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Ventral tegmental area/substantia nigra and prefrontal cortex rodent organotypic brain slices as an integrated model to study the cellular changes induced by oxygen/glucose deprivation and reperfusion: Effect of neuroprotective agents

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ABSTRACT

Unveiling the roles of distinct cell types in brain response to insults is a partially unsolved challenge and a key issue for new neuroreparative approaches. *In vivo* models are not able to dissect the contribution of residential microglia and infiltrating blood-borne monocytes/macrophages, which are fundamentally undistinguishable; conversely, cultured cells lack original tissue anatomical and functional complexity, which profoundly alters reactivity. Here, we tested whether rodent organotypic co-cultures from mesencephalic ventral tegmental area/substantia nigra and prefrontal cortex (VTA/SN-PFC) represent a suitable model to study changes induced by oxygen/glucose deprivation and reperfusion (OGD/R). OGD/R induced cytotoxicity to both VTA/SN and PFC slices, with higher VTA/SN susceptibility. Neurons were highly affected, with astrocytes and oligodendrocytes undergoing very mild damage. Marked reactive astrogliosis was also evident. Notably, OGD/R triggered the activation of CD68-expressing microglia and increased expression of Ym1 and Arg1, two markers of "alternatively" activated beneficial microglia. Treatment with two well-known neuroprotective drugs, the anticonvulsant agent valproic acid and the purinergic P2-antagonist PPADS, prevented neuronal damage. Thus, VTA/SN-PFC cultures are an integrated model to investigate OGD/R-induced effects on distinct cells and easily screen neuroprotective agents. The model is particularly adequate to dissect the microglia phenotypic shift in the lack of a functional vascular compartment.

1. Introduction

The neuroinflammatory response associated to ischemia has widened the interest in this field to cell types other than neurons, including glia. Not only in stroke but also in chronic diseases (e.g., multiple sclerosis, Alzheimer's disease), CNS host resident immune cells (microglia, the brain immunocytes, and astrocytes) react to damage, migrate to the site of injury and secrete inflammatory cytokines and chemokines, that can profoundly influence neuronal viability and recovery. As a result of brain injury, infiltrating blood cells like leukocytes or macrophages can contribute to damage, although, in some instances, these cells can also mediate beneficial effects (Weinstein et al., 2010). In this respect, while classical activation of macrophages leads to the pro-inflammatory M1 phenotype mediating toxicity on neural cells, induction of a second M2

Abbreviations: CTR, control; DIV, days *in vitro*; FJB, Fluoro-Jade B; LDH, lactate dehydrogenase; Iba1, ionized calcium binding adaptor molecule 1; IB4, Isolectin B4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; M/M, macrophages/microglia; OD, optical density; OGD, oxygen and glucose deprivation; OGD/R, oxygen and glucose deprivation and reperfusion; PI, propidium iodide; PPADS, pyridoxalphosphate-6-azo-benzene-2',4'-disulfonic acid; RT, room temperature; PBS, phosphate buffer solution; VPA, valproic acid; VTA-SN, ventral tegmental area/substantia nigra; PFC, prefrontal cortex.

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phenotype by anti-inflammatory cytokines like IL-4, IL-10 or IL-13 (Gordon, 2003; Mosser, 2003) promotes an alternative beneficial anti-inflammatory state favoring scavenging of debris, angiogenesis, tissue remodeling and repair (Capone et al., 2007; Fumagalli et al., 2011; Neumann et al., 2006). Similarly, under certain conditions, microglia can be pushed to both extremes of the M1 and M2 differentiation spectrum (David and Kroner, 2011; Merson et al., 2010; Narantuya et al., 2010). A key issue is now to dissect the mechanisms determining the shift from a detrimental to a beneficial immunophenotype, in order to develop new agents with increased neuroprotective activity. Various reliable *in vivo* models of neurodegeneration (including stroke models) are currently available; however, due to their complexity, these models although they try to reproduce as closer as possible the clinical conditions are not always fully suitable for the analysis of a large number of candidate agents or for detailed mechanistic studies focused on the phenotypic changes of glial cells after insults. Moreover, the relative contribution of macrophages/microglia (M/M) to CNS damage and repair is difficult to be studied *in vivo*, since available markers of activated M/M cells do not distinguish between these two cell types. Obviously, the molecular mechanisms directing phenotypic changes in M/M are much better analyzed in cultured cells; the latter, however, lack the original *in vivo* tissue anatomical and functional complexity, which may profoundly alter their reactivity. In this respect, organotypic cultures of rodent brain slices may represent a useful tool, since they retain essential organizational features of the host tissue, such as neuronal connectivity, relatively well preserved cellular stoichiometry and complex glia–neuron interactions (Carbajal et al., 2011; Hosking et al., 2010).

