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Discriminative behavioral assessment unveils remarkable reactive astrocytosis and early molecular correlates in basal ganglia of 3-nitropropionic acid subchronic treated rats

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ABSTRACT

Reactive astrocytosis seems to be strongly implicated in the development and maintenance of inflammatory and neurodegenerative disorders. We design a new toxic model treatment with 3-nitropropionic acid (3-NP), a mitochondrial complex II irreversible inhibitor, to induce in rats Huntington's disease (HD) like syndrome, characterized by hindlimb dystonia, involuntary choreiform movements and reduced global activity. In an attempt to find out whether molecular and morphological changes in the neuro-glial network could be involved in the pathogenesis of this disease, we developed a protocol of subchronic intra-peritoneal 3-NP intoxication. Moreover we set up specific, highly discriminative, behavioral tests to detect very early mild motor disabilities in 3-NP treated rats. This treatment did not cause severe cell death. However, in the Caudate-Putamen (CPu) of all 3-NP treated animals we found a massive astrogliosis, revealed by increased GFAP levels, paralleled by changes of the glial glutamate transporter GLAST distribution. To these glial changes we detected a transcriptional upregulation of c-fos and Sub-P in the striatal medium spiny neurons (MSN). We propose that this model of 3-NP intoxication along with the designed set of behavioral analyses allow to unmask in a very early phase the motor deficits and the underlying morpho-molecular changes associated to the onset of motor disabilities in the HD-like syndrome. Therefore this model unveil the key role played by the different components of the tripartite synapse in the pathogenesis of the HD, a putative non-cell-autonomous disease.

1. Introduction

The role of non-neuronal cells to the pathogenesis of neurodegenerative diseases has been widely investigated yet it remains poorly understood. Several studies reported that modifications of neuro-glial network strongly contribute to several mental disorders (Musholt et al., 2009) and neurodegenerative diseases (Lobsiger and Cleveland, 2007) such as Alzheimer's disease (Rodriguez et al., 2009), amyotrophic lateral sclerosis (Sheldon and Robinson, 2007), Parkinson's disease (Saijo et al., 2009), and spinocerebellar ataxia type 1 (SCA1) (Giovannoni et al., 2007). Particularly, in an experimental model of SCA1, glial activation, characterized by astrocytic hypertrophy and increased production of glial fibrillary acidic protein (GFAP), correlates with changes of glial glutamate transporter expression (Giovannoni et al., 2007). Glial and microglial activation, through changes of cell phenotype and gene expression, might trigger glial-induced neuronal death (Bal-Price and Brown, 2001), having a key role to the pathophysiology of neurodegenerative disorders (Mrak and Griffin, 2005).

Excitatory amino acids transporter 1 (EAAT1, also known as GLAST) is the major glutamate transporter expressed in the brain (Lehre and Danbolt, 1998). It is not detected in neurons (Ginsberg et al., 1995) but strongly expressed on astrocytes (Chaudhry et al., 1995). Its distribution correlates with the morphological changes reported in the astrocytes during inflammatory (Cavaliere et al., 2007; Xin et al., 2009; Tilleux et al., 2009) or degenerative processes (Dervan et al., 2004) and subsequent changes in neurotransmitter

Abbreviations: 3-NP, 3-nitropropionic acid; i.p., intra-peritoneal; GFAP, glial fibrillary acidic protein; MSN, medium spiny neurons.

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uptake could represent the basis of morphological and functional changes underlying Huntington's disease (HD). Clinical trials in patients with HD (Seppi et al., 2001) and animal models of striatal neurodegeneration (Doble, 1999) designed to slow progression by decreasing glutamatergic neurotransmission have used riluzole as attenuating agent of glutamate release.

