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Comorbid anxiety-like behavior and locus coeruleus impairment in diabetic peripheral neuropathy: A comparative study with the chronic constriction injury model

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ABSTRACT

Anxiety frequently appears in patients with diabetic neuropathic pain, a highly prevalent clinical condition. However, the neurobiological mechanisms of this comorbidity are poorly known. Anxiogenic phenotype has been associated with alterations of the noradrenergic locus coeruleus (LC) after peripheral nerve entrapment. We have examined the sensorial (pain) and affective (anxiety) behaviors, and the LC activity in streptozotocin (STZ)-induced diabetic rats. A comparative study with the chronic constriction injury (CCI) model of sciatic nerve was also carried out.

Diabetic nociceptive hypersensitivity was observed to appear gradually, reaching their maximum at fourth week. In contrast, CCI displayed a sharp decrease in their sensorial threshold at seventh day. In both models, anxiety-like phenotype was evident after four weeks but not earlier, coincident with the LC alterations. Indeed, STZ animals showed reduced LC firing activity, tyrosine hydroxylase, pCREB and noradrenaline transporter levels, contrary to observed in CCI animals. However, in both models, enhanced LC alpha2-adrenoceptor sensitivity was presented at this time point.

This study demonstrated that diabetes induced anxiety-like behavior comorbid with LC impairment at long-term. However, the nociceptive sensitivity time-course, as well as the LC functions, showed distinct features compared to the CCI model, indicating that specific neuroplastic mechanisms are at play in every model.

1. Introduction

A large body of clinical research has demonstrated that emotional distress, such as anxiety, is frequently associated with neuropathic pain (Radat et al., 2013; McWilliams et al., 2003). Diabetes is currently one of the most prevalent conditions in which symptoms of anxiety co-exist with chronic pain. It is estimated that the global prevalence of diabetes rate 8.5% in the adult population during 2014, affecting a total of 422 million people worldwide (Book World Health Organization, 2016). Among the diabetic patients, 14–30% suffers from chronic pain with neuropathic characteristics (Bouhassira et al., 2013; Halawa et al., 2010; Dyck et al., 1993), and over 20% higher prevalence of lifetime diagnosis of anxiety (Jain et al., 2011; Li et al., 2008). The comorbidity between diabetes and anxiety suggests a feedback between the somatic and emotional spheres, but the neurobiological mechanisms underlying this relationship are unclear.

This association between pain-induced anxiety disorders is further supported by preclinical studies demonstrating that prolonged experimental pain caused by mechanical nerve entrapment leads to anxiety-like behavior in rodents (Alba-Delgado et al., 2013; Jiang et al., 2014; Zhang et al., 2014; Leite-Almeida et al., 2012), suggesting that time is a critical factor in these models. However, it is unclear if diabetic pain follows the same temporal evolution than other models of neuropathic pain. Strikingly, we have shown in a neuropathic model caused by nerve constriction that the onset of the anxiety behavior temporally coincides with alterations in the locus coeruleus (LC), the main noradrenergic source in the brain (Alba-Delgado et al., 2013; McCall et al., 2015). This nucleus is widely involved in the transmission of sensory information, including pain (Pertovaara, 2013; Hickey et al., 2014), but also it forms part of central stress response circuit (George et al., 2013; Bravo et al., 2014, 2013). Therefore, the LC may represent a critical hub for

Abbreviations: CCI, Chronic Constriction Injury; ED50, Effective Dose 50; EZM, Elevated Zero Maze test; i.p., Intraperitoneal; i.v., Intravenous; LC, Locus Coeruleus; NAT, Noradrenaline Transporter; pCREB, Phospho-cAMP Response Element-Binding protein; STZ, Streptozotocin; TH, Tyrosine Hydroxylase.

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the modulation of the sensorial and affective features related to neuropathic pain. Several studies have reported modifications of the noradrenergic function in diabetic rats (Figlewicz et al., 1993; Petrisic et al., 1997; Suehiro et al., 2013), but it is unknown if neuropathic animal models with different origins that are usually though to be equivalent in sensorial pain features have the same impact on the LC.

We have used the streptozotocin (STZ)-induced diabetes model, which mimics aspects of type 1 diabetes (Wei et al., 2003), to study behavioral attributes of pain and anxiety, as well as the noradrenergic function of LC neurons. In addition, to better understand the relationship between neuropathic pain of different etiologies and anxiety, we have compared the STZ model with rats submitted to chronic constriction injury (CCI) of sciatic nerve, a model widely used to study neuropathic pain caused by peripheral nerve trauma.

Our results revealed different kinetics in the development of sensorial hypersensitivity between STZ and CCI rats. Despite this, both animal models developed anxiety-like state parallel to noradrenergic impairment of LC neurons at the same time point (four weeks after).

2. Methods

2.1. Animals

Experiments were performed on adult male Sprague-Dawley rats (250–300 g at the beginning of experiments). Animals were housed in groups of four in polycarbonate cages under standard laboratory conditions (22 ± 1 °C, 12 h light/dark cycles) with *ad libitum* access to food and water. All experimental procedures were approved by the Animal Research Ethics Committee at the University of Cadiz (Spain) and they complied with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983). All procedures relating to animal care and use conformed to European Guidelines (2010/63/EU) and Spanish Law (RD 53/2013).

