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Research Article

MicroPET/CT Assessment of Minocycline Effects on Anesthetic-Induced Neuronal Injury in Developing Rats

Abstract

Ketamine is a dissociative anesthetic that is frequently used for the induction and maintenance of general anesthesia in children. It has been reported that blockade of NMDA receptors by ketamine may cause neurotoxicity in neonatal rats when given over a 12 hour period during the brain growth spurt. Noninvasive, quantitative imaging of rodent brains may allow for the detection of functional, morphological and metabolic alterations induced by ketamine. Since it is known that level of the mitochondrial translocator protein (TSPO), formerly known as the peripheral benzodiazepine receptor (PBR) increase in areas of neuronal injury following exposure to neurotoxicants, TSPOs are widely recognized as important targets for imaging using positron emission tomography (PET). In this study, the effect of ketamine on the uptake and retention of [18F]-FEPPA (a TSPO ligand) in the brains of rats and the potential protective effect of minocycline, an anti-inflammatory agent, on anesthetic-induced neuronal cell death were investigated using microPET/CT imaging. On postnatal day 7 (PND 7), rat pups in the experimental group were exposed to 6 injections of ketamine (20 mg/kg at 2 h intervals) with or without minocycline (45mg/kg i.p. 30 minutes prior to, and 4 hours after exposure); control pups received 6 injections of saline. On PNDs 14, 21, 28 and 35, [18F]-FEPPA (18.5 MBq) was injected into the tail vein of treated and control rats and microPET/CT images were obtained over the next 90 minutes. Radiolabeled tracer accumulation in regions of interest (ROIs) in the frontal cortex was converted into Standard Uptake Values (SUVs). In PND 14 and 21 rats the uptake of [18F]-FEPPA was significantly increased and the duration of tracer wash-out was prolonged in ketamine-treated rats. The increased uptake of the tracer was attenuated by the co-administration of minocycline. As expected, no significant difference in radiotracer uptake in the frontal cortex was observed at 28 or 35 days after anesthesia. This preliminary study demonstrates that microPET imaging is capable of distinguishing differences in retention of [18F] -FEPPA in the brains of rodents and suggests that this approach may provide a minimally-invasive biomarker of pathogenic process associated with neurotoxicity induced by ketamine. Minocycline effectively blocks the neuronal injury caused by ketamine anesthesia in the developing rat brain.

Introduction

As a noncompetitive NMDA receptor antagonist, ketamine is a commonly used anesthetic in pediatric patients for the induction and maintenance of general anesthesia, usually in combination with other sedative and anesthetic drugs [1,2]. However, numerous studies of both the developing animal brain and primary cultured neurons have reported that prolonged exposure to ketamine during the brain growth spurt period induces widespread neurotoxicity as shown by increased neuroapoptosis, enhanced neuroinflammation and impaired neurogenesis [3-11]. General anesthesia maintained by ketamine during brain development can result in long-lasting deficits in brain function in nonhuman primates [12]. These observations have raised concerns regarding the use of ketamine-induced anesthesia for surgery or other clinical procedures in early childhood. Recently, retrospective reports have indicated that the development of cognitive abnormalities and learning disabilities in children correlate with anesthetic exposure during surgery before 4 years of age [2,10,13-18]. Thus, practical neuroprotective strategies that can serve to protect the developing brain from ketamine or other general anesthetic-induced neuronal injury and long-lasting cognitive impairments are urgently needed.

Minocycline is a semi-synthetic, tetracycline derivative that has antibiotic activity against a broad spectrum of bacterial types [19-21]. In addition to its broad antimicrobial activity, minocycline effectively crosses the blood-brain barrier and has exhibited neuroprotective effects in various types of experiment models [18-24]. Minocycline is approved by the FDA and indicated up to 200 mg in 24 hours for a variety of

infections. With high bioavailability in humans, minocycline is efficiently absorbed by the gastrointestinal tract the average half-life is around 15 h [25,26]. The mechanisms underlying the neuroprotective effect of minocycline are not clear: minocycline may exert its neuroprotection through inhibition of microglial activation, anti-inflammatory effects, reduction of nitric oxide synthesis and/or prevention of apoptotic cell death [19-22,24,27].