Astrocytic activation in striatum is a common reaction after toxic, ischemic or degenerative process and seems to play a role in the development of HD (Hickey et al., 2008). Huntington's disease, a neurodegenerative autosomic dominant inherited disorder, characterized, in humans, by involuntary choreiform movements and cognitive impairment (Landles and Bates, 2004) caused by a CAG expansion in the coding region of the gene *huntingtin* (1993), is characterized by marked striatal atrophy with neuronal loss, astrocytic activation and intra-neuronal protein aggregates (Aparicio and Lucas, 2002; Paulson and Fischbeck, 1996). The mechanism underlying the selective neuronal death of the GABAergic medium spiny neurons (MSN) in the striatum still remains unknown. One hypothesis suggests that the genetic defect may cause an impairment of energy metabolism that subsequently makes these neurons more vulnerable to slow excitotoxic degeneration (Brouillet et al., 2005).

3-Nitropropionic acid (3-NP) is a suicide inhibitor of succinate dehydrogenase and has been shown to cause a HD-like syndrome in primates (Brouillet et al., 2005) but also in rats (Borlongan et al., 1997a). I.p. 3-NP acute (60–100 mg/kg/die) or chronic treatment (2–5 mg/kg for 7 or 14 or more days) induced striatal lesions that resulted in dystonia and other abnormal motor behaviors mimicking the neurochemical and behavioral features of HD in humans (Bogdanov et al., 1998; Fernagut et al., 2002). Both intoxication protocols have established a link between mitochondrial chain impairment and HD pathogenesis (Browne, 2008). 3-NP acute toxicity induces severe neurological deficits and produces a neuron depleted striatal lesion (Borlongan et al., 1997b) while chronic intoxication has been reported to induce behavioral and neurochemical features (Borlongan et al., 1997b; Palfi et al., 1996) similar to those found in grade 1–2 HD patients (Chyi and Chang, 1999). Unfortunately, both treatments failed to reveal the early molecular and morphological events that lead to the death of the MSN as, in both models, 3-NP treatment produces massive neuronal death, a phenomenon that occurs only in late stage of the human disease (Paulsen et al., 2006).

To identify and better clarify the early modifications of the neurodegenerative process, we developed a new 3-NP treatment protocol which could mimic the phenotype of the human Huntington's disease in rats. This i.p. subchronic administration induced mild behavioral abnormalities depending on mitochondrial chain impairment. We used a new designed behavioral test (progressive elevated beam test) and other well-accepted motor skill test to unmask the early motor deficits induced by 3-NP treatment. In treated animals, we found an intense reactive astrocytosis with no clear sign of neuronal death along with an altered expression of the glial glutamate transporter GLAST. Neurons, however, showed an increased expression of Substance-P (Sub-P) and c-fos. We hypothesize that these molecular changes could determine the functional as structural rearrangement of the tripartite synapse following mitochondrial insult, that is the very early events leading to synaptic dysfunction and, possibly, in later stages, neuronal death.

2. Experimental procedures

2.1. Animals

Adult (250–300 g; Charles River, Calco, Italy) male Sprague Dawley rats ($n = 20$), 3 months old at the beginning of the intoxication, were used. Rats were maintained on a 12/12 h light/dark cycle and allowed free access to food and water. Each animal was housed under specific pathogen-free conditions in iron-sheet cages with solid floors covered with 4–6 cm of sawdust during the experiments. All experimental procedures were performed during the light cycle and were approved by the Animal Ethics Committee of The Second University of Naples. Animal care was in compliance with Italian (D.L. 116/92) and EC (O.J. of E.C. L358/1 18/12/86) regulations on the care of laboratory animals. All efforts were made to reduce animal numbers.

2.2. Treatment

3-Nitropropionic acid (3-NP, Sigma, Italy) was prepared as previously described (Brouillet et al., 1995; Fernagut et al., 2002). In brief, 3-NP was dissolved in a few drops of distilled water and the pH was adjusted to 7.4 with 1 M NaOH and the final volume adjusted with 0.1 M phosphate-buffered saline (PBS), pH 7.4. Rats ($n = 20$) were grouped as follows: animals undergoing subchronic i.p. 3-NP treatment ($n = 12$) and vehicle (saline solution) treated rats ($n = 8$) assumed as control (CTR) animals. We used an injection protocol characterized by three different decreasing 3-NP i.p. doses, in order to maintain a low 3-NP level for all the period of treatment. In particular, the subchronic treatment consisted of twice a day i.p. injections of 3-NP for 18 days. Twice a day injections were divided as follows: 7.5 mg/kg for the first 2 days of treatment, followed by 3.75 mg/kg for 7 days, finally a dosage of 2 mg/kg for the last 8 days of treatment. Animals were sacrificed 4 days after treatment.

