Modulation of Pentylenetetrazol Induced Neuronal Circuit Epilepsy by p2y Purinoreceptor 3 (p2y3) in Sprague Dawley Rats

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ABSTRACT

Epilepsy is a complex neurological disorder which causes abnormal electrical impulses in brain. The role of P2Y purinergic receptor in epilepsy is less known. NF45, a P2Y antagonist receptor, plays a crucial role in pentylenetetrazol (PTZ) kindled epilepsy. This study assessed the locomotion and hippocampal levels of mitochondrial complexes (IV, II, and I) in animal model. The experiment also assessed Thiobarbituric acid-reactive substance (TBARS), mean kindling score, hypertension, anxiety, discrimination ability (SNSE), learning, and memory in eight groups of rats. PTZ – kindling rats showed poor motor activity. PTZ-kindling rats showed increase in emotional tension, anxiety, learning and memory, pro-inflammatory mediators (IL-1β and TNF-α) in hippocampal tissue, neuronal damage (increased sNSE), mitochondrial dysfunction, and oxidative stress. PTZ-kindling augmented TBARS and reduced GSH and CAT. PTZ-kindling significantly decreased locomotion, memory and learning. Neuronal damage and hippocampus swelling were reversed with NF45 in a dose dependent manner. P2y agonist, methylene - ATP, significantly reduced the positive effect of NF45. P2y3 receptors had a significant role in epilepsy kindling. This mechanism has potential in epilepsy therapy and future research.

INTRODUCTION

Epilepsy is caused by abnormal discharges of neurons. It has a global prevalence of more than 5%. It is the third commonest neurological illness next to Alzheimer’s disease and stroke (Beheshti et al., 2013). Symptoms include recurrent seizures, cognitive dys function, memory impairment and attention dispersion. Epilepsy is known for chronic seizures and it can be fatal. At cellular level, there is neuronal death, mitochondrial damage, excotoxicity and oxidative stress. Mitochondrial dysfunction and oxidative stress play a key role in its pathophysiology.

The epileptic seizures can be kindled by repeated subconvulsant stimulus (Kumar et al., 2014). Kindling results due to functional alterations in neural circuit induction.

Pentylenetetrazol (PTZ) is the commonest drug used to induce seizures to study the effectiveness of anti-epileptic drugs in animal model. PTZ induces kindling and helps understand epilepsy.

A powerful endogenous ligand, Adenosine Triphosphate (ATP), activates P2Y receptors. These receptors have trimeric ligand gated-ion channels (Kernan et al., 2012). They create receptors in the form of homomeric or heteromeric by assembling of 3P2Y-7 (P2Y 1-7) receptors. During inflammatory conditions like nociception and neuroinflammation, the P2Y along with its ligand ATP plays an important role. ATP and P2Y receptors are being researched for many diseases and drug development.

Tripolymer (P2Y3R) associated with epilepsy is found in the peripheral nervous system and central nervous system, hippocampus, dorsal root ganglion, and other parts of the brain (He et al., 2021). P2Y increases the membrane permeability to calcium (Ca2+) and potassium (K+). It also causes increased neuronal excitability, inflammatory mediation, and neuropathic pain. P2Y3RS are increased in neuron cell bodies and dendrites in temporal lobe epilepsy. However, its role in kindling epilepsy, learning, memory, anxiety, emotional stress, mitochondrial malfunction, and inflammation is not well understood. Estrogen has been
found to interfere with neuroinflammation, memory, activation of microglial and learning (Wang et al., 2008). Therefore, male rats have been chosen for our study. The study aimed to investigate the pentylenetetrazol (PTZ) induced-kindling in Sprague Dawley rats. It also examined the effect of NF54 on P2Y3 receptor activity. This model could be the first evidence of the roles of P2Y3 and NF45 in pentylenetetrazol (PTZ) induced-kindling in rats.

MATERIALS AND METHODS

Drugs and chemicals
All the chemicals were of analytical grade (AR), and were prepared freshly before use. NF45 and a, b methylene-ATP were procured from Tocris Bioscience, China (Müller, 2015). The enzymatic ELISA kits for Neuron-specific Enolase Antibodies (sNSE), IL-1β, and TNF-α were procured from Invitrogen: Thermo Fisher Scientific. Inc, China. All other chemicals were purchased from Sigma-Aldrich, Hong Kong, China.