In our previous studies, the administration of multiple doses of ketamine (20 mg/kg every 2 hours, 6 times, s.c.) to PND 7 rat pups, induced widespread neuronal damage in the developing brain as indicated by increased caspase-3-, silver stain-, and Fluoro-Jade C-positive cells in neocortical areas, especially in layers II and III of the frontal cortex [11]. MicroPET scans using [18F]-radiolabelled]-N-(2-(2-fluoroethoxy) benzyl)-N-(4-phenoxypyridin-3-yl) acetamide) ([18F]-FEPPA) [28], indicated that anesthetic exposure significantly increased the uptake of [18F]-FEPPA in the temporal and frontal lobes of exposed nonhuman primates in a time-dependent manner. In the current study, we initiated a microPET protocol to measure [18F]-FEPPA uptake as a quantitative marker of neuroinflammation and, presumably, neuronal injury in the rat brain in vivo.

Materials and Methods

Drugs

Ketamine hydrochloride (Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA, USA) for injection was diluted in saline. The purity of ketamine (> 99%) was identified and confirmed via HPLC (High-performance liquid chromatography) and mass spectrometry. Minocycline hydrochloride was purchased from Sigma-Aldrich, USA) and dissolved in sterile saline.

Animals

All animal procedures were approved by the National Center for Toxicological Research (NCTR) Institutional Animal Care and Use committee and conducted in full accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals.

Animals included in current study were obtained from and maintained in the animal facility at NCTR with dam (PND 7-PND 21) or 3 rats/cage after PND 21. The room temperature in the animal facility was kept at 22±2°C. The animals were maintained ad lib on regular rat chow and water under a light/dark cycle of 12hr/12hr. The light cycle for these animals began at 7:00 am. Seven-day-old (PND-7) Sprague Dawley (male and female) rat pups (average body weight 11–18 g) were randomly assigned to control, control plus minocycline, ketamine plus saline, and ketamine plus minocycline groups (n=3-5/group, with the same rats used on PNDs 14, 21,28 and 35). Ketamine hydrochloride or saline (10μl/g) was applied subcutaneously using a 29-gauge needle. Similar to our previous studies [11], doses of ketamine (20 mg/kg/injection) or saline were given in six injections within 12 hours (2-h/interval). In order to maintain body temperature and lessen potential stressors, pups were accommodated with their dam during the 2-h injection interval [11]. Rat pups in the groups with minocycline were given i.p. injections of 45 mg/kg minocycline (100-200μl) in saline 1/2 hr before and 4 hr after the start of ketamine administration. The minocycline solution was prepared within 24h of the experiment and kept on ice between the two injections.

After exposure on PND 7, rat pups in all groups were returned to the rodent facility until microPET scanning on PNDs 14, PND 21, PND 28 and PND 35.

Radiotracer preparation

[18F]-FEPPA was prepared by 3D-Imaging LLC (Little Rock, AR) according to Wilson et al. [29], from the corresponding tosylate as described therein with minor modifications to the procedure. [18F]-FEPPA was produced in > 98% purity at a specific activity EOS of 1.1-3.7 TBq (30-100 Ci)/μmole, as compared to the previously reported value of 11-37 MBq (0.3-1 Ci)/μmole [30]. Specific activity at the time of use was one tenth to two-thirds of the end of synthesis (EOS) value.

MicroPET

MicroPET images of the rat brains were acquired quantitatively using a commercial high resolution small animal PET scanner, Focus 220 (Siemens Preclinical Solution, Knoxville, USA). With 96 lutetium oxyortho-silicate detectors, Focus 220 provides microPET images with a transaxial resolution of 1.35 mm full-width at half-maximum. List mode data were collected in a 128x128x95 matrix with a pixel width of 0.475 mm and a slice thickness of 0.815 mm.