2.3. Behavioral analysis

Progressive elevated beam test: This newly designed behavioral analysis was used to evaluate the appearance of motor performance abnormalities, in the treated animals vs. the controls, by progressively increasing the difficulty in the execution of the task. The aim was to train the animals in crossing three beams of different sizes to reach a platform on which their home cage was placed. Specifically, the beams had a similar length (100 cm) but different width (8, 5 and 3 cm, respectively). Before treatment, animals received 3 days of training (twice per day), after which all rats were able to cross the board without rearing or stopping. Animals were videotaped and analysis was tracked off-line. Each rat was scored for time of crossing and numbers of foot slips for each beam. Occurrence of bradykinesia was quantified by calculating the average velocity of walking along each different bar for treated and CTR animals.

Footprint test: This test was used to assess gait abnormalities in 3-NP treated vs. control animals. After coating the hind feet with a non-toxic black dye and forepaws with a non-toxic blue dye, rats were allowed to walk on a beam (100 cm long, 8 cm width) covered with paper. Step length, paws overlap and gait walking angle were analyzed. Specifically, step length was quantified as the distance between two subsequent feet on the *y*-axis. Paws overlap analysis was carried out by measuring the centre distance between the anterior paw and the correlated rear paw footprints. Gait angle walking was calculated by measuring the angle of the foot in respect to the walking direction.

2.4. Tissue preparation

Rats were deeply anesthetized with an i.p. injection (300 mg/kg body weight) of chloral hydrate and perfused transcardially with saline solution (TRIS HCl 0.1 M, EDTA 10 mM) followed by 4% paraformaldehyde added to 0.1% glutaraldehyde in 0.01 M phosphate-buffer (PB), pH 7.4 at 4 °C. For light microscopy brains were removed and post-fixed 2 h in the same fixative, then soaked in 30% sucrose PBS and frozen in chilled isopentane on dry ice. Serial sections were cut at the cryo-sliding vibratome at a thickness of 30 μm and collected in cold PBS for immunohistochemistry.

2.5. Immunohistochemistry

Brain sections were blocked in 10% normal serum in 0.01 M phosphate-buffer saline (PBS), 0.25% Triton for 1 h at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% normal serum 0.25% Triton. We used rabbit anti Sub-P antiserum (1:5000) and guinea pig anti-GLAST antiserum (1:4000) (Chemicon International, Temecula, CA, USA), rabbit anti c-fos antiserum (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse monoclonal anti glial fibrillary acidic protein (1:400) (SIGMA, Saint Louis, MO, USA) and rabbit anti DARPP-32 antiserum (1:100) (Cell Signaling Technology, USA). Sections were incubated for 48 h at 4 °C. Sections were washed in PBS and incubated with the appropriate biotinylated secondary antibody (Vector Labs, Inc., Burlingame, CA, USA; 1:200) for 90 min at room temperature (RT). Next, they were processed using the Vectastain avidin–biotin peroxidase kit (Vector Labs, Inc., Burlingame, CA, USA) for 90 min, at RT. Sections were washed in 0.05 M Tris–HCl, reacted with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, 0.5 mg/ml Tris–HCl) and 0.01% hydrogen peroxide, then mounted on chrome-alume gelatin coated slides, dehydrated, and coverslipped. Adjacent sections were Nissl-stained.

To analyze the spatial distribution of glial glutamate transporter GLAST, double staining was performed as previously reported (Papa et al., 2003). In brief, sections were incubated with either anti-GLAST and anti-GFAP primary antibodies following 2 h incubation with a solution containing either Alexa fluor 488 antigoat IgG (1:200) and Alexa fluor 555 anti-mouse IgG (1:200; Molecular Probes). Sections were