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Differential Sensitivity of Male and Female Neuronal cells May Play a Role in Onset of Neuronal Diseases

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Abstract
Due to the worldwide increase in the number of older people in both developed and developing countries, there is a public health concern for dealing with age related diseases such as neurodegenerative diseases. There is little known about the difference in neuronal cell responses between genders. Our understanding of the neuronal cell response regarding genders, will be useful for developing more efficient therapies for these diseases such as Alzheimer's disease. To investigate gender differences in neuronal cell response against cell death inducers, we examined the percentage of cell death in male and female mouse primary cortical neuronal cultures. Our findings indicate that there is a difference in cellular response to ethanol (a cell death inducer) that may be the basis of how they behave in vivo of what may be seen in adults, as they age. These observation support this idea that genetic factors, most likely governed by X or Y chromosomes.

Keywords: *Gender, Neuronal cell death, Apoptosis, Neurodegenerative disease*

Introduction

As a consequence of global aging of the human populations, the occurrence of cognitive impairment and dementia is rapidly becoming a significant burden for medical care and public health system. By the year 2020, the WHO predicts there will be nearly 29 million demented people in both developed and developing countries (1). Understanding the reasons for differences between populations in genetic vulnerability and environmental exposures may help to identify modifiable risk factors that may lead to effective prevention of vascular and Alzheimer dementia (1).

Incidence rates for Alzheimer's disease (AD) and vascular dementia appear to behave differently, with an increased risk of AD for women as compared to vascular dementia(2). Women tended to have a higher incidence of Alzheimer's disease in very old ages, and men tended to have a higher incidence of vascular dementia at younger ages (3).

Dementia is a major public health concern in aging societies. Its prevalence increases with age, after 65 years of age, and about 20% of the individuals aged 80 years or older are affected by the disease. The most frequent dementia is AD which is associated with high levels of morbidity, mortality, burden of care and socio-economic costs (4).

Therefore, interventions that delay the onset of the disease are crucial and the identification of risk factors, such as tobacco or alcohol consumption is good candidates.

Many neurodegenerative disorders including AD are characterized by a progressive cell loss

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of a particular population of neurons in specific brain regions (5). Many authors suggest that excitotoxicity is involved in the pathogenesis of most neurodegenerative diseases (6). The regulation of this selective cell killing and the exact cause of neuronal cell death are not fully understood. Cells die and can elicit different signaling pathways for their demise. A marked interest among cell biologists and neuroscientists has been generated by the findings that a deregulated of Cyclin Dependent Kinase 5 (Cdk5) is neurotoxic and has been linked to neurodegenerative diseases, but the roles of its all players remain controversial (7). In particular there is insufficient knowledge about sexual skewing the cell responses in challenge and cell death pathways (8).

Here we report a gender-based difference of neuronal cell response to challenge. These findings will be useful for prevention, diagnosis and treatment of gender related diseases.

Materials and Methods

Mice Swiss Webster mice which had 2.5 months age, were placed together and females were checked for vaginal plugs 12-16 h later and designated as day 0.5. Pregnants were sacrificed by CO_2 and cervical dislocation at specific days (ED=16.5) of gestation. Their embryos were removed, sexed, and decapitated. For each gender 50 embryos were used.

Cell culture and treatment Cortical tissues of embryos were dissected and meninges and blood vessels were removed. Dissected tissues were placed in Earl's balanced salt solution, Ca^{++} and Mg^{++} free. The cells were dissociated by treatment with 0.125% trypsin/EDTA cocktail at 37˚C for 10 min. Tissues were triturated 20 times in a Pasteur pipet, and debris was allowed to settle out. The supernatant, containing single cells was filtered through a 70 µM nylon–mesh strainer. Cells were resuspended in Neurobasal Media with 2% B27 neuronal supplement (Gibco) and seeded onto poly-L-lysine (Sigma) coated coverslip at 200,000 cells/ml or into flasks at 10^6 cells/ml and grown at 37˚ C in 5% CO_2. Media were refreshed every three days. In this media, only neuronal cells can grow, because this media does not have serum. On the 7th day of culture, the cells of each sex were divided into two groups, one is control group without any treatment and one is experimental group which treated with 2% ethanol (AAPER alcohol & chemical CO, absolute,200 proof) per ml of media for 24 h. Cell viability and apoptosis were determined by different methods.

