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Jaewon Jeong April 09, 2019

Intrahippocampal Kainic Acid (IHKA)-induced Seizures in Mice: A Model of New Onset Refractory Status Epilepticus (NORSE)

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Abstract

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The consensus definition of New Onset Refractory Status Epilepticus (NORSE) is new onset of refractory status epilepticus without a clear acute or active structural, toxic, or metabolic cause. Patients with NORSE do not have active epilepsy or other preexisting relevant neurological disorder. Febrile Infection Related Epilepsy Syndrome (FIRES) is a subset of NORSE that requires febrile infection, with fever occurring between 2 weeks and 24 hours prior to onset of refractory status epilepticus. There currently does not exist an animal model to study NORSE/FIRES in detail. This study argues that intrahippocampal kainic acid (IHKA) is a valid model for NORSE/FIRES supported by pathological changes, behavioral manifestations, immune response, and electroencephalography data. Although previously viewed only as a model for Mesial Temporal Lobe Epilepsy (MTLE), the questionable existence of a latent period between the acute epileptic phase and chronic epileptic phase challenges this notion. This study aimed to characterize more thoroughly the features of IHKA that are common to NORSE. Our data suggested that IHKA is a valid model for NORSE/FIRES.

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Intrahippocampal Kainic Acid (IHKA)-induced Seizures in Mice: A Model of New Onset Refractory Status Epilepticus (NORSE)

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KEYWORDS: New Onset Refractory Status Epilepticus (NORSE), Febrile-Infection Related Epilepsy Syndrome (FIRES), Mesial Temporal Lobe Epilepsy (MTLE), Status Epilepticus (SE), Intrahippocampal Kainic Acid (IHKA)

Introduction

The intrahippocampal kainic acid (IHKA) injection in mice causes convulsive status epilepticus, prolonged seizures that last over two hours, and eventually, spontaneous recurrent seizures. Injection of kainic acid (KA) directly into the hippocampus of mice causes pathological changes and a massive immune response (Bouilleret 1999). Anatomical analysis of the hippocampus shows increased astrocytic and glial activation. Electroencephalography (EEG) shows interictal epileptiform discharges and electrographic seizures, the hallmark features that define epilepsy. The intrahippocampal kainic acid (IHKA) model has been found to be more advantageous than other epilepsy models such as systemic administration of kainic acid because it ensures that kainic acid is delivered focally to the hippocampus, and the site of brain to be injected with KA can be easily altered if needed (Bielefeld et al., 2017).

The pathological changes, behavioral manifestations, immune response, and electroencephalography data suggest that IHKA in mice could serve as a model for New-Onset Refractory Status Epilepticus (NORSE) and Febrile-Infection Related Epilepsy Syndrome (FIRES) in human. Patients with NORSE present with new-onset refractory status epilepticus without a clear acute or active structural, toxic, or metabolic cause. The patients with NORSE do not have active epilepsy or other preexisting relevant neurological disorder (Hirsch et al. 2018). Furthermore, the chronic epileptic phase follows the acute epileptic phase without a latent period. FIRES is a subset of NORSE that requires febrile infection, with fever starting between 2 weeks and 24 hours prior to onset of refractory status epilepticus. (Hirsch et al. 2018). FIRES is a rare and devastating epilepsy syndrome that typically affects school-age children. It remains poorly understood how the fever generates such unrelenting repetitive seizures and how the refractory status epilepticus affects the developing brain. Although there have been good

outcomes reported in some children diagnosed with FIRES, the majority of the children develop significant neurological complications including refractory epilepsy with cognitive deficits, a vegetative state, or death. In addition to routinely used anticonvulsants and CNS depressants, anesthetic comas and ketogenic diets have been used to manage young children affected by FIRES. Effective treatments to adequately control seizures and its devastating consequences are lacking in patients with FIRES.

