DOTTORATO DI RICERCA IN BIOLOGIA CELLULARE E DELLO SVILUPPO

Proposta di progetto per una borsa Dottorato Sapienza
Linea di ricerca secondaria (marzo 2019)

Titolo della ricerca: Meccanismi di tossicità neuronale alla base della neurodegenerazione FENIB

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DESCRIZIONE DELLA RICERCA *(max 2 pagine, Arial 12, interlinea singola, esclusa bibliografia)*

This project is a continuation of our research on the neuronal pathways activated by neuroserpin (NS) polymers. In our previous work, we generated mouse neural stem cells expressing wild type or the FENIB variant G392E NS, or green fluorescent protein (GFP) as a negative control. In this model of FENIB, wild type and G392E NS are correctly expressed, and G392E NS forms polymers that accumulate in the ER. RNA sequencing showed 623 up- and 124 down-regulated genes in G392E NS cells, many of them belonging to well-defined signalling pathways. We focused on seven genes of the anti-oxidant response, confirming their overexpression in G392E NS cells and showing that anti-oxidants inhibition leads to apoptotic cell death of G392E NS cells (Guadagno et al, 2017). Our recent results, part of a PhD thesis work ending in October 2019, show alterations of the mitochondrial network in G392E NS cells: wild type NS neurons display a mitochondrial network that extends into neurites, while G392E NS neurons frequently present a perinuclear concentration of mitochondria. Furthermore, in these cells glutathione chelation with diethyl maleate (DEM) leads to mitochondria fragmentation, linking oxidative stress with mitochondrial alterations.

Oxidative stress is involved in several neurodegenerative disorders. In physiological conditions, reactive oxygen species (ROS) are produced as by-products of oxygen-using enzymatic reactions. This is particularly important in neurons, which satisfy their high energy demand by oxidative catabolism of glucose. Cytotoxic ROS are balanced by several antioxidant defence systems that can either scavenge ROS or prevent their formation (Malhotra and Kaufman, 2007). Cells are exposed to ROS throughout their life and they are considered as a key factor for molecular damage and ageing (Dufour and Larsson, 2004), while prolonged exposure to ROS can lead to cell death (Ryter et al., 2007). Disturbance of mitochondrial dynamics also contributes to neuronal dysfunction and death in neurodegeneration, which often involves mitochondrial fragmentation. Fission and fusion defects may limit mitochondrial motility, decrease energy production, promote oxidative stress and impair Ca^{2+} buffering, contributing to intrinsic death pathways (Knott et al., 2008; Lackner and Nunnari, 2009). The accumulation of NS polymers in the ER induces a leakage of ER Ca^{2+}(Davies et al, 2009), which may be part of the signalling pathway that leads from ER perturbation to mitochondrial dysfunction in our model. Moreover, our RNA sequencing analysis has uncovered alterations in genes involved in actin cytoskeleton dynamics, which may also play a role in the mitochondrial phenotype observed in G392E NS neurons.

Obiettivi della ricerca (generale e specifici)

In continuation with our previous work, our general objective is to fully understand the cellular pathways altered by the presence of NS polymers within the ER of neurons, which lead to neuronal death in FENIB.
Our specific aims are:
- To investigate if NS polymer accumulation activates the unfolded protein response (UPR) or the ER overload response (see below) in our neuronal cultures, since UPR activation has only been investigated in non-neuronal models of FENIB so far. In contrast with other neurodegenerative diseases, polymers of NS have so far failed to activate the UPR in cell models of FENIB, but neurons, being postmitotic, may show a different response to that of non-neuronal cells.
- To investigate the relationship between ER stress caused by NS polymer accumulation and mitochondrial alterations in our neuronal model of FENIB, by looking at the distribution of mitochondria associated membrane (MAM) sites, the points of ER-mitochondria communication, in G392E NS and control neurons, by immunostaining against a MAM-resident protein (Sigma-1 receptor) and by transfecting fluorescent reporters specifically designed to investigate the functional state of MAMs.
- To assess the role of Ca^{2+} in the mitochondrial phenotype, by using Ca^{2+} imaging in collaboration with Prof. Duchen (UCL, London).
- To investigate possible alterations of the actin cytoskeleton of G392E NS cells by staining with the actin-polymer specific probe phalloidin-FITC. If a phenotype is found, we will investigate its correlation with mitochondrial distribution by co-staining with Mitotracker®, and the role of cytoskeleton-related hits from our RNA sequencing data.