Cell viability by trypan blue Dying cells, whether necrotic or apoptotic, exhibit alterations in membrane function. The loss of membrane integrity in dead and dying cells allows the preferential uptake of labels like trypan blue (9). At the end of incubation, cells were pelleted and washed with PBS. Well suspended cells (50µl) were mixed with 50 µl of 0.4% trypan blue (Sigma, St Louis, MO, 0.4% trypan blue in 1X PBS, pH 7.4) and incubated at room temperature (RT) for 5 min. Cells were viewed under a microscope and blue stained cells were considered non-viable.

Staining of apoptotic cells with bis-benzimide The nuclear fragmentation of apoptotic cells was assessed by staining the treated cells with the DNA fluorochrome bis-benzimide (Hoechst 33258). Briefly, cells were scraped, pelleted and washed with 1 X PBS and resuspended in 3% paraformaldehyde in 1 X PBS, and incubated with 16 µg/ml bis-benzimide in PBS at RT for 25 min. Ten µl of cell suspension in bis-benzimide was placed on a slide and cells were examined using a Leitz fluorescence microscope. Cells with condensed chromatin or fragmented nuclei were considered to be apoptotic.

DNA fragmentation assay DNA fragmentation was detected by using TUNEL POD (Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling Peroxidase) combined with nonisotopic digoxigenin-11dUTP and terminal transferase according to manufacturer's instructions (Roche Molecular Biochemicals, Germany). Briefly, slides were incubated with

permeabilization solution (0.1% TritonX-100 in 0.1% sodium citrate) on ice for 2 min, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol at RT for 30 min. After 2 rinses in PBS, TUNEL reaction mixture (TUNEL label and TUNEL enzyme, 9:1 v/v) was applied to slides, which were incubated for 30 min at 37° C followed by three washes with 1 X PBS. TUNEL POD was then applied to the slides to bind to the FITC-dUTP which enzymatically added to the free end of the oligonucleotide (as described in TUNEL-POD kit) and the reaction was visualized with DAB (diaminobenzidine, Research Genetics, Huntsville, AL). The cell monolayers were counterstained with methylene blue and mounted with Permount® (Fisher Scientific, Burr Ridge, IL).

Immunohistochemistry *In situ* protein levels of Cdk5 gene were detected by using the ABC (Avidin-Biotinylated-Peroxidase Complex) kit (Vectastain ABC kit, Vector Laboratory, Burlingame, CA). Monolayer of cells were quenched with 0.3% hydrogen peroxide in methanol at RT for 20 min to abolish endogenous peroxidase activity. After three washes with 1X PBS (0.1% Tween 20 in PBS), cells were incubated in blocking solution at RT for 1 h and treated with primary antibody (0.5 µg/ml for Cdk5, 0.1% µg /ml) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) at 4ºC in a humidified chamber overnight. Following three washes with 1X PBS, secondary biotinylated antibody was applied to the cells for 1 h at RT. Cells were then washed with PBST 3 times and incubated with ABC reagent for 2 h at RT. Cells were again washed with PBST 3X before being visualized with DAB, counterstained with methylene blue, and mounted with Permount.

Results

Comparing the sensitivity of male and female neuronal cells to challenge The response of each sex's neuronal cells to ethanol was examined by the determination of cell viability by first using trypan blue dye exclusion and cell count. We found that the neuronal cells from male and female responded similarly to the culturing conditions.

The plates were coated with Poly-L-Lysine and we used neurobasal media with 2% B27 supplement. In these two conditions neuronal cells would settle onto the plate after 24 h. At the first day the cells were undifferentiated (Fig. 1-A) but after 7 d they became differentiated and took the typical shape of neurons with processes resembling axon and dendrites (Fig. 1-B). These cells divided very slowly, but female neuronal cells divided slightly faster than male ones.

There was no difference between the morphology of the male and female cells in cell culture. Under the influence of ethanol the shape of cells was changed and processes of neurons and shape of perikaryon became abnormal and population of cells were decreased in both genders (Fig. 1-C).

Fig 2 shows the effect of ethanol as a cell death inducer on male and female neuronal cells. The percent of cell death is calculated by taking the number of dead cells counted, dividing this number by total number of cell counted and multiplying by 100%. Since these are primary cultures, survival in each set of experiments varies and there is often some cell death in the control culture. The percent of death in male cells was compared with that of female cells. Two way analysis of variance (ANOVA) was used to analyze the differences in cell number between males and females obtained from three independent experiments. We found that male neuronal cells were significantly more sensitive to ethanol than female cells.