Animals
This research was approved by General Hospital of Ningxia Medical University animal ethical committee (Approval No. GHNMU2021-0325). Sprague Dawley adult male rats (weight 250-300g) were housed under the experimental conditions with food, water, temperature (25°C), light (light cycle as 12 h dark and 12 h light), and humidity. Suffering was minimized.

Surgical procedure
Pentobarbital sodium (Nembutal) 40g/kg was used as anesthetic for the animals. Using a stereotaxic surgery, a 10 mm steel guide cannula was inserted in right lateral ventricle of the brain. The cannula was implanted 1.6mm to the middle and 3.4 mm ventrally. A single injection of 3% xylocaine and 2% epinephrine was given. The canula was fixed using cell dentate acrylate, and sealed (Burnstock, 2015; Bele and Fabbretti, 2015). Each animal had the recovery period of 10 days.

PTZ- induced propellent
PTZ (60 mg/kg dissolved in ACSF) was administered to the rats on alternate days to induce kindling. The animals were observed in glass box alone for 30 min. It was continued for 35 days. Modified Racine scale was used to grade intensity of convulsions: 0 for no response; 1 for ear and facial cramps; 2 for myoclonic jerks without rearing; 3 for myoclonic jerks with rearing; 4 for change into position; 5 for change into supine position (Zhou et al., 2016). PTZ injections were stopped for animals with score 5 and they were assigned for comparison purposes. All animals (including score 5) were administered PTZ at a sub convulsive dose (30 mg/kg) during the re-challenge test at the end of the trial to confirm the kindling persistence (Zhou et al., 2016; Bjorling and Wang, 2001).

Behavioural parameters
Assessment of PTZ- induced kindling
To assess the PTZ-induced kindling, the number of days taken for stimulation of each stage in each group was compared with the mean kindling days. The Rotarod apparatus was used to measure grip strength and motor function. The rats were placed individually on the rotarod on day 0 at a speed of (25 rpm) and again on day 35 at the same speed (on the last day of PTZ injection) (Racine 1972; Soni et al., 2015). Each rat, the average fall-off time was recorded. Each rat was subjected to three trials with a 5-min break and a 3-min cut-off time.

PPEN- field test
A wooden box (200Lx200Wx50H) with 25 equal squares was used. On day 35, the rat was placed in the centre of the box, and absolute values of the following variables were scored after 5 min: the number of central squares crossed, faecal boli, and the total number of squares crossed (Zhen et al., 2014). The equipment floor was cleaned after each use before taking in another rat.

Elevated plus maze (EPM)
EPM is used to assess anxiety-related behavior in animal models. The wooden apparatus consisted of a shaped maze elevated 100 cm above the floor with two oppositely positioned closed arms (10 cms), two oppositely positioned open arms (50 cms), and a center area. A raised ledge of 1mm thickness and 5mm height was created to prevent the rats from slipping off (Zhen et al., 2014). The central open platform connected all four arms. On day 35, each rat was positioned in the central platform facing an open arm. The number of entries and duration spent inside the open arms were recorded for each rat. This was correlated with rats’ anxiety.

Task for object recognition (ORT)
An open-field apparatus was used for the object recognition task on day 36. Rats were placed in the apparatus to explore for 2 min. The first trial was started after 24 h with two identical objects (DO1 and DO2) for 3 min. Different colour, textures, and materials (wood and plastic) were offered to the animals (Deshmukh et al., 2009). After 60 min, the second sample experiment was conducted with one familiar (DO1) and one novel object (NO) for 3 min. To avoid the location or object’s possible bias, several combinations of the two objects were used. To
avoid the olfactory clues to next animal, the apparatus was cleaned after every use. In each experiment, the amount of time each animal spent exploring two different objects was recorded. Exploration is defined as contacting an object with the nose or directing the nose towards an object at a distance of less than 2 cm. The time spent examining DO1 and NO was compared to the time spent in discriminating between familiar and novel objects.

