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Article

Seizures Triggered by Systemic Administration of 4-Aminopyridine Lead to Acute Brain Glucose Hypometabolism in Rats, as Assessed by PET Molecular Imaging

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Abstract: 4-aminopyridine (4-AP) is a non-selective blocker of voltage-dependent K⁺ channels used to improve walking in multiple sclerosis patients, and it may be useful in the treatment of cerebellar diseases. In animal models, 4-AP is used as a convulsant agent. Intrahippocampal 4-AP induces acute local glucose hypermetabolism and significant brain damage, while i.p. administration causes less neuronal damage. This study aimed to investigate the effects of a single i.p. administration of 4-AP on acute brain glucose metabolism and to assess the short-term effects on neuronal viability and signs of neuroinflammation. We evaluated 4-AP-induced acute changes in brain glucose metabolism by [¹⁸F]FDG PET neuroimaging evaluated by standard volumes of interest (VOIs) and voxel-based (SPM) analyses. We show that 4-AP induces acute generalized brain glucose hypometabolism, except in the cerebellum, area that was significantly more resilient to this effect. Accordingly, to the appropriate neurohistochemical assays, 4-AP induced hippocampal astrocyte reactivity 3 days after the insult, without changes in signs of neuronal integrity or microglia-mediated neuroinflammation. Thus, acute brain glucose metabolic and short-term neuroinflammatory profiles in response to 4-AP i.p. clearly differ from that reported for intracerebral administration. Our results also suggest cerebellar metabolism as a potential marker of the effects of 4-AP.

Keywords: 4-aminopyridine (4-AP); seizures; 2-deoxy-2-[¹⁸F]fluoro-D-glucose ([¹⁸F]FDG); positron emission tomography (PET); hypometabolism; neuroinflammation; statistical parametric mapping (SPM); hippocampus; cerebellum

1. Introduction

4-aminopyridine (4-AP; fampridine) is a non-selective blocker of voltage-dependent K⁺ channels (K_v) widely used in basic research as a convulsant agent. It increases action potential firing and neurotransmission leading to acute epileptiform activity [1–4].

Interestingly, in the clinical scenario, the depolarizing action endows 4-AP with the therapeutic effectiveness to improve walking in patients with multiple sclerosis when used at low doses [1,5]. In addition, clinical trials and experimental studies suggest that 4-AP might be useful for the treatment of nystagmus, vertigo and various other cerebellar disorders [6].

The depolarizing action of 4-AP stimulates neurotransmitter release and facilitates inward Ca^{2+} currents therefore causing neuronal hyperactivity [2,7–11]. Increased excitatory glutamate-mediated neurotransmission in multiple brain areas such as hippocampus, entorhinal cortex and striatum is one of the main contributors to the epileptiform activity induced by 4-AP [12–14]. This is supported by the protective effects of antagonists of N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, but not by GABAergic drug, against 4-AP-induced epileptiform activity [15–18].

Both intrahippocampal and intraperitoneal 4-AP administration are known to chemically induce seizure activity in rodents and are used as epilepsy models to study neuroprotection [19–21]. Nonetheless, in rodents, some behavioral, electrophysiological, excitotoxic and neuronal damage profiles differ between both routes of administration. Thus, i.p. 4-AP administration results in generalized tonic convulsions characterized by electrical discharges of relatively short duration [2,22] and, usually no extensive neuronal damage is found [17,18,20,23]. By contrast, intrahippocampal injection results in limbic seizures and frequent wet-dog shakes that correlate with hippocampal discharges that rapidly propagate to other structures [22] and encompasses intense brain damage, including loss of pyramidal neurons [17,18,21]. Such neuronal damage is thought to be linked to glutamate-induced excitotoxicity [17,18] through overactivation of NMDA receptors [24]. And, at low doses, i.c.v. administration of 4-AP produces epileptiform activity without affecting glutamate levels [25].

It has been shown that 4-AP has different affinity for the K_v subtypes. Thus, at low concentrations primarily blocks the K_v1 family whereas at high concentrations like those that induce convulsions, it blocks a wide range of K_v channels in neuronal and glial cells [19,26–28]. Therefore, 4-AP-induced effects seem to depend significantly on the route of administration that determines its concentration in the different brain areas as well as the cell types affected.

2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) positron emission tomography (PET) neuroimaging is a minimally-invasive valuable tool for studying regional cerebral glucose metabolism, being a reflection of its functionality. Furthermore, many clinical and basic molecular neuroimaging studies have shown that the brain damage associated either to epilepsy or to seizures is characteristically associated with brain glucose metabolism alterations [20,29–32]. In this context, we have previously reported that intrahippocampal 4-AP administration induces acute brain hypermetabolism that, reflecting increased local neuronal activity, leads to short-term detrimental consequences in hippocampal integrity and neuroinflammation [21].

In the current study our aim was to evaluate the consequences of a single i.p. administration of 4-AP on brain glucose metabolism during the acute depolarizing phase by using in vivo ^{18}F FDG PET. Our second objective was to determine the short-term (3d after seizure) consequences on neuronal viability and signs of neuroinflammation by evaluating different markers of neuronal integrity, brain damage, astrocytic activation and microglia-mediated neuroinflammation.