MicroPET scan

MicroPET images were recorded on PNDs 14, 21, 28 and 35. General anesthesia was initially induced in rat pups using 1.5-2% isoflurane gas delivered through a custom face mask: anesthesia was maintained using 1-1.5% isoflurane throughout the PET imaging procedure. For each imaging procedure, [18F]-FEPPA (18.5 MBq) was injected into the tail vein of each animal. Following the injection, a set of serial microPET images was collected over 90 minutes (18 frames, 5 min each) to assess the uptake of the radiotracer.

MicroPET data analysis

Medical image analysis software, ASIProTM (Concorde Microsystems, Inc, Knoxville, TN) was used in the analyses for each Region of Interest (ROI). ROIs were defined and quantified using ROI tools provided by ASIProTM. As shown in Figure 1, all images were displayed using the same color scale. Based on our histological data, the frontal cortex was the most vulnerable region to ketamine-induced neuronal cell death [11], and thus, was selected as our main ROI. Tracer accumulation in the ROI in the left frontal cortex was converted to Standard Uptake Values (SUVs) which were computed as = average concentration of radioactivity in the ROI (mCi/mL) x body weight (gram)/injected dose (mCi).

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The SUVs calculated for each ROI at different time points are displayed as means ± standard errors (SE). A repeated measures ANOVA profile model was used to determine the effect of ketamine on the uptake and the potential protective effect of minocycline on anesthetic-induced neuronal injury on the basis of the three ketamine treatment groups (minocycline; ketamine; minocycline plus ketamine) and a saline control group. The SUVs were modeled as a linear function of treatment and time profile within each PND time point. A Heterogeneous Autoregressive covariance structure was used to model the within-animal correlations for measurements on the same rat. The SUVs at different time points were compared between ketamine groups and the saline control using Student's t-tests. All statistical tests were two sided and adjusted for multiplicity testing in order to control the nominal Type I Error rate. Dunnett's adjustment method was used for pairwise treatment comparisons while Bonferroni correction was used for comparisons between observation times. Statistical significance was assessed at the 5% level. The SAS System for Windows (v9.3) was used for statistical analysis while Sigma Plot for windows (v11.0) was used to generate graphs.

Results

MicroPET images of rat brain after [18F]-FEPPA administration

On PNDs 14, 21, 28 and 35, dynamic microPET scans were performed on each rat for 90 minutes. Acquisition of microPET data was started following i.v. injection of [18F]-FEPPA. PET image for each scan was constructed in a set of eighteen frames, in which every frame including the total radioactivity of the tissues collected over a 5 min period. Time Activity Curves (TACs) for the ROI in the frontal cortex [31,32], were obtained and tracer accumulation converted to SUVs. Figure 1 show 4 representative microPET images, recorded on PND 14, of the brain from a control rat (saline only), a control rat given minocycline, a ketamine-exposed rat given minocycline and a ketamine-exposed rat given saline. Images in figure 1 illustrate the distribution of radiotracer 0-5 minutes following the [18F]-FEPPA injection. The radioactivity accumulated in the ROI in frontal cortex of ketamine+saline treated rat was significantly higher 0-5 min after injection of radiotracer compared to the radioactivity in the same ROI of brains of saline, saline + minocycline, and ketamine+minocycline treated rats.

Dynamic [18F]-FEPPA uptake in the brain at different time points

On PND14, about one week after the anesthetic exposure, SUVs in the ROI of frontal cortical area were higher in the ketamine+saline treated animals than in the saline controls and the minocycline +saline and ketamine+minocycline animals at almost every time points, indicating a significantly enhanced uptake and retention of [18F]-FEPPA in subjects that exposed to ketamine (Figure 2). Co-administration of minocycline effectively blocked this increase and there was no significant effect of minocycline on SUVs when given alone.

On PND 21, 2 weeks after anesthetic exposure, SUVs of [18F]-FEPPA in the same ROI were still significantly higher in the ketamine tested animals than in all other groups at most of the time points, showing a prolonged increase of [18F]-FEPPA uptake and retention in treated subjects (Figure 3). Compared with controls, there was no significant difference in SUVs detected in animals treated with ketamine plus minocycline.