Similar to the patients with NORSE, the mice, before the IHKA surgery, do not have any active epilepsy or preexisting relevant neurological disorder. The mice develop sudden status epilepticus after the injection of kainic acid into the hippocampus. Previous studies have shown that the IHKA injected mice develop chronic epileptic phase that directly follows acute epileptic phase without any delays, a key characteristic of patients with NORSE (Twele et al., 2015, Ndode-Ekane et al., 2013). Previous research has shown that the epilepsy developed via IHKA injection does not respond to many commonly used antiepileptic drugs (AEDs) such as levetiracetam and valproate at a low dosage, but only respond at a toxic dosage for humans, proving the refractory nature of the epilepsy (Duveau et al. 2016). These characteristics suggest that IHKA could be a model for NORSE/FIRES.

Neuronal excitability and epileptogenesis have been found to be greatly impacted by neuroinflammation. Increased activation of microglia, astrocytes and proinflammatory cytokines has been observed in patients with epilepsy (Choi et al. 2008). The Blood Brain Barrier (BBB) regulates entry of cells and molecules into the CNS and provides a distinctive niche protected from cells and molecules found in the general circulation that could be harmful to the brain. (Choi et al. 2008). Activation of key immunomodulators is related to increased permeability in the BBB. The compromised BBB allows infiltration of peripheral monocytes and

proinflammatory molecules into the brain. The intrinsic immune response in the brain to neurological insults can acutely promote recovery of the brain (Correale and Villa 2004), but infiltrative neuroinflammation can exacerbate the injury (Elenkov et al. 2005). Previous studies show robust immune responses in the brain decrease seizure threshold, enhance neuronal excitability, and regulate epileptogenesis. (Xu et al., 2013). This suggests that neuroinflammation and inflammatory pathways such as interleukin-1 receptor/toll-like receptor (IL-1R/TLR) may serve as a novel therapeutic target for treatment of epilepsy. Therefore, our overall goal is to establish IHKA as a model for NORSE and explore potential roles of neuroinflammatory pathways in the pathogenesis of epilepsy.

Materials and Methods

Animals

All mice were purchased from Jackson Laboratory (Bar Harbor, ME) including (1) C57BL/6J males and breeding pairs of (2) B6.129P-Cx3cr1tm1Litt/J (Cx3Cr1GFP/GFP transgenic mice) and (3) B6.129(Cg)-Ccr2tm2.1Ifc/J (Ccr2RFP/RFP transgenic mice). Cx3Cr1GFP/GFP mice were bred with Ccr2RFP/RFP mice to produce F1 animals heterozygous for each fluorescent protein (Cx3Cr1GFP/+:Ccr2RFP/+), which still enables sufficient fluorescence for detection. Cx3Cr1GFP/+ transgenic mice have a green fluorescent protein (GFP) allowing visualization of brain microglia. Ccr2RFP/+ transgenic mice have a monomeric red fluorescent protein (RFP) sequence replacing the coding sequence of the chemokine (C-C motif) receptor 2 allowing the visualization of monocyte recruitment.

Intrahippocampal Kainic Acid (IHKA) Surgery

Animals (~P40) were fixed into the stereotaxis with incisor bar and non-piercing ear bars securing the head into position. A 2cm incision was made over the midline of the scalp. The periosteum was moved away from the surgery site, and the skull was marked using the stereotaxic arm holding a sterile surgery marker for injection site into the right dorsal hippocampus, specifically the CA3 according to coordinates (in reference to Bregma) AP(anterior-posterior) = -1.7 mm, ML (mediolateral)= -1.7 mm, DV (dorsoventral)= 1.8 mm. Once marked, a hand drill was used to make a 0.5 mm hole in the skull for stereotaxic placement of a guide cannula. Once in place the 0.5 uL neurosyringe loaded with 150 nL of a 1mg/mL solution of freshly prepared Kainic acid dissolved in 0.9% bacteriostatic saline, or equivalent volume of saline (for controls) was injected into area CA3 of the hippocampus over a 3-minute

period. After the injection of kainic acid at right CA3/dorsal hippocampus (coordinates: AP -1.7 mm, ML -1.7 mm, DV -1.8 mm from bregma), cortical electrodes (coordinates: AP: -0.9 mm, ML: +/- 3.0 mm from bregma) and depth electrodes (coordinates: AP -1.7 mm, ML -1.0 mm, DV -1.3mm from bregma) were implanted. The rostral-caudal incision was then sutured closed using nylon black monofilament nonabsorbable sterile 4-0 USP 1.5 metric 3/8c 19mm in an interrupted suture placement method.

