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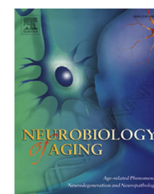

A comparison of frailty of primary neurons, embryonic, and aging mouse cortical layers

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A B S T R A C T

Superficial layers I to III of the human cerebral cortex are more vulnerable toward Aβ peptides than deep layers V to VI in aging. Three models of layers were used to investigate this pattern of frailty. First, primary neurons from E14 and E17 embryonic murine cortices, corresponding respectively to future deep and superficial layers, were treated either with $A\beta_{1-42}$, okadaic acid, or kainic acid. Second, whole E14 and E17 embryonic cortices, and third, in vitro separated deep and superficial layers of young and old C57BL/6J mice, were treated identically. We observed that E14 and E17 neurons in culture were prone to death after the Aβ and particularly the kainic acid treatment. This was also the case for the superficial layers of the aged cortex, but not for the embryonic, the young cortex, and the deep layers of the aged cortex. Thus, the aged superficial layers appeared to be preferentially vulnerable against Aβ and kainic acid. This pattern of vulnerability corresponds to enhanced accumulation of senile plaques in the superficial cortical layers with aging and Alzheimer's disease.

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1. Introduction

Impairment of cortical functions may occur not only during development (Rakic, 2009) but also later in aging, in relation with maintenance and degradation of the cortical networks (de Haan et al., 2012).

In normal aging, synaptic and dendritic loss occurs initially mainly in the frontal cortex, leading to gradually altered executive functions. Impaired vascularization, causing excitotoxicity and cell death, is implicated in the absence of neurofibrillary tangles (Buckner, 2004). There is also a progressive accumulation of extracellular amyloid β or Aβ peptides (Benilova et al., 2012), starting in the frontal cortex. Intracellular accumulation of abnormally phosphorylated tau proteins, forming neurofibrillary tangles (NFT), appears progressively in the aging hippocampal region but is then found in all neocortical regions affected by Alzheimer's disease (AD) in the presence of Aβ deposits (Braak and Braak, 1996; Braak et al., 2011).

Furthermore, lesions are differentially distributed inside cortical layers. Aβ deposits in aging and AD are preferentially localized in superficial layers II to III, compared to deep layers V to VI and to molecular layer I of the association cortex (Duyckaerts et al., 1986; Esiri and Chance, 2006). NFT are generally not observed in the inferior temporal cortex (ITC) or in the superior frontal cortex (SFC) of aging brains, whereas they are present in layer III of the ITC and rarely in the same layer of the SFC of patients with very mild AD. They are frequent in layer III as well as in layers V/VI in the ITC and SFC of severe AD (Morrison and Hof, 1997). A slightly preferential accumulation of NFT is generally observed in layer V compared to layer III of the association cortex (Leuba et al., 2009; Pearson et al., 1985). The differential vulnerability of cortical tissues and layers in normal and pathological aging is still an unsolved problem,

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particularly in relation to cell viability and death. It represents 1 of the major challenges in AD research.

In their seminal work, Romito-DiGiacomo et al. (Romito-DiGiacomo et al., 2007) showed that primary neurons, originating from either E14 or E17 embryos and grown for 4 to 7 days in culture, correspond to neurons of either the deep or the superficial cortical layers of adult animals, respectively. This observation allows the modeling of differential cortical vulnerability in culture dishes. We decided to investigate cell death in E14 and E17 neurons after $A\beta_{1-42}$ peptide treatments, similarly to what Romito-DiGiacomo et al. did, as well as differential viability, measured as intracellular ATP levels. This was done not only after Aß treatment, but also in the presence of okadaic acid (OA) and kainic acid (KA). These treatments reproduce alterations occuring in aging and AD: OA mimics increased phosphorylation of protein tau, that is, decreased Tau-1 and increased AT-8 immunoreactivities, and KA mimics cell death by excitotoxicity (Wang et al., 2005). All treatments influence the plasticity and the reduction of synapses (Wei et al., 2010). Importantly, we further investigated if the vulnerability of E14 versus E17 neurons in culture was similar to that of homogenized cortical tissues from E14 versus E17 embryos and from in vitro separated deep versus superficial layers of young and aged adult mice. Cux2, well known for its preferential expression in superficial cortical layers (Belgard et al., 2011; Cobos et al., 2006; Gingras et al., 2005), was used as a control for correct laminar separation and the postsynaptic marker PSD-95 to detect synaptic alterations (Leuba et al., 2008a, 2008b). Thus, these experiments were designed for reproducing the differential vulnerability of cortical layers in vitro leading to a better understanding of this phenomenon in aging and AD.

