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The neuronal basis of copper induced modulation of anxiety state in rat

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ABSTRACT

Recently, studies have provided strong evidence indicating the involvement of trace elements in the physiopathology of psychiatric disorders, particularly anxiety. We aimed, through the present study, to describe the effect of acute exposure to Cu (10 mg/kg BW) on anxiety state together with the serotoninergic and dopaminergic systems in rat by means of neurobehavioral tests (elevated plus maze, dark light box) and immunohistochemistry using anti-serotonin (5HT) and anti-tyrosine hydroxylase (TH). Our data report that Cu enhanced 5HT innervation in the dorsal raphe nucleus (DRN) together with a loss of TH expression within the ventral tegmental area (VTA), Substantia nigra compacta (SNc) and their subsequent outputs including the medial forebrain bundle (MFB) and striatum. In the elevated plus maze Cu significantly increased the time and the number of entries into the open arms, and raised the time spent in the Dark Box indicating a clear reduced anxiety state induced by Cu. The present data show for the first time a powerful neuro-modulatory potential of Cu in rat which involves primarily a dysfunction of 5HT and DA neurotransmissions.

1. Introduction

Anxiety is a feeling manifested by a repulsive state of inner disorder, goes along with nervous behavior. Anxiety disorders are the most common mental health disorders, characterized by an extreme or pathological anxiety states as the primary disturbance in mood, in the company of amplified fear and overstated acute stress responses (Ninan, 2001), more prevalent than both affective and substance abuse disorders (Shearer, 2007). Anxiety disorders can negatively affect quality of life, mobility, education, employment, social functioning, health care, and physical well being, and are significantly related to other physiological dysfunctions especially thyroid disease, respiratory disease, gastrointestinal disease, arthritis, migraine headaches, and allergic conditions (Sareen et al., 2006). Numerous studies have shown the implication of various neurotransmitters in the pathophysiology of anxiety disorders, especially serotonin (5-HT) and dopamine (DA) (Stein et al., 2002), glutamate (Carlsson, 2001) and γ-aminobutyric acid (GABA) (Lydiard, 2003). Furthermore, dopaminergic and serotonergic systems have a significant role in the neurotransmission implicated in stress response (Puglisi-Allegra and Cabib, 1990; Le Moal and Simon, 1991).

Currently, several studies have provided evidence concerning the involvement of essential elements (Zn, Mg, Li, Ca, Cu, Mn) in depression and anxiety (for review see Młyniec et al., 2014a,b), and their influence on the neurotransmission implicated in emotional processes, such as serotonergic, dopaminergic, glutamatergic, noradrenergic, and GABAergic systems (Frederickson et al., 2000; Piotrowska et al., 2013).

Copper (Cu) is an essential trace element that ensures the well-functioning of different biological systems including central nervous system (CNS). Such role of Cu as a vital element resides in its function as a cofactor for structural and catalytic properties of different essential enzymes comprising the free radical scavenger superoxide dismutase (Cu, Zn-SOD), dopamine monooxygenase, cytochrome oxidase, lysyl oxidase and ceruloplasmin. Whereas, Cu is required for numerous physiological functions especially: cellular respiration, antioxidant defense, neurotransmitter function (Uauy et al., 1998; Turnlund, 1999; Letelier et al., 2005). Cu has suspected as a key element in the pathophysiology of several neurodegenerative disorders, such as Alzheimer's, Parkinson's diseases and amyotrophic lateral sclerosis (Bains and Shaw, 1997; Cookson

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and Shaw, 1999; Sayre et al., 2000; Strausak et al., 2001) by acting as a neuromodulator of several neurotransmission systems including particularly serotonin and dopamine. Therefore, it is of crucial importance to assess the neurobehavioral resounding of Cu administration with a focus on the anxiety state.

In view of the above mentioned, we aimed, through the present investigation, to assess the anxiety state in rats acutely treated with Cu at a dose of 10 mg/kg, together with the brain innervation including serotonin in the Dorsal Raphe Nucleus (DRN) and dopamine in Substantia Nigra pars compacta (SNc), Ventral Tegmental Area (VTA) and the medial forebrain bundle (MFB).

2. Material and methods

2.1. Animals

Our animals were supplied by the central animal care facilities of Cadi Ayyad University, Marrakech, Morocco. All animals were housed at a constant room temperature (25 °C), with a 12-h dark–light cycle and free access to food for all studied groups in a special room for animal housing. All animals were treated according to the European decree, related to the ethical evaluation and authorization of projects using animals for experimental procedures, 1 st February 2013, NOR: AGRG1238767A. Thus, all efforts were made to minimize the number and suffering of animals used.

2.2. Chemicals

Cu (II) acetate trihydrate was supplied by (Riedel-de Haen, Seelze, Germany; Code No. 25038, Lot No. 83370).

2.3. Cu administration

In the present investigation we used 4 months adult male Wistar rats weighing 200–250 g. Animals were randomized into 2 groups. Group I: of control rats (**C**) ($n = 12$,) injected i.p. with physiological saline solution (0.9% NaCl, 5 ml/kg. i.p) for 3 consecutive days. Group II: of Cu intoxicated rats (Cu) ($n = 12$,) injected i.p. with Cu at a dose of 10 μg/g B.W. 5 ml/kg for 3 consecutive days. The experimental protocol and the doses of Cu used are based on literature reports (Wenk and Suzuki, 1982). Injections were performed between 10 and 11 a.m.