2. Results

The administration of 4-AP was accompanied by intense seizure behavior. In fact, the dose of 5 mg/kg triggered generalized convulsions in all rats between 5 and 10 min after injection, which is in line with our previous work [20]. Besides, no mortality was recorded at this dose. When the ^{18}F FDG brain uptake was analyzed by SUV, 4-AP induced an acute hypometabolism of approximately 28% in all areas studied ($p < 0.01$) except in the cerebellum ($p = 0.067$) (Figure 1A-B-C).

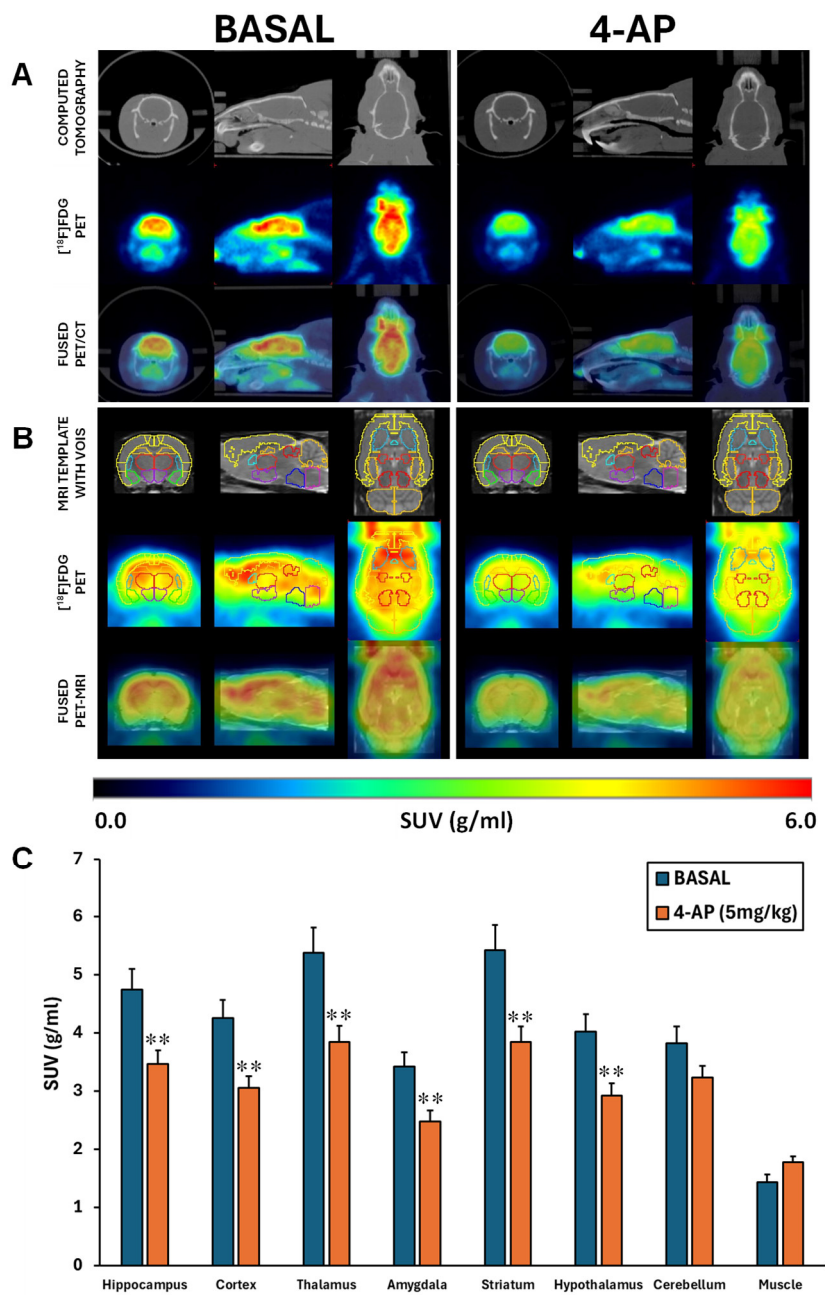


Figure 1. Systemic 4-AP administration (5 mg/kg, i.p.) induced generalized acute brain hypometabolism except in the cerebellum. **(A).** Representative images in coronal, sagittal and trans-axial views showing [¹⁸F]FDG uptake under basal and 4-AP treatment conditions. Upper row: representative CT images; Mid row: [¹⁸F]FDG PET images and bottom row: [¹⁸F]FDG PET/CT fused images. **(B)** Coronal, sagittal and trans-axial views of [¹⁸F]FDG uptake under basal and 4-AP treatment conditions shown as MRI brain template with VOIs (upper row), [¹⁸F]FDG PET with overlapped VOIs (mid row) and [¹⁸F]FDG PET/MRI fused images (bottom row). **(C)** Brain [¹⁸F]FDG uptake under basal and 4-AP treatment conditions are shown as SUV units (mean ± SEM, n=13 rats/group). Significant differences between 4-AP and BASAL conditions are depicted as: **p<0.01, paired t-tests.

These results coincided with those obtained when the SUV differences were analyzed by SPM (Figure 2A). However, when the SPM analysis was done after normalization of the [¹⁸F]FDG uptake by the whole brain, the reduced uptake in clusters localized in telencephalic and diencephalic regions was maintained but a statistically significant increase of the glucose metabolism in the cerebellum is perceivable (Figure 2B).