Stato delle conoscenze
The serpinopathies are a group of protein conformational diseases that comprises a series of heterogeneous pathologies caused by a common molecular mechanism: the polymerisation and deposition of mutant members of the serpin (serin proteinase inhibitor) superfamily (Gooptu and Lomas, 2009). The serpins are a large and conserved group of proteins that control the activity of serin proteases in the organism. Their suicide inhibitory mechanism is based on the flexibility of the metastable serpin molecule, which renders serpins vulnerable to mutations that increase their natural instability. Several such mutations have been identified as the cause of different pathologies, where mutant serpins form chains of polymers that accumulate within the endoplasmic reticulum (ER) of the cell of synthesis. This causes, for each mutant serpin, a loss-of-function phenotype due to the lack of active serpin in the extracellular place of action, and in some cases a gain-of-toxic-function phenotype due to intracellular polymer accumulation in the tissue of synthesis (Gooptu and Lomas, 2009).
The most striking serpinopathy is the autosomal dominant dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB) (Davis et al., 1999). This neurodegenerative condition has been described in patients carrying point mutations in neuroserpin (NS), an extracellular inhibitor of the serin protease tissue plasminogen activator, which is produced mainly in neurons and secretory cells of neural origin (Miranda and Lomas, 2006). The hallmark of the disease are intraneuronal inclusion bodies composed almost exclusively of NS and found mainly in the cerebral cortex, *substantia nigra* and hippocampus (Davis et al., 1999; Davis et al., 2002; Coutelier et al., 2008). Four of these mutations (Ser49Pro, Ser52Arg, His338Arg and Gly392Glu) were predicted to drive NS polymerisation at a rate directly proportional to the number of inclusions in the brain and inversely correlated to the age of onset of FENIB (Davis et al., 2002). Our studies demonstrated that each mutation caused intracellular polymerisation and ER retention of NS to a degree that correlated with the predicted polymerisation propensity (Miranda et al., 2004 and 2008). This work also

demonstrated a direct link between polymer accumulation and toxicity in a fly model of FENIB (Miranda et al., 2008), but the mechanism of this toxicity is still poorly understood.
After synthesis, serpin molecules fold in the ER and the monomeric serpin molecules are secreted. If there is a folding problem, the monomeric misfolded protein is recognised by the ER quality control surveillance system and gets degraded by ER associated degradation (ERAD) (Qu et al., 1996; McCracken et al., 1996; Miranda et al., 2004; Kroeger et al., 2009; Ying et al., 2011). We believe that a significant proportion of the mutant serpin monomers achieve a near native conformation, becoming invisible to the ER quality control, escaping ERAD and becoming available for polymerisation. Polymers are highly ordered and stable, and accumulate within the ER forming the inclusion bodies typical of the serpinopathies, without triggering the unfolded protein response (UPR), an ER stress signalling pathway activated by misfolded proteins (Davies et al., 2009). Instead, polymer accumulation activates the ordered protein response (OPR) characterised by NFkB signalling (Davies et al., 2009). It has also been reported that accumulation of another serpin, alpha-1 antitrypsin (AAT) within the ER leads to activation of caspases 9 and 12 in mouse and caspase 4 in human cells (Hidvegi et al., 2005; Rudnick et al., 2004), but the link between serpin polymers and toxicity is still poorly understood. Several cell models of FENIB reported so far have failed to show a clear toxicity phenotype, despite the fact that NS polymer accumulation in humans causes dementia in all cases known to date. In contrast, our recently published model of mouse neural stem cells differentiated to neurons shows apoptotic cell death caused by NS polymers upon reduction of the antioxidant defence (Guadagno et al., 2017), making it an optimal model to study cellular toxicity due to NS polymerisation.

Metodologie

Our methods are based in:

- Cell culture of neural progenitor cells and *in vitro* differentiation to neurons by use of purified signaling molecules; treatment of cultured cells with pharmacological compounds; assessment of cell growth, morphology and survival.
- Cell transfection and downstream analysis of luminescent reporters of UPR and ER overload response activation, and fluorescent reporters of the functional state of MAMs.
- Polyacrylamide gel electrophoresis (denaturing and non-denaturing) and western blot analysis of protein content in culture media and cell lysates.
- Immunofluorescence analysis with epifluorescence and confocal microscopy, of cells stained with organelle markers and by immunostaining against proteins of interest.
- ELISA quantification of monomeric and polymeric neuroserpin by use of house-made monoclonal antibodies.
- Real time RT-PCR quantification of gene expression.

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