Post-Operative Care

Animals were placed in a clean recovery cage that has no conscious animals in the cage with them. Antibiotic and Bupivicane (local anesthetic) were placed on top of the surgical site to prevent infection and pain. The mice were be monitored every 15 minutes until the animals were able to walk. The mice were injected with 1mL of saline to prevent dehydration. A heating pad under half of the recovery cage were used to prevent hypothermia with the animal having the ability to move away from the heat when fully awake. Wet food in a portion cup was placed with the mice. The mice were checked daily for signs of lack of feeding, lethargy, lack of grooming, and spontaneous recurrent seizures.

EEG Recording

The animals were connected via implanted head mount/pedestal to the EEG acquisition system (Biopac). A wired cord was inserted into the pedestal to establish connection between the implanted electrodes and the EEG amplifiers. Animals were anesthetized very briefly using the open-exposure method of isoflurane. After animals were unconscious and no tail pinch response was evoked, they were quickly connected to the tethered system and placed into the modified video-EEG recording cages. These modified cages are plastic housing cages converted into

appropriate EEG monitoring cages with holes drilled for water bottle placement, ventilation and connection to the acquisition system. Video and depth EEG were recorded using a Biopac Systems MP-160 system with AcqKnowledge 5.0 software for acquisition and analysis. Potential seizure epochs were identified using the seizure detection algorithm with a criterion of 20 spikes per 10-second epoch, and then verified or rejected by visual inspection.

Immunohistochemistry (IHC)

The animals were sacrificed at 7 days post-surgery to be processed with IHC. Immunohistochemistry and fluorescence were performed on brains perfused with ice cold phosphate buffered saline, followed by 4% paraformaldehyde. Once fixed and cryoprotected, tissue was processed and sliced into 40 μm frozen sections using a freezing microtome. Tissue was incubated in primary antibody overnight, followed by horseradish-peroxidase secondary antibody and diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) visualization process with nickel II sulfate intensification. Primary antibodies for immunohistochemistry include anti-GFAP (glial fibrillary acidic protein) to visualize astrocytes and anti-IBA-1 (ionized calcium-binding molecule 1) to image activated microglia.

Results

Optimization of Kainic Acid dosage to decrease mortality rate (Figure 1.)

Kainic acid injection at dosage of 200 nl of 1 mg/ml caused status epilepticus in 80% of injected animals but also caused unacceptably high (50%) mortality rate. Decreasing the dosage to 150nl of 1mg/ml led to around 20% mortality rate while inducing status epilepticus consistently (73%). Therefore, 150nl (.15μg) was used in all subsequent experiment.

Acute Seizure post IHKA surgery

The mice were observed for 3 hours post IHKA surgery. The mice showed irregular behaviors upon emerging from anesthesia. First behavioral change was a pause, standing still with stiff whiskers and tonic tail. These behavioral arrests alternated with nearly continuous, repetitive spinning for over one hour. Clonic jerks, tonic posturing, loss of balance, violent jumps, and generalized tonic clonic seizures were also observed. These behaviors lasted at least 2-3 hours.

Electroencephalography shows interictal discharges and spontaneous recurrent seizure (Figure 2, 3)

Electrographic seizures were observed as early as 1 hour post-surgery (1 hour was chosen to allow the mice to recover from the surgery). By 7 days all animals exhibited interictal spikes and EEG seizures that lasted 10-60 seconds. Animals recorded once a week for the following three weeks continued to show spontaneous EEG seizures that increased only slightly in frequency. In contrast, one out of 4 animals injected intrahippocampal PBS exhibited interictal discharge but none of these controls exhibited seizures. One animal exhibiting interictal discharge may be due to the surgery itself, as EEG implantation could cause neuroinflammation and hyperexcitability in hippocampus (Balzekas et al., 2016).