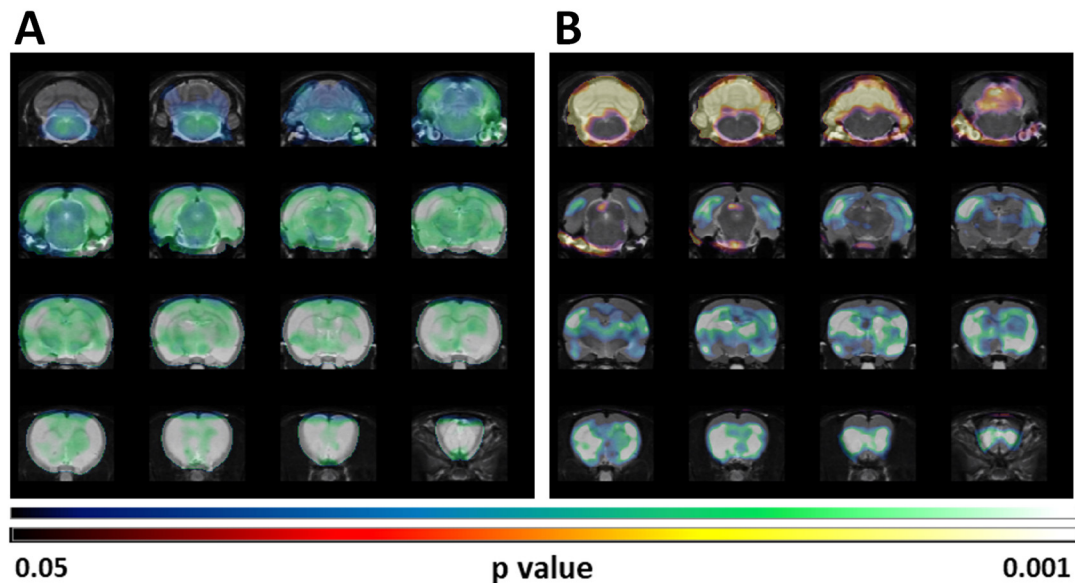


Figure 2. Statistical parametric map (SPM) analysis for FDG-PET metabolic patterns resulting from the comparison between the BASAL and the 4-AP groups are shown overlaid on a rat T2-MRI template in coronal view from olfactory bulb (bottom row) to cerebellum (upper row). **(A)** SPM images obtained after SUV normalization show extensive cerebral hypometabolism (note the lack of effect in most of the cerebellum). **(B)** SPM images obtained after normalization to whole brain $[^{18}\text{F}]$ FDG uptake showing hypometabolism involving telencephalic and diencephalic structures as well as an statistically significant increase of metabolism in the cerebellum. Upper scale: Color code bar for clusters showing statistically significant hypometabolism (4-AP vs BASAL); lower scale: Color code bar for clusters showing statistically significant hypermetabolism (4-AP vs BASAL). Results are shown at $p < 0.05$ (uncorrected for multiple comparisons) with a minimum cluster size of 100 voxels.

Considering the 4-AP was administered systemically, we also evaluated the SUV $[^{18}\text{F}]$ FDG uptake in the forepaw muscle. Although not significant, our results suggest that 4-AP might acutely increase the muscle glucose metabolism ($p=0.057$; Figure 1B).

We also evaluated the short-term effects of 4-AP on neuron integrity, neurodegeneration, astrocyte reactivity and microglia-mediated neuroinflammation in hippocampus, 3 days after 4-AP induced seizures. Our results revealed that 4-AP had no detrimental effects either on hippocampal neurodegeneration (Nissl and Fluoro-Jade C stainings; Figure 3) or on microglia-mediated neuroinflammation when evaluating the optical density of different brain areas (Figure 4A-B; $p>0.05$ in all areas studied).

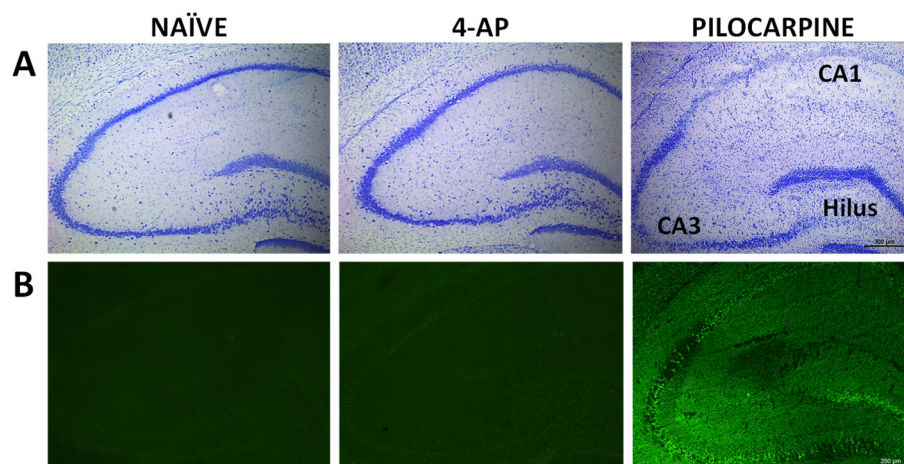


Figure 3. 4-AP-induced seizures did not have short-term effects on hippocampal integrity when evaluated 3 days after the insult. **(A)** Representative Nissl (blue toluidine) and **(B)** Fluoro-Jade C fluorescence staining micrographs from NAÏVE and 4-AP rats containing hippocampal CA1, CA3 and dentate gyrus/hilus are shown. Micrographs corresponding to rats exposed to pilocarpine-induced status epilepticus (PILOCARPINE) have been included for comparison purposes to serve as references images that clearly depict intense hippocampal neuronal loss and neurodegeneration.