All procedures were conducted according to the experimental design (Fig. 1) during the light phase (between 9:00 AM and 4:00 PM), minimizing as possible the animal stress. The rats were distributed on six independent experimental sets: (i) nociceptive behavioral tests; (ii) open field test; (iii) elevated zero maze test; (iv) electrophysiology; (v) immunofluorescence; (vi) western blot, quantification of β-cell mass and insulin levels. The exact number of animals per group for each group is detailed in the corresponding Figure of Results. All animals were submitted to the von Frey test to check their nociceptive thresholds the day before the corresponding assay.

2.2. Drugs

The following drugs were used: ketamine (Merial Laboratorios SA, Spain); xylazine (Bayer Hispania SL, Spain); chloral hydrate (Fluka Chemie AG, Switzerland); streptozotocin (STZ; Sigma Chemical, Spain); RX821002 (alpha2-adrenoceptor antagonist; Sigma Chemical, Spain); UK14,304 (alpha2-adrenoceptor agonist; Tocris Bioscience, UK). All drugs were dissolved in saline solution (0.9% NaCl).

2.3. Pain models

2.3.1. Streptozotocin-induced diabetes

Diabetes was induced in rats by a single injection of STZ (70 mg/kg, intraperitoneal, i.p.) (Ahlgren and Levine, 1993) freshly dissolved in 0.9% sterile saline (Ahlgren and Levine, 1993; Chen et al., 2011; Ciruela et al., 2003). The STZ solution was discarded if bubbling was observed. Control rats received an equal volume of saline vehicle. Blood sugar levels were measured 48 h after injection and rats with values >250–300 mg/dl were included in the experimental group. Diabetes was successfully induced in 85.7% of the rats injected. None of them were treated with insulin.

Since administration of STZ causes destruction of pancreatic β-cell (Szkudelski, 2001), the percentage of reduction of β-cell mass after STZ administration was also quantified (Jimenez-Palomares et al., 2012). Briefly, the pancreas was removed, weighed, and divided into head (next to duodenum) and tail (next to spleen) sections. Each section was fixed in Bouin's solution (Sigma Chemical, Spain) for 24 h. Fixed tissues were washed extensively, immersed in Neutral Buffer Formalin for 2 days, dehydrated and embedded in paraffin. Twenty-five to thirty paraffin slices (5 μm thickness) were collected at 100-μm intervals for each section. Four slices per pancreas were used to quantify the β-cell mass. Rehydrated slices were incubated overnight at 4 °C with mouse anti-insulin primary antibody (1:1000; I2018 Sigma Chemical, Spain). Next, they were incubated for 1 h at room temperature with the goat anti-mouse linked to biotin antibody (1:200; B7401 Sigma Chemical, Spain). β-cells were reveled with HRP-conjugated streptavidin (1:100; S2438 Sigma Chemical, Spain), and counterstained with hematoxylin. All images were acquired using a microslide scanner (Nikon Super CoolScan 5000 ED, Japan), and quantified using ImageJ software (National Institutes of Health, USA). β-cell mass was estimated following the equation: pancreas section weight × mean of β-cell area. The β-cell area of a slice was calculated by dividing the sum of all insulin-positive cells areas in this slice by the total scanned pancreas area of the slice (Bernal-Mizrachi et al., 2001).

To assess the metabolic status as an index of diabetes progression, the body weight, the water consumption (in 24 h period) and the glucose levels were monitored weekly in non-fasting conditions. To measure blood glucose levels, blood samples were taken from an incision made at the tip of the tail and glucose was measured using an Opium Xceed glucometer (Abbot Cientifica SA, Spain). Plasma concentrations of insulin were also measured in blood samples before and 4 weeks after STZ injection. The samples were collected in ice-chilled heparinized tubes (Microvette, Germany). Plasma was immediately separated and stored at −80 °C. Plasma samples were analyzed using an ELISA

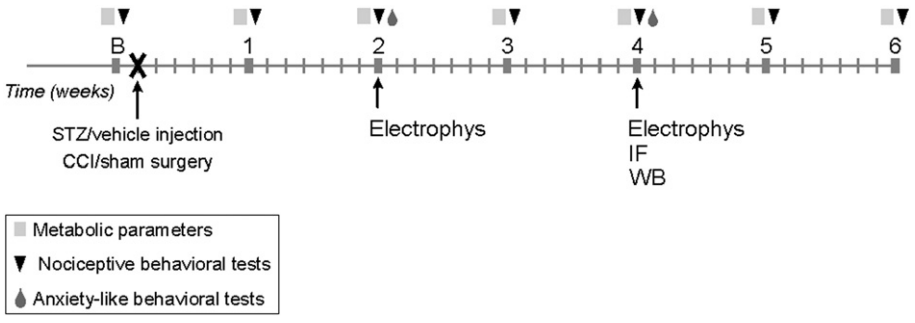

Fig. 1. Experimental design. Scheme illustrating the time course of experimental schedule and the protocols carried out for streptozotocin (STZ), vehicle-treated, chronic constriction injury (CCI) and sham rats. The basal values (B) for metabolic and nociceptive behavioral tests were measured the previous day of the pain model induction. Then, weekly measures were taken. Anxiety-like behaviors were measured by elevated plus maze and open field tests at two and four weeks. In vivo electrophysiological recordings of locus coeruleus neurons (Electrophys) were executed at two and four weeks. Immunofluorescence (IF) and western blotting (WB) from LC samples were realized the fourth week.