Microglial activation, astrocyte level, and monocyte infiltration (Figure 4, 5, 6)

The microglial activation and astrocyte level were compared between the intrahippocampal PBS and the intrahippocampal kainic acid group. The mice with kainic acid injection had significant increase in microglial activation compared to the PBS group as demonstrated by IBA-1 staining (Figure 2. C and D; Figure 3. C and D). Astrocyte level did not differ greatly but there was a slight increase in astrocyte level in the kainic acid group demonstrated by the GFAP staining (Figure 2. A and B; Figure 3. A and B). Confocal image of the kainic acid injected brain were taken to visualize microglia and monocytes. Green fluorescent proteins (GFP) shows microglial activation and red fluorescent protein (RFP) shows monocyte infiltration (Figure 4. A and B).

Figures

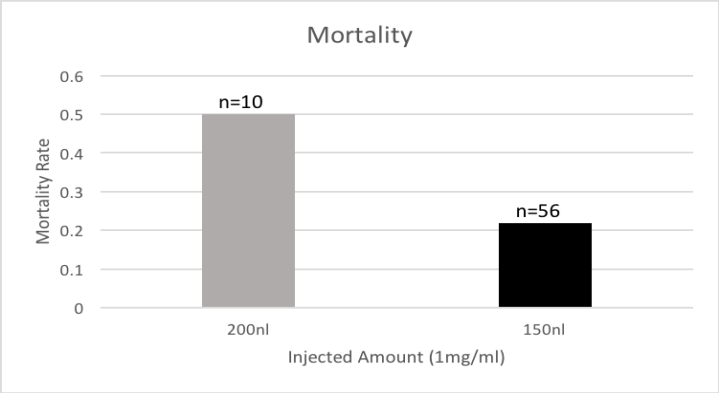

Figure 1. Mortality rate with different kainic acid dosages.

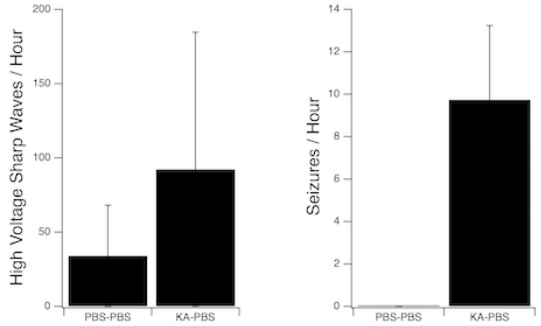

Figure 2. Left: High Voltage Sharp Waves (HVSW) in Intrahippocampal pbs (control) vs. Intrahippocampal kainic acid (experimental) at 7 days post intrahippocampal surgery. Right: Frequency of seizures at 7 days after intrahippocampal surgery.

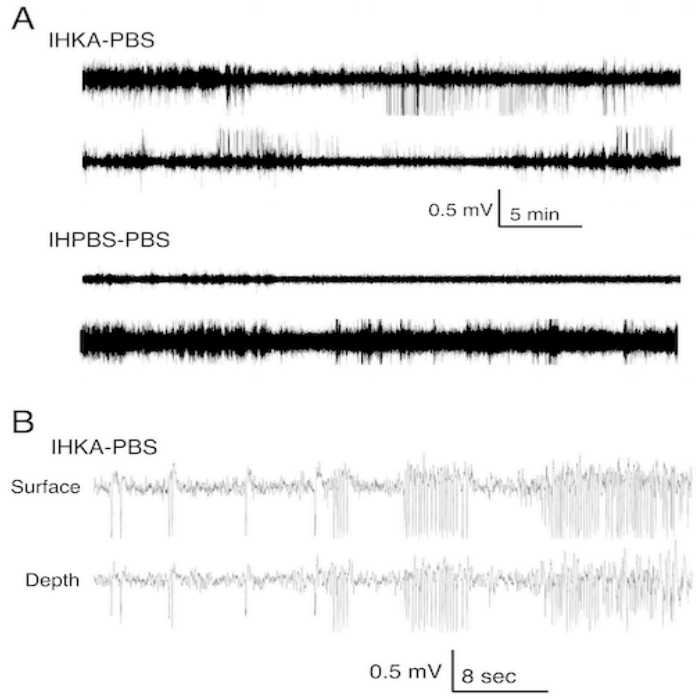

Figure 3. A: Raw EEG traces from two animals in IHKA and two animals in IHPBS. B: Top animal traces expanded comparing surface and depth electrodes.