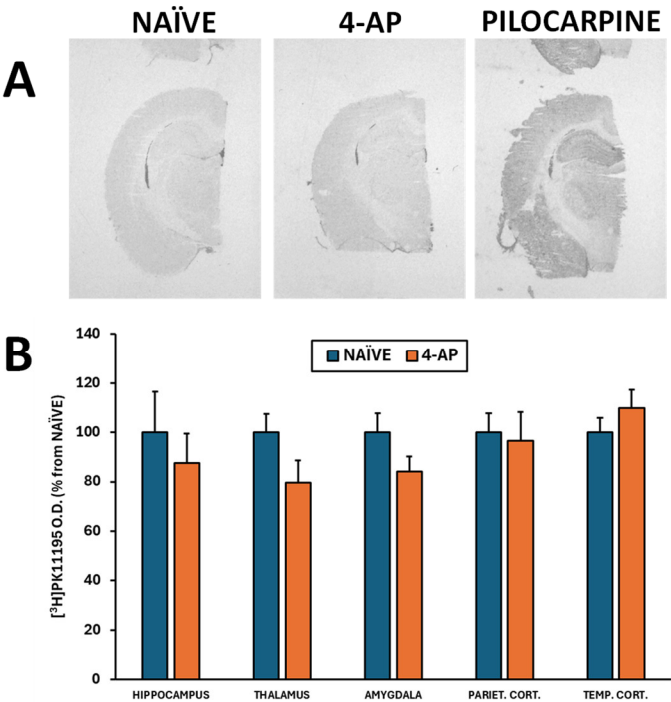


Figure 4. Systemic 4-AP (5 mg/kg, i.p.) administration did not result in signs of microglia-induced neuroinflammation as measured 3 days after the insult by the [3H]PK11195 binding in major brain regions involved in convulsant activity. **(A)** Representative [3H]PK11195 autoradiograms corresponding to the NAÏVE (n = 6) and 4-AP (n = 13) groups. An autoradiogram corresponding to rats exposed to pilocarpine-induced status epilepticus (PILOCARPINE) has been included for comparison purposes to serve as a reference image that clearly shows signs of microglia-induced neuroinflammation, especially in hippocampus and cortex. **(B)** [3H]PK11195 binding expressed in O.D. as % from NAÏVE group in various brain regions (not significant; $p > 0.05$ in all areas studied). Data are shown as mean \pm SEM.

By contrast, astrocyte reactivity was detected based on a significant increase of approximately 22% ($p < 0.05$) on GFAP fluorescence signal in the hippocampus (Figure 5).

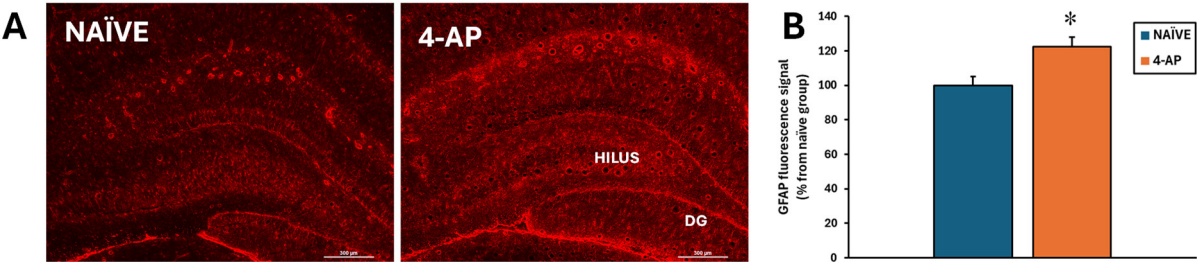


Figure 5. Systemic 4-AP administration (5 mg/kg, i.p.) resulted in short-term hippocampal astrogliosis. **(A)** Images showing representative GFAP immunofluorescence micrographs at the hilus/dentate gyrus area of NAÏVE (n = 6) and 4-AP-treated rats (n = 13). **(B)** Bar plot corresponding to quantitative data from GFAP immunofluorescence intensity as marker of astrogliosis on the hilus. Data are expressed

as percentage of the signal obtained in the NAÏVE group and shown as mean \pm SEM. * $p < 0.05$ NAÏVE vs. 4-AP; unpaired t-test.

3. Discussion

In this study we have evaluated brain glucose metabolism by longitudinal [^{18}F]FDG PET neuroimaging in rats under baseline conditions as well as, in the same rats, immediately after i.p. 4-AP administration (5 mg/kg). This dose and route of administration resulted in convulsive seizures and acute generalized brain glucose hypometabolism except in the cerebellum. Besides, 4-AP resulted in short-term astrocyte reactivity measured 3 days after the insult without apparent effects on microglia-mediated neuroinflammation.