At PNDs 28 and 35, the uptake of [18F]-FEPPA in the ketamine-exposed rats was similar to that of control animals and no significant differences were observed at any time points (Figures 4,5).
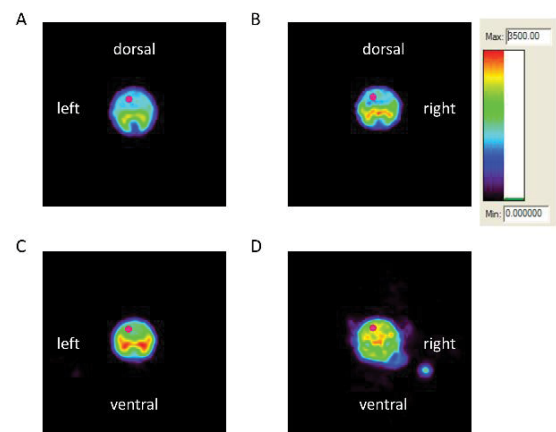

Figure 1: A set of representative microPET images (transaxial plane) of a control rat given minocycline (A), a control rat given saline (B), a ketamine-treated rat given minocycline (C) and a ketamine-treated rat given saline (D) obtained on PND 14, one week after 8 hour exposures on PND 7. Compared with the control rat given saline only (B), the 18F-FEPPA uptake was significantly increased in the ketamine exposed rat pup (D). Co-administration of minocycline effectively blocked this increase in the uptake of the [18F]-FEPPA induced by ketamine (C): there was no significant effect of minocycline on the uptake of the [18F]-FEPPA when given alone. Purple circle mimic the ROI in left frontal cortex.
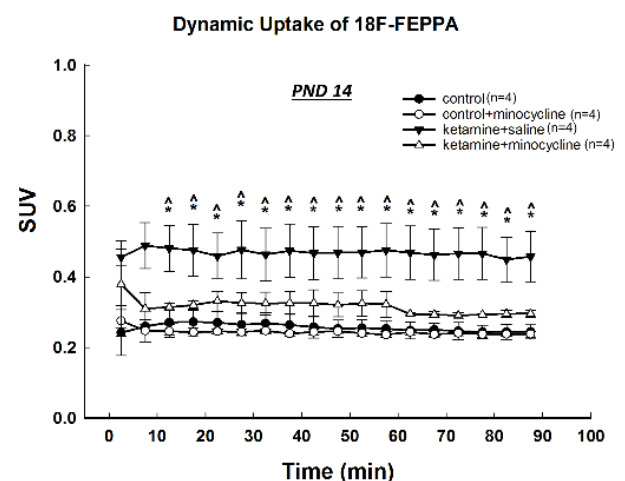

Figure 2: Graphic showing the dynamic uptake of [18F]-FEPPA expressed as SUV vs. time in the ROI in the left frontal cortex of a control+saline group, a control+minocycline group, a ketamine+saline and a ketamine+minocycline group at PND 14 (n=4/group). Asterisks denote significantly higher accumulation of [18F]-FEPPA in ketamine+saline treated rat compared with the control (saline only) animals: *p<0.05. Carets denote significantly higher accumulation of [18F]-FEPPA in ketamine+saline treated rats compared with the ketamine+minocycline animals: ^p<0.05.

