Epilepsy & Behavior 52 (2015) 180–186

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Benzodiazepines induce sequelae in immature mice with inflammation-induced status epilepticus

Keisuke Nakajima [a,b], Shinobu Hirai [a], Tomohiro Morio [b], Haruo Okado [a,*]

[a] Department of Brain Development and Neural Regeneration, Neural Development Project, Tokyo Metropolitan Institute of Medical Science, Japan
[b] Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, Japan

ARTICLE INFO

Article history:
Received 3 July 2015
Revised 9 August 2015
Accepted 10 August 2015
Available online xxxx

Keywords:
Status epilepticus
Children
Inflammation
Benzodiazepine
GABA-A receptor

ABSTRACT

Objective: Since benzodiazepines (BZPs) became clinically available for the treatment of status epilepticus (SE) in children, the incidence of neurological sequelae has increased. However, the cause–effect relationship is poorly understood. In this paper, we examined the effect of BZPs on an inflammation-induced SE (iSE) animal model.
Method: Inflammation was induced by injecting poly(I:C) (pIC 10 mg/kg, postnatal day 12–14), seizure was induced by injecting pilocarpine hydrochloride (PILO 200 mg/kg, postnatal day 15) into C57BL/6J mice, and the pIC + PILO mice were used as the iSE model (miSE). The GABA-A receptor agonist midazolam (MDL 0.5 mg/kg) was used to inhibit seizures. Sequelae were evaluated by performing behavior and immunohistochemical analyses in the chronic phase.
Result: The exploratory activity of mice in the miSE plus MDL group increased significantly, indicating that hyperactivity was newly induced by MDL in miSE mice. The contextual fear memory of the miSE mice was also significantly increased and that of miSE treated with MDL returned to the normal level. The parvalbumin-positive GABA neurons were decreased in number by pIC + PILO which was rescued by MDL. Apoptosis marker ssDNA-positive cells were increased by pIC + PILO which could not be rescued by MDL. Therefore, we propose that BZP-dependent therapy for SE needs to be rethought from the perspective of using other treatment approaches.

1. Introduction

Since benzodiazepines (BZPs) became clinically available for the treatment of childhood status epilepticus (SE), the incidence of acute death has fallen markedly. Indeed, BZPs are now the preferred drugs for treatment of childhood SE [1]. However, the increasing use of BZPs has led to a concurrent increase in the incidence of serious sequelae [2]. It is, therefore, tempting to conclude that these severe sequelae are caused by BZPs. To examine this hypothesis, we generated an animal model of inflammation-induced SE (iSE) and used it to examine the effects of a BZP, midazolam (MDL). We regard this model, which exhibits a combination of inflammation and seizure, as a suitable model for iSE because most childhood SE cases present with inflammation [3]. The majority of childhood iSE cases are of viral etiology. These infections induce the production of inflammatory cytokines, including IL-1β, which cause fever and seizures [4]. Here, we selected the TLR3 ligand, poly(I:C) (pIC), which mimics viral infection [5]. It works mainly via two pathways in the animal and human body. One is the IFN-β pathway that relates to the apoptosis. The other is the inflammatory cytokine pathway activating the IL-1β, which leads to the seizure [6]. It was reported that maternal immune activation with pIC decreases the PV-positive cells of postnatal mice. These mice display schizophrenic disorder-like behavior [7,8]. It is possible that pIC increases various cytokines, which impair the PV-positive GABA neurons, resulting in abnormal behavior. It is clear that pIC represents one part of the innate immune response to viral infection, and there are other pathways to elicit the inflammation by viral infection [9,10]. We also used pilocarpine hydrochloride (PILO). Pilocarpine hydrochloride is a muscarinic receptor agonist that acts mainly on the hippocampus region, CA1, and is often used as a model of temporal lobe epilepsy [11]. Pilocarpine hydrochloride also stimulates the hippocampal GABAergic interneurons, which control the severity of the seizures [12]. Therefore, we used a combination of pIC and PILO to induce iSE in our mouse model. The doses of pIC and PILO were decided by past studies [13–16]. Midazolam was then administered 1 h after PILO-induced seizure. To examine whether a period of MDL treatment is critical, we used single or multiple MDL injections during the acute phase, and induced sequelae (e.g., cognitive deficit or aberrant movement patterns) were evaluated during the chronic phase. The dose of MDL was decided by clinically used concentrations.