3.1. VOIs Analysis Showed That 4-AP Administered i.p. Was Followed by Acute Generalized Brain Glucose Hypometabolism

Our results showing acute brain hypometabolism in response to 4-AP i.p. injection (Figure 1), was at first surprising considering two-fold. On one hand, all rats responded with a marked convulsive activity, and on the other hand, we had previously reported that, when administered in the hippocampus, 4-AP (7 μg in 5 μl) induced acute local hypermetabolism around the injection site, reflecting increased local neuronal activity and leading to detrimental consequences in hippocampal integrity and neuroinflammation. Likewise, local hypermetabolism at the intracortical injection site of 4-AP in rats has also been found when measured by the uptake of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) a fluorescent deoxyglucose substitute [33].

However, we have also previously shown that systemic 4-AP (3 mg/kg, i.p.) had no effects on short-term brain glucose metabolism, as measured 3 days after the 4-AP administration [20]. Supporting the hypometabolic response describe herein, hypometabolism in response to 4-AP-induced seizures has been also reported in mouse hippocampal slices [29]. Thus, it seems the metabolic responses to 4-AP go in the opposite direction depending on the dose and route of administration. Furthermore, when 4-AP is administered systemically it may induce a dose-dependently transient brain hypometabolism.

Despite these differences, 4-AP is used either by systemic or by intracerebral route as a model to induce seizure activity in rodents [3,20,21]. However, the results highlight that the brain glucose metabolic profile clearly differs between both routes of administration. This route-dependent difference in metabolic profile is in line with the differences reported regarding the differential behavioral, electrophysiologic as well as neuronal damage profiles which characterize these routes [2,22].

3.2. Cerebellar Metabolism is Increased in Response to 4-AP as Evaluated by SPM Normalized to Whole Brain [^{18}F]FDG Uptake

We also evaluated brain glucose metabolism by SPM after normalizing the values to the whole brain [^{18}F]FDG uptake. In our current study this analysis detected significantly hypometabolic clusters that overlapped with the regions detected by traditional VOIs analyses. These clusters included telencephalic as well as diencephalic structures (Figure 2). Interestingly, this type of analysis highlighted a significant increase in cerebellar uptake (Figure 2). This type of normalization of regional values *vs* the whole brain reference value allows detecting the impact of localized changes, minimizes individual differences and avoids the impact of peripheral uptake.

In addition to the central convulsive activity, 4-AP is known to have noticeable peripheral effects increasing synaptic neuromuscular transmission and directly increasing skeletal muscle twitch tension [34–36]. It has been suggested that the latter may contribute to their therapeutic effects in multiple sclerosis patients [36]. Considering that in our study 4-AP was administered systemically, and to determine whether the actions of 4-AP on skeletal muscle might have interfered with brain [^{18}F]FDG uptake, we measured glucose metabolism in the forepaw muscle. Our results showed that there is a visually apparent but statistically non-significant increase in muscle glucose metabolism (Figure 1B), a result that would be in concert with its effects increasing muscle contraction, but that

also indicates that brain hypometabolism found in our study might not be attributed to [^{18}F]FDG uptake being deviated from brain towards muscle.

One limitation associated with the SPM analyses after normalizing the values to the whole brain [^{18}F]FDG uptake has been raised, in particular, when studying diseases characterized by whole brain hypometabolism. It has been noticed that this analysis may lead to underestimation of reduced metabolism in some regions as well as to overestimations in others, which might be inconsistent with the clinical or experimental conditions [37–39].

However, in our case, and independently of the type of analysis (VOIs and SPM analyses), our data suggest that the cerebellum might be more resilient to 4-AP-induced hypometabolism. 4-AP has been shown to increase Purkinje cells activity in brain slices of the rat [40,41], and consequently to increase the GABAergic neurotransmission that restores cerebellar inhibition, effect that has been related to the therapeutic effects of 4-AP on cerebellar disorders [42]. In a clinical case study evaluating brain glucose metabolism by [^{18}F]FDG PET, treatment with 4-AP lessened the cerebellar hypometabolism associated to downbeat nystagmus. The authors suggested that this effect might indicate an improvement of the cerebellar inhibition [43]. Besides, 4-AP has been reported to exert a robust neuroprotective effect on apoptotic cerebellar granule cells [44]. Furthermore, 4-AP (3 mg/kg, i.v.) has been shown to increase blood flow to various brain regions being particularly significant in the cerebellum [45]. Thus, our results regarding the effects of 4-AP on cerebellar metabolism might be related to some of the above-mentioned actions of 4-AP.

3.3. Systemic 4-AP Administration Does Not Alter Hippocampal Integrity

4-AP (5 mg/kg, i.p.) administration had no detrimental short-term effects on hippocampal neuronal integrity and survival as measured 3 days after the insult (Figure 3). This result agrees with a previous study where we showed that 4-AP i.p. at a dose of 3 mg/kg did not induce signs of neurodegeneration or neuronal death [20].

Many studies support the neuroprotective effects of K^+ channels blockade [28,46]. For example, 4-AP (5 mg/kg, i.p.) abolishes the kainate-induced hippocampal neuronal cell death, as measured 3 days after kainate injection [47]. Both, in vivo as well as in vitro stroke models have shown that potassium channel blockade attenuates ischemia-induced neuronal death and apoptosis [27]. Likewise, studies in cell cultures have reported that 4-AP prevents cerebellar granule neuronal cell death and apoptosis induced by low K^+ (5 mM) serum-free conditions [44].