2.4. Elevated plus maze test

The maze is made with black wood and elevated 50 cm above the floor. It consists of two open arms 10 cm width and 50 cm length connected perpendicularly to two closed arms of equal dimensions with a 100 cm^2 square center region. The closed arms have black walls 30 cm in height. Testing was conducted in a quiet room. Each animal was gently placed in the central square facing toward a closed arm and allowed to explore for 5 min as described by Braun et al. (2011), An entry was defined as placing all four paws within the boundaries of the arm. Animal movements are then recorded by video camera for 5 min and the% of time spent in the open arms (total time in open arms/total time spent in closed + open arms) together with the number of open arms entries are measured as indicators of anxiety state. Anxiolytic activity was indicated by increased time spent in open arms or in a greater number of open arm entries. Between trials, the maze was cleaned with 70% ethanol to remove all traces (urine and defecations) of the precedent animals.

2.5. Dark light box

This task consists of a rectangular box (44 cm × 8.5 cm × 25 cm) divided equally into a light (open compartment) and a Dark (Closed compartment) connected by a door (17 cm in height). Each animal was placed facing the side away from the door and then released. During 10 min, the time spent in dark and light compartments, respectively, was measured to determine the degrees of anxiety (Miller et al., 2011). The box was carefully cleaned with 70% ethanol before each animal was introduced.

2.6. Immunohistochemistry

At the end of the experiment, animals were sacrificed 24 h after last Cu injection between 10 and 12 a.m. for the immunohistochemical study, rats were anesthetized intraperitonially with urethane (40 mg/kg i.p.) and perfused transcardiacally with chilled physiological saline (NaCl 0.9%) (Sigma-aldrich, St.Louis,MO, USA, Cas No. 7647-14-5, lot No. BCBH2237V) and paraformaldehyde (4%) (Panreac Quimica SA, Barcelona, Spain, catalog No. 141451.1211, lot No. 0000078736) in phosphate buffered saline (PBS, 0.1 M, pH 7.4) (Riedel-de Haen, Seelze, Germany). Brains were removed and post-fixed in the same fixative for 12 h at 4 °C, then dehydrated through a graded ethanol series (70–100%), passed through serial polyethylene glycol (Merck-Shuchardts, Hohenbrunn, Germany, Cas No. 25322-68-3) (PEG: 20 to 100%) solutions and embedded in pure PEG. Coronal sections of 20 μm thickness were cut with a microtome according to the rat brain in stereotaxic coordinates (Paxinos and Watson, 2006) and collected in phosphate buffered saline (PBS). Sections were taken throughout the dorsal raphe nucleus, midbrain through substantia nigra compacta (SNc) and ventral tegmental area (VTA) (Bregma – 5,3 mm), the medial forebrain bundle (MFB) (Bregma 0,80 mm) and the dorsal striatum (Bregma 0,20 mm). The slices were selected and preincubated during 2 h in PBS with 0.3% triton and 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, USA, CAS No. 9048-46-8, Lot NO. 078K0729) under agitation, the slices are then incubated overnight at 4 °C in a solutions of polyclonal 5-HT antibody (Sigma-Aldrich, St.Louis, MO, USA, Catalog No. S5545, Lot No. 127H4813) and monoclonal TH antibody (Santa Cruz, CA, USA; catalog No. SC-25269), diluted 1/1000 containing PBS (0.1 M, pH 7.4), Triton (0.3%), and BSA (1%). The slices are then washed three times with PBS (0.1 M, pH 7.4) containing BSA (1%) for 5 min then incubated with the secondary antibody (rabbit anti-immunoglobulins, 1/500) (Vector Labs, Burlingame, CA, USA, Catalog NO. BA-1100, lot No. WO611) for 2 h at room temperature. After three washes, the slices were incubated for 1 h 30 min in PBS buffer containing Triton (0.3%) and the Avidin-biotine peroxidase complex (Kit ABC 1/500) (Vector Laboratories Burlingame, Californie, USA, Catalog No. PK-6101). 5-HT and TH was revealed following the enzymatic reaction of the peroxidase in presence of the 3,3-diaminobenzidine (0.03%) (Sigma-Aldrich; Oakville, Canada, CAS No. 868272-85-9) and hydrogen peroxide (0.006%) in Tris buffer (0.05 M pH 7.5). The sections were then collected, dehydrated and mounted in Eukit for optical microscopy observation. The specificity of the immunoreactive materials was tested following subjection of the slices to the same immunohistochemical procedure as described above and using the preimmune serum or omitting of the primary antibodies. These tests showed that the primary antibodies used against 5-HT and TH display specific labeling (Sansar et al., 2012; Elgot et al., 2012; El Hiba et al., 2013; Benammi et al., 2014).

2.7. Immunolabeling quantification

Quantification of TH-immunoreactivity (TH-IR) was performed according to the protocol published by Vilaplana and Lavialle

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