Discussion

Ketamine is commonly used to induce and maintain general anesthesia in pediatric patients [33]. Ketamine is thought to exert its anesthetic effect through blockade of NMDA receptors, resulting in inhibition of neuronal activity in CNS. Prolonged exposure to ketamine causes widespread apoptotic neurodegeneration in the developing animal brain [3,7,9,34-37]. Previous studies in our lab demonstrated that neuronal apoptosis in several major area of developing brain can be induced by multiple ketamine injections (20 mg/kg given every 2 hours for 6 times) to PND 7 rats, especially in the frontal cortex [11,34]. Ketamine-induced neuronal apoptosis has also been shown to occur in newborn rhesus monkeys [38],
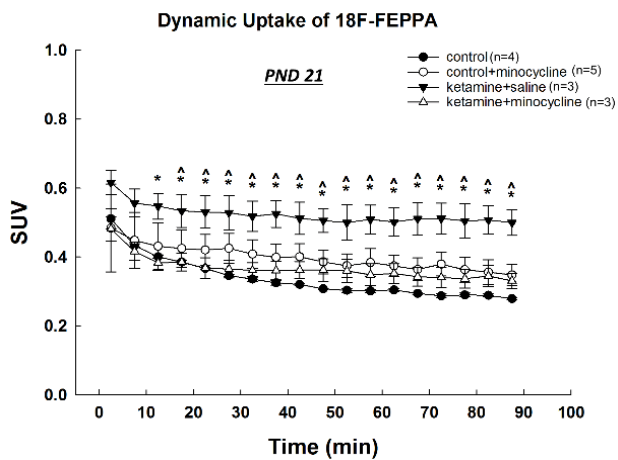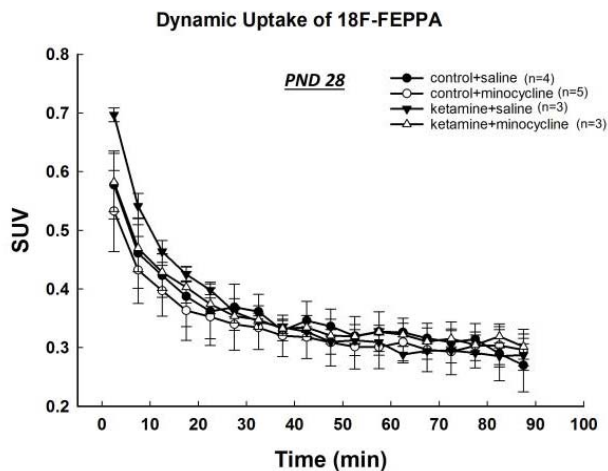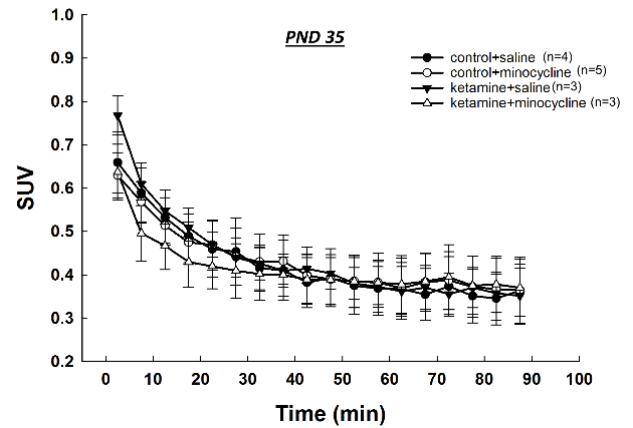
Figure 5: Graphic showing the dynamic uptake of [18F]-FEPPA expressed as SUV vs. time in the ROI in the left frontal cortex of the control (saline only) group, the control+minocycline group, the ketamine+saline and the ketamine+minocycline groups at PND 35 (n=3-5/group). There were no significant differences between any of the groups.

in fetal NHP brains [34], and primary cultured rat neurons [6,39]. Early exposure to ketamine leads to persistent cognitive deficits, including impaired learning and memory in rodent and nonhuman primate models [12,40].

In the present study, microPET imaging using [18F]-FEPPA was used to repeatedly detect and quantify, in the same animal, aspects of neuronal activity thought to be associated with brain cell damage caused by ketamine-induced general anesthesia during early development. As a specific ligand for the Translocator protein (TSPO), a biomarker of activated microglia [41-43], the synthesis of [18F]-FEPPA has proven efficiently with high radiochemical yields. [18F]-FEPPA was applied successfully in previous nonhuman primate and rodent studies [29,44-50]. In response to a variety of neuronal insults, glial cells are activated and the expression of TSPO in those cells, especially microglia and astrocytes, is significantly upregulated. The upregulation of TSPOs is associated with several neurological and psychological disorders including multiple sclerosis, cerebral ischemia and stroke, epilepsy, brain injury, brain infection, neurodegenerative diseases and schizophrenia [43,51-56]. By monitoring the increased expression of TSPO in the same animal at different time points, anesthetic-induced neurotoxicity can be investigated dynamically via microPET imaging using [18F]-FEPPA [47-49].