3.4. Effects of Systemic 4-AP Administration on Short-Term Neuroinflammation

Gliosis is a histopathological feature of epilepsy, including microglia and astrocyte activation [48–51]. Furthermore, complex and reciprocal regulatory interactions between microglia and astrocytes exist and, the temporal order of events is still unclear [52,53]. Nonetheless, it seems that the inflammatory effects of microglia may precede those of astrocytes [54–56].

Herein we show that systemic 4-AP administration did not result in microglia-mediated neuroinflammation analyzed by [^3H]PK11195 autoradiography (Figure 4). By contrast, intrahippocampal 4-AP injection induced microglia-mediated neuroinflammation measured by in vitro [^{18}F]GE180 autoradiography [21]. In other models of microglia activation, 4-AP has been shown to inhibit K^+ outward current in rat [57] and to lessens the microglial production of neurotoxins and the consequent neuronal damage [58,59]. Thus, again it seems that despite being able to induce seizures the effects of 4-AP, when administered systemically, clearly differ from those observed after intracerebral administration.

Astrocytes have been shown to be key players in the etiology and pathogenesis of epilepsy [49,51] and 4-AP has been also reported to non-selectively block K^+ in astrocytes [26]. In our current study, astrocyte reactivity was observed 3 days after i.p. 4-AP-induced seizures. This result agrees with our previously reported data obtained in rats (4-AP, 3mg/kg, i.p.) [20] as well as by data reported in mice (4-AP, 5.6 mg/kg, i.p.) [60]. Likewise, pre-treatment with 4-AP in rodent C8D1A cultured astroglial cells increases LPS-stimulated astrocyte reactivity and production of pro-inflammatory cytokines [61].

Even though astrocyte reactivity is commonly associated with neurodegeneration, in our study, as depicted in Figure 5, we found astrocyte reactivity in the absence of hippocampal damage. Herein, it is interesting to notice that 4-AP directly acts on both neurons and astrocytes, the latter critically modulating the effects of 4-AP on neuronal integrity and survival [62]. Our result suggests that the astrocyte reactivity could be consequence of the direct action of 4-AP on astrocytes and not as a response to neuronal damage. At this point, we cannot exclude the possibility that the astrocyte reactivity observed 3 days after the insult might in turn result in longer-term changes in hippocampal integrity.

4. Materials and Methods

4.1. Animals

Thirteen adult Sprague-Dawley male rats (Charles Rivers, Spain) were housed in pairs in standard cages on a ventilated rack (Tecniplast, Buguggiate, Italy). The animal room was under controlled conditions of temperature ($22 \pm 2^\circ\text{C}$) and was under a light/dark cycle of 12 h (8 am – 8 pm). Rats were left undisturbed for a minimum of 5 d to allow adaptation to the new environment. Animals had free access to food chow and water. Access to food only was restricted for the 12h before the PET scan procedures to ensure low blood glucose plasma concentrations for minimizing any eventual interference with the radiotracer tissue uptake.

All procedures were carried out in accordance with animal welfare regulations of the European Union (2010/63/UE) and Spain (RD53/2013) and was approved by the Animal Research Ethical Committee of the Complutense University of Madrid and by the Autonomous Community of Madrid (PROEX 222.1/20). All efforts were made to minimize the suffering of the animals.

4.2. Experimental Design

Longitudinal brain glucose metabolism activity was evaluated by [^{18}F]FDG PET. By obtaining longitudinal measurements within the same animal, we could reduce the number of animals needed without put at risk statistical power. Thus, on experimental day 1, we evaluated the basal metabolic activity (BASAL group) and on experimental day 7, the acute metabolic effects of 4-AP (Merck; 5 mg/kg, i.p. in 0.9% NaCl at a volume of 1 ml/kg) in the same animals (4-AP group) were evaluated 1 min after [^{18}F]FDG injection.

In parallel, a group of rats were kept naïve throughout the whole experiment to serve as a control group for histological studies (NAÏVE group; $n = 6$). All animals were sacrificed on experimental day 10. The experimental design is schematized in Figure 6.

