.001	+6.7%	0.92

Since intersubject variability of this imaging method is small (in the order of 10%), this approach allows sensitive measurement of changes in absolute CB1R binding. Although a slight variability in CB1R binding between different cyclic phases in female rats has been described in certain brain areas (Rodríguez de Fonseca et al., 1994), the low intersubject variability indicates that this effect is only a minor potential bias and is unlikely to have affected the results of the study. Also a possible effect of pentobarbital anesthesia on CB1R binding through endocannabinoid-GABAergic interactions cannot be excluded. This possible bias is unlikely to have influenced the results since identical amounts of pentobarbital anesthesia were used in each of the experimental conditions. Modeling of tracer kinetics indicated that the area-under-the-curve macro-parameter SUV is a good index of CB1R binding at the measured scanning interval (Burns et al., 2007).

We assume that the direct binding affinity at the rat CB1R will be similar to the reported direct binding affinity at the human CB1R since human and rat sequences are 90% identical in terms of nucleotides and 98% in terms of amino acids (Gérard et al., 1990). Since VPA does not show high affinity at the CB1R *in vitro*, the reported changes in CB1R binding after chronic administration of this drug, are not likely to result from a direct interaction of the drug with the receptor. It is however possible that VPA influences the levels of endocannabinoids, thereby reactively upregulating CB1R binding. Direct measurements of endocannabinoid release e.g. by microdialysis and signal transduction studies could further clarify this issue. Possible increased CB1R binding for VPA may also result from changes in the transcription or translation of the CB1R or from a reduced internalization of the receptor caused by this anticonvulsant drug, both resulting in an increased receptor density at the synaptic cleft. Indirect interactions through changes in other neurotransmitter system that are under modulatory control of the CB1R, such as GABA and glutamate, and play an important role in the excitatory or inhibitory tone of the neuron, may also be responsible for indirect changes of the endocannabinoid levels and/or CB1R expression (Marsicano et al., 2003; Lutz, 2004).

An increase in cerebral blood flow (CBF) caused by VPA, resulting in a higher amount of tracer in the brain and thereby a higher degree of tracer binding to the available CB1Rs is less likely to explain these results. Direct measurements of flow-stimulating effects using IV administration of acetazolamide, inducing increases in CBF up to 70%, have shown only moderate changes in [^{18}F]MK-9470 SUV values (unpublished results). Furthermore, it has been demonstrated previously that VPA does not affect the regional CBF (Oliver and Dormehl, 1998) or even produces average decreases of 14.9% (Gaillard et al., 1996).

Since we did not find significant changes in CB1R binding after the chronic administration of LEV, it is less likely that this drug exerts its antiepileptic function through interaction with the endocannabinoid system. A slight increase in the endocannabinoid levels caused by LEV, that is sufficient to cause an increased cannabinoid transmission, but insufficient to cause a change in the CB1R binding, may however stay unnoticed using the currently applied radioligand.

In this study, we administered the anti-epileptic drugs chronically during 2 weeks. It is however not clear whether a similar upregulation would also be found after a single acute administration of these drugs. Further experiments evaluating the dynamic pattern of changes in CB1R binding after a single exposure of the drugs are needed.

The increase in CB1R binding and possibly signaling after chronic administration of VPA may represent a supplementary and new mechanism of action by which this drug exerts its anticonvulsant function, since activation of CB1Rs has been shown to decrease excitability and excitotoxicity on-demand (Lutz, 2004). The molecular endocannabinoid-related mechanism by

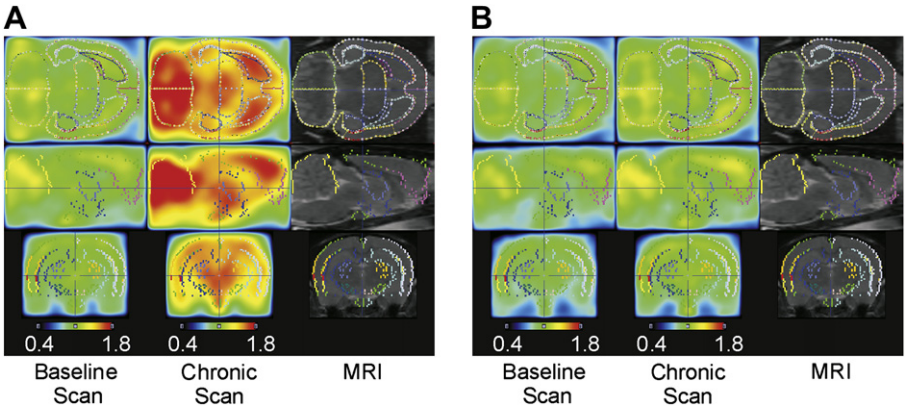

Fig. 2. Average cross-sectional SUV images of baseline and chronic (day 14) scans after VPA (A) or LEV (B) treatment coregistered to MRI, with volume-of-interest-overlay.

which VPA increases the CB1R binding in the brain needs to be elucidated.

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