According to our PET imaging data obtained on PNDs 14 and 21, treatment of rat pups with multiple doses of ketamine on PND 7 increased microglial activation in the frontal cortex for at least two weeks. This effect had abated completely 3 weeks after exposure. These observations demonstrate that seemingly adverse effects of ketamine-induced anesthesia persist long after animals have recovered from anesthesia when levels of ketamine are no longer detectable.

Although previous studies and the current data provide evidence of general anesthesia-induced developmental

Figure 3: Graphic showing the dynamic uptake of [18F]-FEPPA expressed as SUV vs. time in the ROI in the left frontal cortex of the control+saline group, control+minocycline group, ketamine+saline and ketamine+minocycline group at PND 21 (n=3-5/group). Asterisks denote significantly higher accumulation of [18F]-FEPPA in ketamine+saline treated rats compared with the control (saline only animals): *p<0.05. Carets denote significantly higher accumulation of [18F]-FEPPA in ketamine+saline treated rats compared with the ketamine+minocycline animals: ^p<0.05.

Figure 4: Graphic showing the dynamic uptake of [18F]-FEPPA expressed as SUV vs. time in the ROI in the left frontal cortex of the control (saline only) group, the control+minocycline group, the ketamine+saline and ketamine+minocycline groups at PND 28 (n=3-5/group). There were no significant differences between any of the groups.

neurotoxicity, it is simply not possible to eliminate the need for general anesthesia in pediatric and neonatal patients. Thus, practical strategies that can protect the developing brain from general anesthesia-induced neuronal injury and possibly the long-lasting cognitive impairments that may ensue are urgently needed. In addition to other agents that have been shown to be effective in ameliorating general anesthetic-induced neurotoxicity such as L-carnitine, acetyl-L-carnitine, and 7-nitroindazole [32,57,58], the neuroprotective effect of minocycline was investigated in the present study.

Minocycline is an antibiotic effective against a broad spectrum of bacteria and can easily traverse the blood-brain barrier, probably because of its high lipophilicity [19-21]. Recently, minocycline has been reported to have other properties in addition to its antibiotic activity including neuroprotection [19-22,24,27,59]. One of the possible mechanisms underlying the neuroprotective effect of minocycline is its ability to inhibit microglial activation [21,59-61]. Microglial activation in response to neuronal insults enhances the clearance of necrotic and apoptotic neurons and the survival of surrounding healthy neurons. However, microglia activation can also be toxic to the CNS due to the associated release of free radicals and inflammatory cytokines [59,62,63]. Based on our previous microPET data showing general anesthesia related retention of FEPPA indicating microglial activation, we sought in the present study to assess the protective effects of minocycline using microglial activation as the metric.

The results presented here indicate that microPET imaging is capable of distinguishing differences in the retention of [18F]-FEPPA in the brains of rodents with a history of general anesthesia. This approach seemingly provides a minimally invasive way in which to monitor pathogenic processes associated with the developmental neurotoxicity caused by general anesthetics such as ketamine. The data presented here also suggest that minocycline can protect the neonatal brain from ketamine-induced neuronal damage, presumably-- at least in part-- by inhibition of microglial activation. That minocycline might be employed as a neuroprotective agent in pediatric patients and infants in need of general anesthesia should be seriously considered. However, much yet need to be described concerning the precise timing and dosing that would be appropriate for minocycline in the pediatric setting. Toward this end, further animal studies that focus on examining other neuroprotective mechanisms underlying the effects of minocycline would be helpful as would a thorough assessment of its potential adverse effects.

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