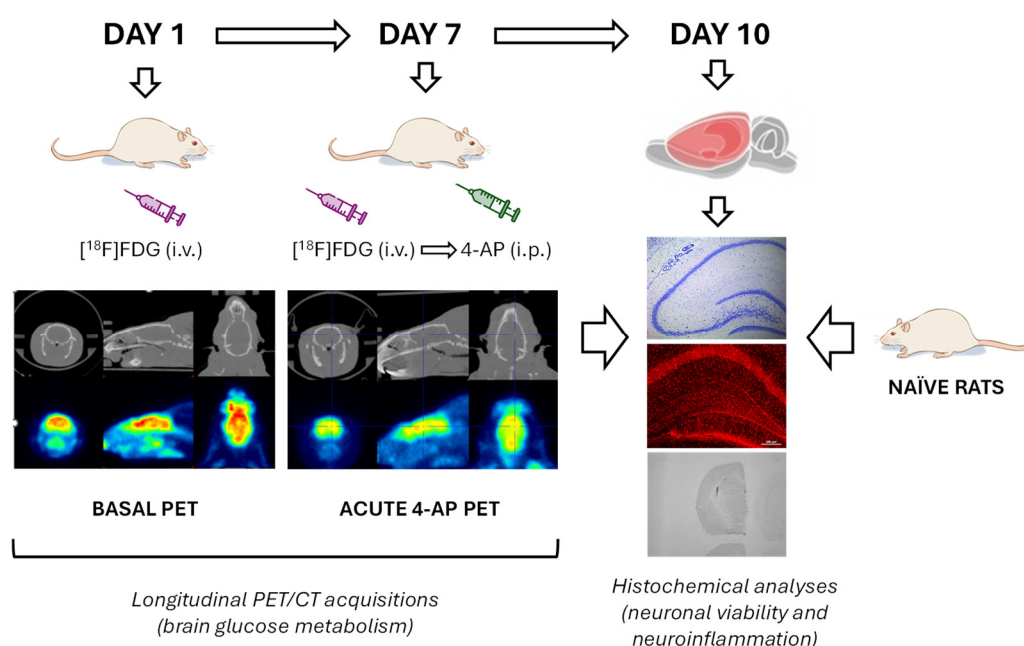


Figure 6. Schematic representation depicting the experimental design and procedures.

4.3. $[^{18}\text{F}]\text{FDG}$ PET/CT Imaging

Brain glucose metabolism was evaluated by in vivo $[^{18}\text{F}]\text{FDG}$ PET imaging as previously reported [21,63]. Briefly, the rats were fasted overnight before the procedure to minimize the eventual competition between circulating glucose and $[^{18}\text{F}]\text{FDG}$. For basal metabolic activity evaluation (day 1), $[^{18}\text{F}]\text{FDG}$ was injected into the tail vein (13.05 ± 0.05 MBq in approximately 0.2 ml of 0.9% NaCl; Curium Pharma, Madrid, Spain). On experimental day 7, the acute metabolic effects of 4-AP in the same animals (4-AP group) were evaluated by administering 4-AP (Merck; 5 mg/kg, i.p. in 0.9% NaCl at a volume of 1 ml/kg) 1 min after $[^{18}\text{F}]\text{FDG}$ injection.

Immediately after the radiotracer was injected, the rats were returned to their cages for a 30 min period to ensure the brain uptake of the radiotracer. After this uptake period, rats were anesthetized by inhalation of isoflurane/oxygen and placed on the bed of the tomograph. Image tomographic acquisitions were done using a dedicated-small animal dual PET/CT scanner (Albira PET/CT dual scanner, Bruker NMI, Germany). The acquisitions consisted of a 20 min static PET scan followed by a high-resolution computed tomography (CT) scan.

In both cases, once the acquisitions were finished, PET images were reconstructed by using a maximum likelihood expectation maximization algorithm, applying decay, random and scatter corrections (Bruker, Germany).

4.4. PET Image Analysis by VOIs Analysis

The CT image of the skull of each rat was manually co-registered to a magnetic resonance imaging (MRI) rat brain template that containing the pre-defined brain regions allows for the obtention of a fitted spatial mathematical transformation. For each animal, the later CT transformation was applied to the corresponding PET image, allowing for the correct matching between the PET image and the MRI template as previously described (Jupp and O'Brien, 2007). Then, this co-registered PET image was overlaid onto the MRI template, allowing for the quantification of the tracer regional uptake values (kBq/ml; PMOD 4.1, PMOD Technologies Ltd., Zurich, Switzerland). Afterwards, the standard uptake value (SUV) was calculated applying the following equation: $[\text{Tracer uptake (kBq/ml)} * \text{BW (g)}] / (\text{Tracer dose administered (kBq)})$, that corrects by the differential individual body weights and $[^{18}\text{F}]\text{FDG}$ doses administered.

4.5. PET Evaluation by Statistical Parametric Mapping (SPM)

In experimental PET imaging studies, the regions that eventually show effects are often unknown in advance and they might not correspond to the predefined anatomical VOIs. Thus, different from the traditional quantification using VOIs (see section 4.4), SPM includes the whole brain in the analysis, without any preconception about the structures involved in the experimental paradigm [64]. This approach might unveil differences in subregions that are not evident in the VOI analysis. Besides, when the regional uptake is normalized to whole brain, it allows to compare relative regional changes without interferences of changes in blood brain flow and tracer peripheral uptake.

SPM comparisons were performed using MATLAB software (The MathWorks, Natick, Ma, USA) and SPM12 (Wellcome Trust Center for Neuroimaging, UCL, London, UK). To evaluate the effects of acute 4-AP injection, differences between BASAL and 4-AP-treated rats were calculated by paired t-test, setting a significance level threshold of 0.05 (uncorrected for multiple comparisons). The minimum cluster size of voxels was set to 100. The t-value threshold was set to show voxels with a correspondent p value lower than 0.05. Parametric t maps resulting from the comparison between the basal and the 4-AP groups were loaded in PMOD and fused to a rat brain T2-MRI template.

4.6. Brain Tissue Collection and Processing for Neurohistochemical Assessments

All rats were sacrificed by decapitation on experimental day 10 (3 days after 4-AP-induced seizures). Brains were collected, cut longitudinally into two halves and stored at -80°C. Twenty-five μm thickness coronal slices at -20 °C were obtained using a cryostat (Leica CM1850, Leica Biosystems, Germany). Sections containing the dorsal hippocampus were thaw-mounted onto Superfrost Plus slides (Thermo Scientific, Germany), dried on a hot plate and stored into slide boxes at -80 °C until the day of the assays.