Morris water maze (MWM) test

A circular water tank (60 cm H and 190 cm W) was used. The water temperature was maintained at 25 °C. Four quadrants (D, S, M, V) were divided and labelled in the water tank. A colourless escape of 10 cm diameter was created 5 cm below the water surface in the v quadrant. This was the target quadrant. From day 37 to day 40, a 4 days acquisition trial was performed (Zhen et al., 2014). Four inter trial sessions were conducted with 5 min break. In each trial, the rat was placed in a different quadrant and allowed to reach the escape platform. If the rat did not locate the platform within 120 sec (the cut-off period), it was gently guided to the escape. On the fifth day, a retrieval experiment was undertaken. The escape platform was removed in this. The crossings over the former platform position, and the time spent in the target quadrant were recorded (day 41).

Collection of samples

PTZ re-challenge test was conducted on day 42. Each rat was killed on day 43. Four ml of blood was taken from each sedated rat via heart puncture. The hippocampus was dissected and the rats were decapitated.

Estimation of IL-1β and TNF-α

The dissected hippocampus was homogenised with T-PER buffer containing protease inhibitors. The lysates were centrifuged and the supernatant was collected for ELISA analysis. The concentrations of IL-1β and TNF-α were measured using the ELISA kit according to the manufacturer’s instructions.

Estimation of thiobarbituric acid-reactive substance (TBARS)

The hippocampus homogenate was used to assess lipid peroxidation. Using a homogenizer, the tissue homogenate was brought to the ratio of 10 ml of phosphate buffer (pH 7.2) to 2 ml of wet tissue. To 0.1 ml of the homogenate, 1.5 ml of thiobarbituric acid (0.8 %) 1.5 ml of acetic acid (20%), and 0.2 ml of sodium do decyl sulphonate (8.2 %) were added. The solution was mixed with 5 ml of distilled water, and heated at 96°C for 60 min. This mixture was added with n-butanol, pyridine and 1 ml of distilled water, and was vigorously agitated. The resulting mixture was centrifuged at 4000 rpm for 15 min. The absorbance of the organic layer was measured using 532 nm spectrophotometer against a reagent blank (all reagents except homogenate). The malondialdehyde (MDA) equivalents of the sample were estimated using the extinction coefficient 1.56 X 105 M-1 cm-1.

Estimation of catalase (CAT) activity

The chance approach was used to determine CAT’s activity. A 10% W/V homogenate of the hippocampus was made in phosphate buffer. The homogenate was centrifuged, and the supernatant was utilised to conduct the enzyme test. Test cuvette comprised of Tris PH 7.0, 10 Mm H2O2, and (50 Mm) EDTA (5M M Buffer). Reference cuvette comprised of distilled water and Tris EDTA. Both cuvettes were incubated at 37°C for 10 min (Deshmukh et al., 2009). The test homogenate was added to the reference and test cuvettes. Catalase was assessed using the rate of dissociation of H2O2. The rate of change (per min) of absorbance was measured at 240 nm for 4 min. The catalase activity was calculated using a molar extinction coefficient of 4.36 Mm-1 cm-1.

Estimation of reduced glutathione (GSH)

GSH assay was performed in hippocampus tissue homogenates per Moron. 600 ul homogenate was added with 100 ul 5% trichloroacetic acid and centrifuged at 2000 rpm for 15 min. Separated supernatants were diluted in 0.51 M sodium phosphate buffer (PH 7.0). Then, we added 2.0 ml of 0.6 mM 5,5 Dithiobis (2-Nitrobenzoic acid). The coloured complex produced by GSH AND DTNB was measured spectrophotometrically at 412 nm against a reference cuvette containing 0.1 ml of 5% TCA. A GSH standard curve was constructed for each experiment. The level of GSH was expressed as GSH/MG protein (Luck, 1965).

Mitochondrial complex I, II and IV estimations

Hippocampus tissue was homogenized using the isolation buffer with EDTA (325 mM maltose, 95 mM sucrose and 1% EDTA). At 13,000 x g, the homogenate was centrifuged for 5 min at 4°C. The pellets were re-suspended with the isolation buffer with EDTA, and again
centrifuged at 13,000xg for 5min. The final supernatant solution was transferred to a new container and treated with isolation buffer with EDTA. It was centrifuged for the third time at 13,000xg for 10min. The final solution was re-suspended with the isolation buffer, and the Lastly, rat brain mitochondria were isolated (Moron et al., 1979).