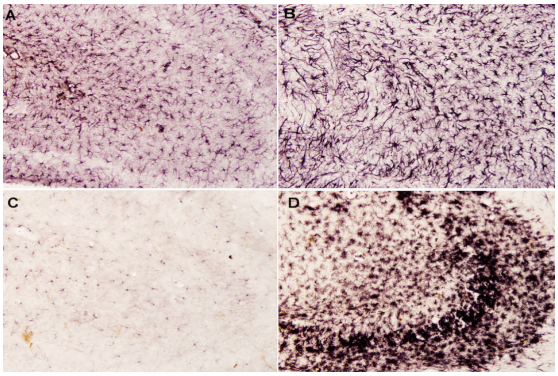

Figure 4. Picture of CA3 of the hippocampus. A. IHPBS-GFAP B. IHKA-GFAP C. IHPBS-IBA1 D. IHKA-IBA1.

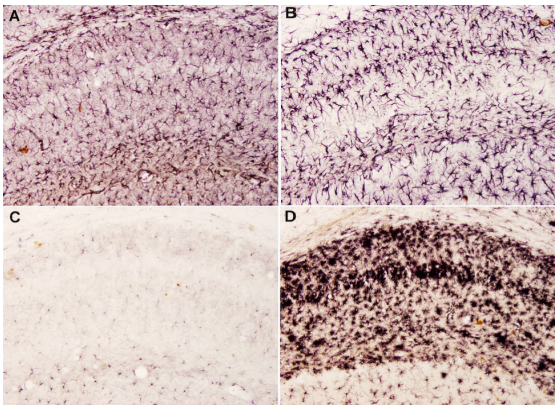

Figure 5. Picture of CA1 of the brain A. IHS-GFAP B. IHKA-GFAP C. IHS-IBA1 D. IHKA-IBA1.

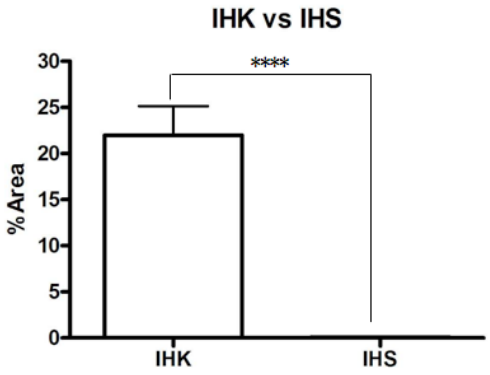

Figure 6. Quantification of IBA1 (CA1) (n=4)

Microglia was increased significantly

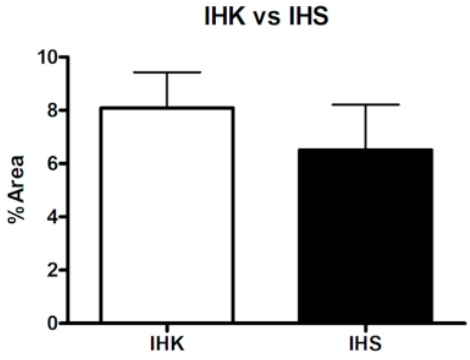

Figure 7. Quantification of GFAP (CA1) (n=4)

Astrocyte was increased but not significantly

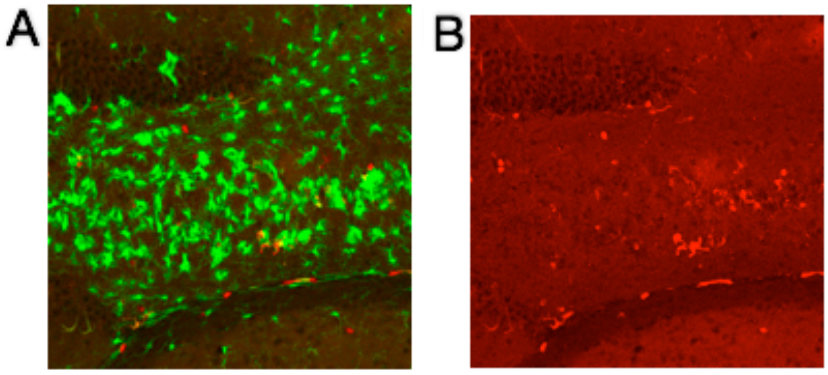

Figure 8. Confocal image of hilus. The GFP (green fluorescent protein) shows microglial activation. The RFP (red fluorescent protein) shows monocyte infiltration.