4.7. Nissl Staining

Qualitative evaluation of neuronal viability and hippocampal disruption was done with toluidine blue staining. Thus, slides were stained in 0.5% toluidine blue for 2 min, decolorized and dehydrated in graded ethanols: (i) 96% ethanol (10 s) and (ii) 100% ethanol (2x10 s). Then, the sections were cleared twice (2 min and 5 min) in Neo-Clear™ xylene substitute (Merck-Sigma). Finally, the slides were mounted with DPX mounting medium (Herter, Barcelona, Spain). The images were captured with a digital camera (Leica DFC425, Leica, Germany) coupled to a microscope (Leica DM 2000 LED, Leica, Germany).

4.8. Fluoro-Jade C Labeling

Fluoro-Jade C staining was performed following the protocol previously described [65]. Thus, the brain sections were immersed for 2 min in 0.0001% Fluoro-Jade C (Millipore, Darmstadt, Germany) in PBS. Then, the slides were air-dried and cover-slipped with DPX mounting medium (Herter, Barcelona, Spain). The images were captured with a digital camera (Leica DFC3000G) coupled to a microscope (Leica DM 2000 LED) by using the FITC filter. At the hippocampal CA1, CA3 and hilus, the fluorescence signal was measured using ImageJ 1.46r software. The average value for each rat was calculated and the results were expressed as percentage *vs* the naïve group.

Images from Fluoro-Jade C were acquired using a Leitz Laborlux S microscope (Leica, Germany). The filters used were as follows: FITC filter for Fluoro-Jade C. The hippocampal subareas CA1, CA3 and hilus of each brain slice were manually delimited by a blind operator and the mean fluorescence signal value of those regions were obtained. The average value for each animal was obtained from 4 different brain slices containing dorsal hippocampus. Both delimitation, and quantification steps were performed with ImageJ software (NIH, available at the following website: <https://imagej.nih.gov/ij/>).

4.9. Microglia-Mediated Neuroinflammation by [³H]PK11195 Autoradiography

As a marker of microglia-mediated neuroinflammation, we evaluated the translocator protein (TSPO; 18 kDa) by [^3H]PK11195 autoradiography following the protocol previously reported [66] with minor modifications. The slides were dried on a hot plate at 37°C (10 min), preincubated with 50 mM Tris-HCl pH 7.4 at RT (15 min) and then incubated with 1 nM [^3H]PK11195 (Perkin Elmer) in preincubation buffer. After 60 min incubating, the samples were washed in an ice cold preincubation buffer (2x5 min) and dipped in ice-cold distilled water. The slides were air-dried and exposed to Kodak BioMax MR autoradiography film (Carestream, U.S.A.) for approximately 2 months. After manual development, the film was placed onto a light box (Kaiser Prolite 5000, Kaiser Fototechnik, Germany) and the images were captured with a camera (Leica DFC425) coupled to a stereomicroscope (Leica MZ6). The optical densities of the selected brain regions were used as index of neuroinflammation degree.

4.10. Reactive Astrogliosis

Glial fibrillary acidic protein (GFAP) immunofluorescence was performed as previously reported [21,67]. Briefly, the slices were fixed in 4% formaldehyde, washed and then blocked and permeabilized with 3% BSA, 0.1% triton X-100 in TBS (60 min). The slides were incubated overnight with the anti-GFAP-Cy3 antibody (1:500, Sigma Aldrich) in 1% BSA in TBS at 4 °C. Afterwards, they were washed in 0.1% Tween 20 dissolved in TBS (3 x for 5 min each) and cover slipped with Mowiol mounting medium. The images were captured and evaluated using the same optical systems used for Fluoro-Jade C, but in this case using the TRITC filter. For each brain section containing the dorsal hippocampus, the fluorescence intensity was measured using ImageJ 1.46r software. As with the Fluoro-Jade C measurement. The results were expressed as percentage *vs* the NAÏVE group.

4.11. Statistics

Data are shown as mean \pm SEM. The longitudinal data corresponding to within-individual differences in brain glucose metabolism between BASAL and 4-AP-treated groups were evaluated using paired t-test. The data corresponding the neurohistochemical studies was compared between the NAÏVE and 4-AP groups by unpaired t-test. The analyses were carried out using the SigmaPlot 11.0 (Systat Software Inc., IL). The statistical significance level threshold was $p < 0.05$.

5. Conclusions

Summing up, even though 4-AP is relatively selective for A-type K^+ channels at low concentrations, the selectivity is lost when used at higher doses. Thus, despite that 4-AP acts as a convulsant agent independently of the route of administration, acute generalized brain glucose hypometabolism and lack of neurodegeneration in response to 4-AP i.p. is clearly in opposition to the acute local hypermetabolism and extensive neuronal damage reported when 4-AP is administered intracerebrally. Therefore, it is evident that the route of administration and the dose have a significant impact on the 4-AP concentrations that reach the brain and, according to its potential beneficial or deleterious consequences. In addition, our study further points towards cerebellum glucose metabolism as a potential marker of systemic 4-AP-induced effects.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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