The present work takes advantage of brain organotypic slice co-cultures of the ventral tegmental area/substantia nigra (VTA/SN)-complex and the prefrontal cortex (PFC), that have been previously characterized in terms of cytoarchitectural organization of the VTA/SN-complex, innervation pattern of the target areas by dopaminergic fibers (Franke et al., 2003) and expression and localization of receptors participating to post-injury events, such as purinergic P2 receptors (Heine et al., 2007). In these cultures, the lack of a functional vascular compartment allows to dissect in detail the effects and roles played by CNS resident cells after injury, i.e. excluding blood immune cells and focusing on residential microglia. Specifically, we were interested in validating these cultures for the analysis of agents able to counteract damage induced by a paradigm of hypoxic/hypoglycemic ischemia. Moreover, since these cultures are obtained from the brains of neonatal mice, they may as well represent a model to recapitulate the cellular changes that also occur during delivery-associated lack of oxygen leading to permanent brain ischemic damage in infants. As a first step to this aim, we evaluated the effects induced by oxygen and glucose deprivation followed by reperfusion (OGD/R) on neurons and all types of glial cells (astrocytes, oligodendrocytes and microglia), by paying special attention to the time-dependent phenotypic changes of microglia. As a second step, we tested the effects of two different pharmacological agents, the widely used anti-epileptic drug valproic acid (VPA), whose neuroprotective properties have recently attracted the interest of basic scientists (Bachmann et al., 2005; Marinova et al., 2009) and the non-selective purinergic P2Y/X antagonist phosphate-6-azo-benzene-2',4'-disulfonic acid (Abbracchio et al., 2006). The latter was chosen based on previous data indicating that a pathological exacerbation of purinergic (ATP)-induced effects contributes to ischemic damage (Burnstock, 2011), and that blockade of these effects by P2 receptor antagonists induces neuroprotection (Cavaliere et al., 2005; D'Ambrosi et al., 2009; Lammer et al., 2006). Moreover, as mentioned above, various P2X/Y receptors subtypes are indeed present in the VTA/SN-PFC co-cultures on neurons and glial cells (Heine et al., 2007), thus making this purinergic agent an appropriate tool for our purposes.

2. Materials and methods

2.1. Preparation of VTA/SN-PFC organotypic co-cultures

VTA/SN-PFC organotypic co-cultures were prepared from P3-P5 CD1 mice (Charles River Laboratories, Calco, Italy) and cultured according to the static culture protocol modified from Franke et al. (2003). Briefly, mouse pups were decapitated, the brains were removed from the skull under sterile conditions and kept at 4 °C. The brains were then embedded in 1.5% low melting agarose, fixed onto the specimen stage of a vibratome with cyanoacrylate glue and mesencephalic and forebrain coronal sections of 300 μm thickness were cut in cold (4 °C) preparation medium (MEM + Hepes 25 mM + Glutamax 2 mM + penicillin/streptomycin 1:100 + fungizone 1:100). In the preparation of organotypic slice cultures of the ventral mesencephalon we did not attempt to separate VTA and the SN. For further discussion this area will be named VTA/SN-complex. Immediately after vibratome sectioning, the slices were separated into the different brain areas, transferred into Petri dishes filled with cold (4 °C) preparation medium. Selected sections of each portion were placed side by side on moistened membranes of tissue culture inserts (0.4 μm, Millicell-CM, Millipore, Vimodrone, Italy) using a dissection microscope, thus obtaining VTA/SN-PFC organotypic co-cultures. These were put in six-well plates, each filled with 1 ml of culture medium with serum (preparation medium 25% + Basal Medium Eagle 25% + heat inactivated horse serum 25% + 20% glucose, 0.78 ml each 50 ml + Glutamax 2 mM + penicillin/streptomycin 1:100 + fungizone 1:100 + sterile water to 50 ml; set to pH 7.2) and organotypic slices were placed in humidified CO_2 enriched atmosphere at 37 °C. After 24 h, culture medium was fully changed with culture medium without serum (Neurobasal medium + B27 supplement 1:50 + Glutamax 2 mM + penicillin/streptomycin 1:100 + fungizone 1:100; set to pH 7.2). Medium was completely changed three times a week. Co-cultures were maintained for 10–15 days *in vitro* (DIV).

2.2. OGD/R induction, pharmacological treatments, and tissue processing

OGD was induced as previously described (Cho et al., 2007) by culturing brain slices in phenol red-free DMEM lacking of glucose and maintained in a hypoxic chamber (Billups-Rothemberg, California, USA), equipped with an anaerobic kit (Anaerogen™Compact, Oxoid Ltd, Hampshire–England), flushed with N_2 (20–25 l/min) for 10 min, then sealed and placed at 37 °C for 1 h. OGD was terminated by returning slices to a normoxic incubator and to standard preconditioned and warmed culture medium (reperfusion phase, R) for 1-6-18-24-48 h (OGD/R1-6-18-24-48). In control conditions, brain slices were placed in phenol red-free DMEM medium (glucose 4.5 g/l) and maintained in a normoxic incubator.

For pharmacological treatments, drugs were added for the entire reperfusion time (VPA, 250–500 μM; PPADS, 100 μM). At the end of reperfusion, cultures were fixed with 4% paraformaldehyde, 4% glucose, 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature (RT), washed with PBS and finally cryoprotected with a sucrose gradient (4–10–20–30%) for at least 48 h at 4 °C. After embedding in Tissue Tek OCT compound (Sakura), 20 μm-thick slices were sectioned with a cryostat and mounted on glass slides.

2.3. Viability and cytotoxicity assays

Culture supernatants from each condition were collected for lactate dehydrogenase (LDH) release measurement using the