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Anticonvulsant effect of neural regeneration peptide 2945 on pentylenetetrazol-induced seizures in rats

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ABSTRACT

Neuron regeneration peptides (NRPs) are small synthetic peptides that stimulate neural proliferation, migration, and differentiation with no apparent toxicity and high target specificity in CNS. The aim of this study was to investigate the effect of NRP2945 on seizure activity induced by pentylenetetrazol (PTZ) in rats. Using behavioural assessment and electrocorticographical recordings, the effects of different doses of NRP2945 (5–20 μg/kg) were tested on seizure attacks induced by PTZ injection. In addition, the effect of NRP2945 was evaluated on the production of dark neurons and expression of $GABA_A$ receptor α and β subunits and GAD-65 in the hippocampus and somatosensory cortex of the rat brain. Intraperitoneal injection of NRP2945 at 20 μg/kg prevented seizure attacks after PTZ injection. NRP2945 at doses of 5 and 10 μg/kg significantly decreased the total duration of seizure attacks and reduced the amplitude, duration and latency of epileptiform burst discharges induced by PTZ. In addition, the peptide significantly inhibited the production of dark neurons in the hippocampus and somatosensory cortex of epileptic rats. NRP2945 also significantly increased the expression of $GABA_A$ receptor α and β subunits and GAD-65 in the hippocampus and somatosensory cortex compared with PTZ treated rats. This study indicates that NRP2945 is able to prevent the seizure attacks and neuronal injuries induced by PTZ, likely by stimulating $GABA_A$ and GAD-65 protein expression and/or protecting these components of GABAergic signalling from PTZ-induced alteration. Further studies are needed to elucidate the potential role of NRP2945 as an antiepileptic drug.

1. Introduction

Neural regeneration peptides (NRPs) are small synthetic peptides that promote proliferation, migration, differentiation and survival of neural precursors and neural stem cells under pathological conditions affecting the central nervous system (Gorba et al., 2006; Singh et al., 2010). The discovery of NRPs dates back to experimentation with a newly developed in vitro model of traumatic brain injury (Sieg et al., 1999). Administration of biochemically fractionated cell culture supernatant derived from hippocampal organotypic tissue cultures (OTCs) led to a neuronal bridge formation in thalamocortical OTC co-cultures within 3–4 days (Landgraf, 2005). The first completely identified gene that encodes for bioactive peptides displaying potency for neuronal survival-promoting activity was experimentally shown to be encoded on chromosome 12 in mice and is coding for a 135 amino acid long protein (Gorba et al., 2006). NRP2945 derives from the human NRP gene located at position 7q31.35 also known as calcium- dependent activator of protein secretion 2 (CAPS2; Sadakata et al., 2004) and represents amino acid position 40–50 within the CAPS2 sequence (Speidel et al., 2003). NRPs have both neuroregenerative and anti-inflammatory effects (Gorba et al., 2006; Landgraf, 2005; Sieg and Antonic, 2007). Several studies using human primary brain cells, human embryonic neural stem cells, and animal cells subsequently confirmed that NRPs at

Abbreviations: DAB, 3-3′-diaminobenzidine; NRPs, neuron regeneration peptides; OTCs, organotypic tissue cultures; PFA, paraformaldehyde; PTZ, pentylenetetrazol; PBS, phosphate buffered saline.

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sub-nanomolar concentrations have both neuroprotective and anti-inflammatory effects suitable for the treatment of neuroinflammatory diseases (Gorba et al., 2006).

Over the past two decades, an increasing amount of evidence indicates that activation of inflammatory processes occurs during epileptogenesis which contribute in concert to the adverse outcomes (Vezzani and Rüegg, 2011). Increased levels of inflammatory molecules and upregulation of their receptors in glial and neuronal cells as well as microvascular endothelial cells have been demonstrated in human brain tissues of medically refractory epileptic patients; suggesting that proinflammatory pathways are activated in seizure foci (Aronica et al., 2012; Choi et al., 2009; Vezzani et al., 2013). It is proposed that some patients with chronic epilepsy might benefit from anti-inflammatory or immunomodulatory therapies. Neuroprotective and anti-inflammatory drugs significantly decreased expression of inflammatory molecules and cell loss in the brain and can slow epileptogenesis (Dedeurwaerdere et al., 2012). Therefore, preventing the activation of inflammatory cascades represents a promising strategy to improve treatment of epilepsy.

Several screening models are currently used to investigate compounds for anticonvulsant activity. Seizure induction by pentylenetetrazol (PTZ) application, as a model for acute, generalised clonic type seizures (absence or myoclonic; Ferrendelli et al., 1989; Meldrum, 2002), is commonly employed in anticonvulsant screening, structure-activity research, drug design, and other approaches for antiepileptic drug development (Kupferberg, 2001). Pretreatment with well-established anticonvulsants, such as clonazepam, carbamazepine, valproate or ethosuximide (15–30 min before PTZ injection), significantly increased the seizure threshold in rats (Löscher et al., 1991). This study was designed to evaluate the effect of NRP2945 on behavioural and histopathological consequences of PTZ-induced convulsions in adult rats.