Abbreviations: BZP, benzodiazepine; pIC, poly(I:C); PILO, pilocarpine hydrochloride; MDL, midazolam; mMDL, multiple MDL; Ct, control; SE, status epilepticus; iSE, inflammation-induced SE; miSE, model of inflammation-induced SE; PV, parvalbumin; ssDNA, single-stranded DNA.

* Corresponding author at: Department of Brain Development and Neural Regeneration, Neural Development Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6, Kamikitazawa, Setagaya-ku, Tokyo, Japan. Tel.: +81 3 5316 3100; fax: +81 3 5316 3150.
E-mail address: okado-hr@igakuken.or.jp (H. Okado).

http://dx.doi.org/10.1016/j.yebeh.2015.08.012
1525-5050/

2. Methods

2.1. Animals

Mice (C57BL/6J) were housed under a normal light/dark (12 h/12 h) cycle and allowed access to water and food ad libitum. All studies conformed to the animal care regulations set down by the Tokyo Metropolitan Institute of Medical Science. We have obtained permission from an animal use committee.

2.2. Induction of inflammation and seizure

The time courses of experiments are shown in Figs. 1A and 2A, respectively. To generate the iSE model, mice received 10 mg/kg of poly(I:C) (Sigma-Aldrich) for 3 consecutive days (postnatal day 12 (P12)–14), followed by 200 mg/kg of pilocarpine hydrochloride (Wako) on P15 (group of pIC + PILO, n = 12). Postnatal day 15 (P15) corresponds to a human age of 3–4 years. Another group received PILO alone (group of PILO, n = 8), and a third received pIC alone (group of pIC, n = 9). Mice that received PILO also received a subcutaneous dose (1 mg/kg) of scopolamine methylbromide (Sigma-Aldrich) 30 min prior to the PILO injection to reduce the peripheral effects of the drug. Control (Ct) mice (group of Ct, n = 12) received normal saline at the time when the other mice received PILO. All injections were made intraperitoneally (i.p.) to animals. We observed these mice for 30 min after seizure onset. After that, we observed the mice intermittently until 5 h from seizure onset. Five to ten minutes after PILO injection, head nodding or forelimb jerk started, and after two hours the seizure involved the whole body; then after several hours, the seizure behavior waned. We could not find any obvious difference in the severity of seizure related to administration of pIC. We did not perform quantitative analysis. The mice were then subjected to various behavioral tests during the chronic phase of the disease (P30–40).

2.3. Treatment of miSE mice with MDL

For Experiment 2, we regard the mice treated with pIC and PILO as the model of iSE (miSE). Then, 1 h after the administration of miSE, the mice received either a single midazolam dose (Astellas Pharma) (group of miSE + MDL, n = 11) or multiple midazolam doses (group of miSE + mMDL, n = 12) to inhibit seizures. The mice received normal saline (group of Ct, n = 12), single MDL dose (group of MDL, n = 9), or multiple MDL doses (group of mMDL, n = 15) to compare the effect of the BZPs in the miSE. The single MDL dose was 0.5 mg/kg i.p., and multiple MDL doses were administered at 0.5 mg/kg i.p. on three consecutive days.

2.4. Behavioral testing

Behavioral tests were performed at P30–40. Mice were subjected to an open-field test, a Y-maze test, an elevated plus maze test, and a contextual fear conditioning test. The animals were monitored by motion capture software (CompACT VAS Ver.3.0, and EthoVision XT 8.5) paired with video cameras.