**Assessment of complex I (NADH dehydrogenase) complex**

This method involves reduction of cytochrome C and catalytic oxidation of NADH to NAD+. The NADH dehydrogenase was spectrophotometrically measured. The reaction mixture comprised of 10.5mM cytochrome C glycoll glycine buffer. This reaction mixture was added with requisite amount of extracted mitochondria to initiate a reaction. Absorbance was assessed at 550nm for 2min (Berman and Hastings, 1999).

**Assessment of complex II (succinate dehydrogenase) activity**

It was measured spectrophotometrically using artificial electron acceptor potassium ferricyanide. Oxidation of the succinate was done with the reaction mixture (0.5M phosphate buffer with PH 9.0, 7.1 % BSA, 0.9M succinic acid, and 0.09 M potassium ferrocyanide). The reaction was started by adding solubilized hippocampal mitochondrial cells, and the absorbance was monitored at 480nm for 5 min (Berman and Hastings, 1999).

**Compound IV (cytochrome oxidase) activity evaluation**

In the hippocampal mitochondria, the activity of cytochrome oxidase was assayed. The mixture of the assay contained 0.5mM cytochrome C (reduced form in 95mM buffer of phosphate). The reaction was initiated by adding the hippocampal mitochondrial extract in solubilized form to the mixture. The absorbance was measured photometrically at 550nm for 5 min (Sottocasa et al., 1967).

**Experimental protocol**

The animals were segregated into 8 groups with 12 rats in each group(12/group). Same persons handled the treatments for all groups of animals. Infusions were given via the surgically implanted cannula in the rats brains.

** Shan method for group I**

The cannula was implanted surgically in the animals brains. No other treatments were given. Animals were assessed for behavioural parameters from day 35 onwards (Taiwe et al., 2015).

**Group II – Vehicle control (ACSF)**

7 µl of artificial cerebrospinal fluid was infused from day 1 to day 42 via the surgically implanted cannula. The animals were assessed for behavioural parameters from day 35 onwards.

**Group III – NF45 /SE**

The rats were administered NF45 (90nM prepared with ACSF) from day 1 to the final day of experiment (day 42) using the cannula. They were assessed for their behavioural parameters from day 35 onwards.

**Group IV- Pentylene tetrazole (PTZ) kindling**

PTZ (60 mg/kg) infusion was started from day 1 till kindling occured (max. 35 days). They were assessed for behavioural parameters from day 35 onwards.

NF45 dose 1, 2, 3 for group V, VI and VII, respectively

PTZ was infused from day 1 till kindling occured (max. 35 days). The rats were infused with 7µl of NF45 in three separate dosages (30Nm, 60nM, and 90Nm) from day 1 till the last day (day 42). They were assessed for behavioural parameters from day 35 onwards.

**Group VIII- α-β-methylene- ATP + NF45 (dose 3)+ PTZ**

The rats were infused with 7µl of alpha beta methylene ATP, PTZ 90Nm, and NF45 from day 27 to the end of the experiment. They were assessed for behavioral parameters from day 35 onwards.

**Statistical analysis**

The results were expressed as mean ± S.D. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple range test. P≤0.05 was considered to be statistically significant.

**RESULTS**

**Effects of NF45on the mean kindling score**

The NF45, vehicle control, sham control did not show any significant change in parameters. NF45, a P2Y3 antagonist, had an effect on the mean score of PTZ-kindling. PTZ kindled rats demonstrated generalised tonic clonic seizures (GTCs) in stage 5 seizure by day 23. PTZ kindled mice with NF45 (dosage 30nM) reached stage 5 on day 35. Higher dosage of NF45 (60nM) delayed response, and the rats reached stage 4 on day 35. Further higher dose (90nM) treatment caused rats to reach stage 4 or 5 on day 35. The mean kindling score was reduced by NF45. The three doses of NF45 increased the number of days required for the activation of various stages of PTZ kindling. In contrast, three doses of NF45 increased the time required for PTZ-induced GTCs. Alpha beta methylene ATP therapy completely eliminated GTCs (Fig. 1).
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Fig. 1. The P2Y3 antagonist NF45 has an effect on the mean kindling score of PTZ-kindling animals. (A) Seizure stage for each group. (B) Days of stimulation for each group at various phases. (C) Each group’s average kindling score.