2. Materials and methods

Adult male Wistar rats (200–250 g, $n = 40$) were housed under controlled conditions (ambient temperature = 22 °C, humidity = 40%; 12-h light/dark cycle) with food and water ad libitum. Animals were age- and weight-matched and randomly divided into three groups: (i) Control group: animals underwent all of the surgical procedures and treated with saline intraperitoneally (i.p.) but did not receive PTZ (Santa Cruz, Heidelberg, Germany) or NRP2945 ($n = 8$). (ii) PTZ group: rats received saline 30 min before i.p. injection of PTZ (50 mg/kg dissolved in 0.9% saline; PTZ + saline, $n = 8$). (iii) NRP2945 group: animals received i.p. injection of NRP2945 (5, 10 or 20 μg/kg) 30 min before i.p. injection of PTZ (PTZ + NRP2945; $n = 24$, eight rats for each concentration). NRP2945 was generously donated by CuroNZ Ltd, Auckland, New Zealand. All experiments were carried out according to the protocol approved by the Animal Ethics Committee of Shefa Neuroscience Center, Tehran, Iran.

2.1. Behavioural tests

After each PTZ-injection, the convulsive behaviour was assessed for 30 min, and resultant seizures were scored as follows: 0 = normal behaviour; 1 = immobility; 2 = rigid posture; 3 = repetitive scratching, circling, or head bobbing; 4 = forelimb clonus, rearing, and falling; 5 = repeated occurrence of level four behaviour, and 6 = severe tonic-clonic behaviour or status epilepticus (Karimzadeh et al., 2013; Morrison et al., 1996). Latency of seizure onset was defined as the average length of time in seconds between PTZ administration and initially detectable seizure. A generalised seizure was characterised by symmetric forelimb and hind limb tonus, followed by hind limb clonus and subsequent jumping activity. Since animals occasionally had more than one seizure, the seizure duration was assessed as the sum of durations of these multiple seizures.

2.2. Surgical procedure and electrocorticogram (ECoG) recording

Animals were anaesthetised with chloral hydrate 3.5% (dissolved in normal 0.9% saline; 1 mL/100 g, i.p., Sigma-Aldrich) and the head of each rat was placed in a stereotaxical instrument (Stoelting Instruments, Wood Dale, IL, USA). Epidural recording electrodes were stereotaxically implanted above the left and right somatosensory cortices (3.0 mm posteriorly to Bregma and 3.0 mm laterally from the midline) under continuous chloral hydrate anaesthesia. The reference electrode is located at the nasal bone. ECoG was recorded via monopolar silver electrodes connected to an amplifier (EXT-02 F, NPI, Tamm, Germany; with band-pass filters at 0.5–30 kHz, sampling rate 10 kHz) and stored by a digital oscilloscope. Rats were allowed to recover 1 week after surgery. ECoG recordings were performed for 30 min after receiving saline or NRP2945 (5, 10, 20 μg/kg) and 60 min after the injection of PTZ. Latency, duration, amplitude, and frequency of epileptic spikes activity were calculated using AxoScope 10 software (Axon Instruments, Foster City, CA, USA).

2.3. Stereological methods and physical dissector

The volume-weighted mean volume of normal neurons was calculated directly by point-sampled intercept on 10 uniform systematically and randomly sampled microscopic fields of the pyramidal layers of hippocampal areas CA1 and CA3 as well as the somatosensory cortex. A lattice of test points on lines was superimposed randomly onto the traced nuclear profiles in each particular field. Nuclei of neurons were marked and two isotropic lines from randomly selected directions were centred on this neuron and superimposed. The intersection of each line with the outer surface of the respective neuron's soma was marked. These lines produced point-sampled intercepts, the lengths of which were measured and cubed. The mean was multiplied by $\pi/3$, and finally all intercepts were averaged to give an estimate of the volume-weighted mean neuronal volume. For quantitative analysis of dark neurons, the physical dissector method was used. Ten pairs of sections, with 8-mm distance, were collected from each brain. The first section of each pair was designated as the reference and the second one was used for comparison. On each pair of sections, at least 10 microscopic fields were selected by uniform systematic-random sampling in every area of interest. Using the unbiased frame and physical dissector counting rule, the counting of dark neurons in each field was carried out (Jafarian et al., 2010; Sadeghian et al., 2012).

2.4. Histological studies

Rats were sacrificed after 90 min of PTZ injection. Animals were deeply anaesthetised with chloral hydrate (3.5%; Sigma-Aldrich) and perfused transcardially with 200 mL of saline followed by 200 mL of a solution containing 1% paraformaldehyde (PFA). After perfusion, all rats were decapitated and the brains were removed. The brains were kept in 1% PFA for at least 10 days and were then processed for histological studies as follows. The brain was cut into transverse sections of 8 μm thickness. Sections were stained with toluidine blue. The sections were selected by uniform systematic random sampling in every area of interest. Different areas (hippocampal CA1 and CA3 areas and somatosensory neocortex) were studied under a light microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera. Digital photographs were taken using a 40 × oil immersion objective lens (Olympus). The magnification was calculated using an objective micrometre.