Discussion

The mice that underwent the IHKA surgery showed irregular behaviors that signified acute seizures. The mice showed tonic tail and spinning as common irregular behaviors and a few showed generalized tonic clonic seizures. Electroencephalography data showed spontaneous recurrent seizures developed directly following status epilepticus (Twele et al., 2015; Ndode-Ekane et al., 2013). Histology data showed increased microglial activation, astrocyte level and monocyte infiltration, indicative of neuroinflammation.

The IHKA model has historically been viewed as a model of MTLE (Lévesque et al. 2013). Mesial temporal lobe epilepsy (MTLE) is the most common form of focal epilepsy in adult (Falconer et al., 1964; Margerison et al., 1966). One characteristic of MTLE is progressive loss of cells with gliosis leading to hippocampal sclerosis with selective neuronal loss in CA1/CA3 of hippocampus (Berkovic et al., 1991). Injection of kainic acid into the hippocampus of mice results in similar morphologic characteristics of hippocampal sclerosis observed in MTLE patients (Riban et al., 2002). Also, in a MTLE patients, a delay exists between initial injury such as status epilepticus and occurrence of spontaneous recurrent seizures, therefore, existence of latent period between the acute and the chronic epileptic phase. Whether this latent period exist in the IHKA model has been questioned.

Data suggests that IHKA model could be a model of NORSE. Consistent with NORSE patients, the mice prior to the IHKA surgery had no active epilepsy or other preexisting relevant neurological disorder. The epilepsy developed by this surgery presents new-onset of refractory status epilepticus without a clear prior structural, toxic, or metabolic cause (Hirsch et al. 2018). The mice after the IHKA surgery develop status epilepticus acutely, followed by spontaneous recurrent seizures without a latent period, which is also consistent with the NORSE patients.

Validating a mouse model of NORSE will facilitate investigation into the underlying mechanisms and identification of novel therapeutic treatments. Continuing ongoing studies in our lab aim to further validate IHKA as a NORSE model.

A key finding from this study is the possibility of using inflammatory pathways as therapeutic targets to treat patients with NORSE. Increased microglia activation, astrocyte level, and peripheral monocyte infiltration all suggests that neuroinflammation occurs. One of the primary immune pathways activated following tissue injury and prolonged seizure is IL-1R/TLR pathway (Vezzani et al., 2013). Activated IL-1R/TLR pathway is proinflammatory affecting the neuronal network hyperexcitability (Vezzani et al., 2007). We have tried to measure interleukin levels in the IHKA models. Although elevated level of interleukin-1 beta was detected in the animal serum, we were hesitant to make conclusions due to technical difficulties that rendered comparisons to control unreliable. Further studies will address this question as well as investigation of other proinflammatory pathways.

Finding treatments for NORSE is critical as an effective treatment there does not exist. Notably, available evidence suggests that IL-1R/TLR pathway is a particularly promising therapeutic target for NORSE/FIRES. Anakinra, a human recombinant form of interleukin-1R antagonist (IL-1Ra), has been used successfully as a treatment for children who present with FIRES, a subtype of NORSE (Kenney-Jung et al., 2017). This further supports anti-inflammatory treatments broadly, and IL-1R/TLR specifically, as avenues for treatment of NORSE. However, anakinra is not widely used for treatment of NORSE or FIRES (van Baalen et al., 2017). We believe anakinra is potentially one of the most effective treatments that could be used, and we plan to test the effects of anakinra in the IHKA model. If anakinra is found to be effective in IHKA, this would further support both IHKA as a model of NORSE and a critical role of

neuroinflammation in this form of epilepsy, as well as indicating anakinra as a possible first line of treatment for NORSE patients.

In summary, our studies indicate IHKA could be an important new model for NORSE, for which no other model exists. This will give scientists and clinicians opportunities to explore NORSE in more detail to understand its pathogenesis. Furthermore, our data indicate that excessive neuroinflammation could have an important role and be a viable therapeutic target for NORSE and other epilepsy. Our future work will build on this project to open new avenues in epilepsy research and therapy development.

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