Effect of NF40005 on motor using rotarod test

Compared to sham control, the PTZ kindling lowered the motor activity. NF45 significantly increased motor activity. Trisodium further eliminated the effect of PTZ-kindling (Fig. 2A).

Effect of NF45 on locomotion and emotional tension using an open field test

As compared to the sham control animals, the PTZ kindling animals showed reduction in locomotion (noted as number of squares crossed reduced) and increased in emotional tension (increased in defecations). NF45 reversed these changes and trisodium was significantly reduced (Fig. 2B).

Effect of NF45 on anxiety using elevated plus maze

PTZ-kindling caused a significant increase in number of entries, and time spent in closed arms. There was a reduction in number of entries and time spent in open arms as compared to the sham control animals. Daily dose of NF45 increased in number of entries, and time spent in open arms, and decreased these in closed arms. Treatment with alpha beta methylene ATP enhanced NF45 effects (Fig. 3).

Fig. 2. The P2Y3 antagonist NF45 has an effect on the mean kindling score of PTZ-kindling animals. (A) Seizure stage for each group. (B) Days of stimulation for each group at various phases. Group I, No treatment; Group II, infused with artificial cerebrospinal fluid; Group III, administered with NF 45; Group IV, administered with PTZ; Group V, administered with PTZ+30nm NF45; Group VI, administered with PTZ+60nm NF45; Group VII, administered with PTZ+90nM NF45; Group VIII, α-β- methylene-ATP+90nM NF45+PTZ.
The P2Y3 antagonist NF45 has an effect on the anxiety behaviour of PTZ-kindling animals. (A) The amount of time spent in open and closed arms. (B) Frequency in the open and closed arm. For details of groups, see Figure 2.

**Effect of NF45 on discrimination using an object recognition task**

In test 1 (T1), the rats were exposed to 2 familiar objects (DO1 and DO2) and the exploration time was found similar in all groups. In test 2 (T2), rats were exposed to one familiar and one unknown object. PTZ kindling animals did not distinguish between familiar and novel objects. NF45 increased the rats’ discrimination between familiar and novel object and increased the time spent with novel object. These were abolished further by trisodium (Fig. 4).

**The effect of NF45 on learning and memory using the Morris water maze**

Sham control animals showed reduction in escape latency time (ELT) on day 4 (day 40) as compared to day 1 (day 37). PTZ kindling mice showed an excessive rise in ELT on day 1 as compared to day 4. NF45 lowered the ELT in PTZ kindling rats on day 4. Alpha beta methylene ATP prevented increase in ELT. PTZ kindling animals demonstrated a decline in normal memory. This was assessed by the time spent in the target quadrant and the number of platforms crossed on day 5 (day 41). The PTZ kindling spent more time in the target quadrant (V). NF45 and, more so, alpha beta methylene ATP significantly reduced the time spent in the target quadrant (Fig. 5).

**Effect of NF45 on hippocampal IL-1B and TNF-α levels**

The PTZ kindling animals had higher hippocampus IL-1B and TNF-alpha levels compared to sham-controlled...
NF45 reduced hippocampus 1L-1B and TNF levels. Alpha beta methylene ATP brought the levels to negligible (Fig. 6).

**Effect of NF45 on hippocampal sNSE, TBARS, CAT and GSH levels**

PTZ-kindling significantly increased hippocampal NSE, TBARS, and decreased hippocampal CAT and GSH. Reverse was observed in sham-controlled animals. The continued injection of NF45 in PTZ kindling rats reduced the quantity of sNSE and TBARS and increased hippocampal CAT and GSH. It was also eliminated by using alpha beta methylene ATP as a therapy (Fig. 7).

**Effect of NF45 on hippocampal mitochondrial complex I, II and IV levels**

PTZ-kindling significantly reduced hippocampal mitochondrial complex (I, II, and IV) levels as compared to the sham controls animals. NF45 with PTZ-kindling increased the hippocampal mitochondrial complex (I, II, and IV). These were significantly reduced on treatment with alpha, beta methylene ATP (Fig. 8).