2.4.1. Open-field test

A field size was 50 × 50 × 40 cm. Each mouse was placed in the middle of the open field and allowed to explore the arena freely for 300 s × 6 sessions. The percent of time that mice stayed in the center (center staying %) was measured to assess anxiety. Moreover, total moving time (sec) and total moving distance (cm) were measured to assess the amount of movement [17].

2.4.2. Y-maze test

The three trough-shaped arms (95 mm in width, 395 mm in length, 120 mm in depth) were separated. A mouse was placed in one arm of the apparatus and was allowed to explore the maze for a period of 10 min. Arm choices were manually recorded during this time. Number of entries to the arm were measured and taken to reflect the level of anxiety. The alternation rate was attributed to working memory [18].

2.4.3. Elevated plus maze

The elevated plus maze test consisted of four arms situated at 90° angles to each other. Closed arms had 20-cm high walls. Open arms had a lip around the edge. At the start of the test, the mice were placed in the center of the arms. They were allowed to explore the maze for 10 min. The anxiety score was defined as time spent in the open arms (sec), and total moving time (sec) was measured to evaluate the amount of activity [17].

2.4.4. Contextual fear conditioning text

During the first day, mice were conditioned to the circumstance (cube box, brightness: 100 lx, tone: 65 Hz, odor: EtOH) by the electric shock (3 times, 0.2 mA, 1 s). The next day, mice were set into the same condition and an altered condition (triangle pole box, brightness: 30 lx, tone: 75 Hz, odor: none) to evaluate the contextual fear memory by freeze time %. We used the immature mice so that conditioned electric shocks were more mild (0.2 mA) than used in a previous study (0.5 mA) [19].

2.5. Brain section

After behavioral testing in chronic phase (postnatal day 30–40), mice were anesthetized deeply with pentobarbital and perfused with phosphate-buffered saline (PBS), pH 7.4, and subsequently with 4% paraformaldehyde (PFA) containing 0.2% saturated picric acid in PBS. Extracted brain tissues were fixed in the same fixative solution overnight and put in PBS containing 20% sucrose at 4 °C. After the brain was frozen rapidly on dry-ice, the tissue was sliced into 50-μm thick sections by Microtome (Yamato Kohki Industrial). Sliced sections were stored at 4 °C in the PBS until use.

2.6. Immunohistochemistry

Following incubation in HistoVT one solution (Nacalai tesque) to reactivate the antigens for 30 min at 70 °C, sliced sections were treated with 0.2% Triton X-100 in PBS containing 1% block Ace (Yukijirushi) for 30 min at RT and were incubated overnight at RT with primary antibodies in PBS containing 0.4% block Ace: Anti-NeuN (Millipore, 1 : 500), anti-ssDNA (Dako, 1 : 200), and anti-PV (Swant, 1 : 500); after washing with PBS 3 times, sections were incubated with secondary antibodies: antimouse 488 and antirabbit cy3 (Jackson Immuno Research). Nuclei were stained with DAPI/Topro-3. Evaluation utilized a FV500/FV1000 (Olympus micro systems). The PV- and ssDNA-positive cells in the dentate gyrus of the hippocampal formation were evaluated by averaging the findings of two individuals.

2.7. Statistical analysis

All data are expressed as the mean ± s.e.m. and were analyzed using one-way ANOVA with Dunnett's post hoc comparison. All the statistical analyses were performed using JMP10.0 (SAS Institute Inc.).

3. Results

3.1. Experiment 1 (exp1): pIC plus PILO to generate a mouse model of iSE

3.1.1. Result of open-field test in exp1

There were no differences in the amount of exploratory activity (as evaluated by total moving time and distance) between the PILO, pIC, and pIC + PILO groups and the Ct group (Fig. 1, B1/B2). The center staying time (%) for mice in the PILO and pIC + PILO groups was higher