2. Methods

2.1. Mice, tissue samples, and dissections

Animals were manipulated in accordance with Swiss Veterinary regulations and institutional approval. C57BL/6J mice (Janvier, Le Genest-St-Isle, France) were sacrificed at embryonic days E14 and E17, at 1 to 3 months (young adult), and at >15 months (aged adult). Brains were quickly removed and cortices dissected under binocular microscope. Cortical neurons of embryos were grown in neurobasal medium with B27 supplement 2%, 0.5 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all chemicals from Life Technologies/Invitrogen) for 6 to 8 days (medium changed once) according to a standard protocol (Brewer et al., 1993). Alternatively the same embryonic tissue was immediately homogenized (~5 seconds with Polytron) in neurobasal medium.

The medial cortices of young and aged adult mice were separated in superficial (I to III) and deep (V to VI) layers at the level of layer IV, weighted and immediately homogenized in neurobasal medium. The medial cortex corresponds to the sensorymotor cortical regions obtained after removal of the most rostral and caudal parts of the cortex. Neurons in culture and homogenized tissues were used both in a cell viability and in a cell death assay, as well as in Western blot experiments. Chemical treatments were applied as described below.

2.2. Tissue and cell treatments

The homogenized cortical tissues and primary neurons grown in neurobasal medium with B27 supplement (see before) were either not treated or were treated as follows: (1) with okadaic acid (OA; Sigma) for 3 hours at 100 or 500 nmol/L; (2) with kainic acid (KA; Sigma) for 24 hours at 100, 500 μmol/L, and 5 mmol/L; (3) with $A\beta_{1-42}$ (Axxora) monomers or polymers for 24 hours at 40 and 80 μmol/L. Polymerization is favored by leaving Aβ monomers (2 mmol/L) 5 days at 37 °C in H_2O, before storing them at 4 °C (Lorenzo and Yankner, 1994). "Polymers" are defined here as samples with an enriched ratio of Aβ polymers versus Aβ monomers. All treatments were carried out in triplicate in an incubator at 37 °C, 5% CO_2, and 85% humidity.

2.3. Cell viability assay

For assessing viability of primary neurons or cortical tissues, 20,000 cells per well were grown in 2 mL of neurobasal medium for 6 to 8 days, as described before, or 1 mg freshly homogenized tissue was resuspended in 1 mL of the same medium. Neurons and tissues were treated in 1 mL with OA (3 hours), KA (24 hours), or Aβ monomers and polymers (24 hours). Photographs of the primary neurons were taken before and after treatments (Nikon Colpix 995), and the samples were then left 30 minutes at room temperature. A 100-μL quantity of cell suspension or tissue supernatant was agitated for 2 minutes with 100 μL of CellTiter-Glo Reagent prepared according to the manufacturer's instructions (CellTiter-Glo Luminescent Cell Viability Assay, Promega) to induce cell lysis and to release the cell ATP content, as an indicator of metabolic activity. After a 10-minute incubation at room temperature, luminescence (in relative light units) was recorded with a standard luminometer (Promega, GloMax 20/20). Medium without cells or tissue samples resulted in background luminescence.

2.4. Cell death assay

For assessing cell death of primary neurons or of the cortical tissues, 20,000 cells per well were grown in 2 mL of neurobasal medium for 6 to 8 days, as described before, or 1 mg freshly homogenized tissue was resuspended in 1 mL of the same medium. After the treatments (see previously), 50 μL of substrate prepared according to the manufacturer's instructions (Cytotox-Glo Cytotoxicity Assay, Promega, 0.5 ml of Assay buffer with 8.0 mg AAF-Glo substrate at 37 °C) was added to 100 μL of medium from each neuronal or cortical sample, mixed briefly and left at room temperature for 15 min. Luminescence (in relative light units) was recorded with a luminometer (GloMax 20/20, Promega) and corresponds to the amount of cell death proteases released in the medium by the cells killed after membrane deterioration by the treatments. Medium without cells or tissue samples resulted in background luminescence.

2.5. Western blots

Approximately 1 to 2×10^6 primary cortical neurons grown for 6 to 8 days were resuspended in a standard lysis buffer and sonicated (Bandelin Sonopuls HD200, 1 × 10 seconds). Cortical tissue was homogenized and then collected in the same way. Protein concentration was determined by the BCA protein assay (Pierce, Thermo Scientific). Proteins were denatured in Laemmli loading buffer.

A 20-μg quantity of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Ready gel 10%, Mini-Protean Tetra System, Bio-Rad, 60 V for 1 hour, then 90 V for 1 hour) and then electroblotted (overnight, 35 V; Mini Trans-Blot electrophoretic transfer cell, Bio-Rad) to a nitrocellulose membrane (Protran BA85, Schleicher and Schuell). A 10-μL quantity of Seeblue Plus 2 prestained standard (Invitrogen) was loaded as molecular weight standard. The membrane was incubated overnight at 4 °C with the primary antibody in TBS with 0.1% Tween and 5% BSA.

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