![Fig. 6. Effect of the P2Y3 antagonist NF45 on learning and memory of PTZ-kindling animals, as assessed on Morris water maze. (A) PTZ impaired learning of rats and NF45 mitigated this damage in PTZ-kindling animals. (B, C) In a dose-dependent manner, NF45 raised the TSTQ and number of crossings in the platform area of PTZ-kindling animals on day 5. For details of groups, see Figure 2.](image)

![Fig. 7. Effect of the P2Y3 antagonist NF110 on PTZ-kindling animals' hippocampal IL-1β and TNF-α levels. (A) In PTZ-kindling animals, NF45 significantly reduced hippocampal IL-1β. (B) In PTZ-kindling rats, NF110 dramatically lowered hippocampus TNF-α levels. For details of groups, see Figure 2.](image)
DISCUSSION

PTZ (60 mg/kg on alternate days) significantly enhanced pro-inflammatory mediators (raised TNF-alpha and IL1β and IL6), mitochondrial dysfunctions, brain damage, discrimination ability, memory, as well as increased emotion tension, learning, locomotion, and learning ability (Taiwe et al., 2015). NF45 treatment corrected motor activity, locomotion, memory, learning, neuronal damage, oxidative stress, and hippocampal inflammation. NF45 corrected and greatly reversed by P2Y agonist, alpha beta methylene ATP.

Our study found that PTZ kindling reached stage 5 in 23 days. PTZ treatment caused epileptic seizures between day 23 and 27. NF45 prevented induction after 35 days (Vezzani et al., 2008). The P2Y agonist, abolished the beneficial effects due to NF45. The P2Y3 significantly increased temporal lobe epilepsy. The P2Y3 receptors sped up continuous repeated firing upon activation. Upregulation of P2Y3 receptor expression has been found in both epileptic humans and rats (Wang et al., 2015). According to our findings, NF45, a P2Y3 antagonist, significantly reduced PTZ kindling.

The rot rod test was used to evaluate the motor function and grip strength of the rats. The open field test was used to evaluate animal movement and emotional tension Racine (1972). Elevated Plus Maze has been used for assessment of anxiety in animal model by many researchers. Morris water maze has been used for the assessment of learning and memory in animals (Soni et al., 2015). The motor function, locomotion, anxiety, grip strength, emotional status, learning and memory was impaired by the administration of NF45 and the P2Y receptors. The P2Y receptors are known for functions like co-transmission, trophic signaling, and neuro-modulation (Zhen et al., 2014). P2Y receptors have been linked with various neurological conditions; Alzheimer’s disease, multiple sclerosis, depression, and anxiety. The study assessed the learning and memory, motor strength, grip, anxiety, and P2Y receptor modulation with NF45.
The pro-inflammatory cytokines (TNF-α and IL-1β) have pro-epileptic properties and play a role in the induction and maintenance of seizures. These cytokines lower the seizure threshold to cause sporadic spontaneous epilepsy. The P2Y receptors have also been linked to other inflammatory mediators as prostaglandins, sympathetic amines, and bradykin. NF45 suppressed P2Y3 receptors by preventing PTZ-induced hippocampal inflammation.

Seizures were caused by free radicals, which were previously thought to be a by-product of regular cellular metabolism. Lipid peroxidation is caused by a decline in the body’s endogenous antioxidant system, which attacks phospholipids in the cell membrane, as well as an increase in free radicals.

**CONCLUSION**

PTZ kindling caused impaired behavior such as learning, memory, motor activity, hippocampal inflammation, and oxidative stress. NF45, a strong P2Y3 antagonist, reversed the effects of PTZ-kindling. The administration of P2Y agonist, trisodium completely abolished the beneficial effects of NF45. Thus, P2Y3 receptors and antagonists are a potential therapeutic mechanism for developing newer drugs for epilepsy. We recommend further research to understand the drug’s characteristics, and mechanisms in the anti-epileptic process.

**ACKNOWLEDGEMENT**

None.

**Funding**

None.

**IRB approval**

This research was approved by General Hospital of Ningxia Medical University Animal Ethical Committee (Approval No. GHNMU2021-0325)

**Disclosure statement**

The authors report no competing interests.

**Data availability statement**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**Statement of conflict of interest**

The authors have declared no conflict of interest.

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