Apoptotic nature of cell death under ethanol influence To determine if the cell death in both genders was apoptotic, we used the Hoechst dye and *in situ* DNA fragmentation. Hoechst staining was used to examine nuclear fragmentation, one of the most reliable indications of regulated apoptotic cell death (Fig. 1-

D). We found a direct correlation between the incidence of trypan blue positive cells and the appearance of fragmented nuclei by Hoechst staining. These data were further confirmed by *in situ* DNA end labeling (Figs. 1-E, F, G, H). These observations indicate that cell death in these developed cultures is mostly by apoptosis.

Correlation of Cdk5 and neuronal cell death under ethanol influence Cdk5 expression is another mark of apoptosis. Therefore we examined the expression of Cdk5 by immunohistochemistry. The number of cells in 3 randomly selected fields (40X) of each slide was counted using a Leitz microscope. We found that Cdk5 was expressed in control cells, representing the few cells that die normally due to the placement in cell culture, but under the influence of ethanol, the expression was increased and was greater in male neuronal cells than in female ones. Thus, the level of Cdk5 expression corresponds with the level of cell death induced by ethanol.

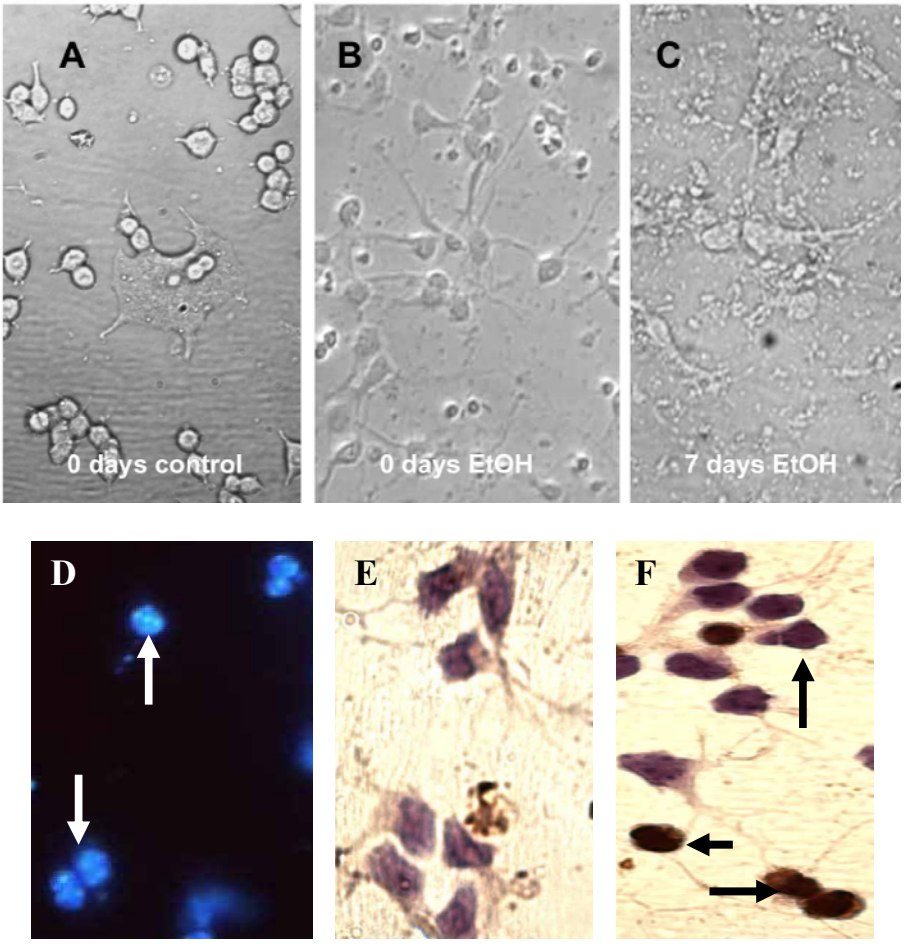

Fig. 1: Neuronal cell culture from cerebral cortex of male and female mice embryos. Upper row: A: The first day of culturing, the cells are undifferentiated; B: 7 days of culture. Neuronal processes can be seen in these cells, indicating differentiation; C: neuronal cells after 8 days in culture followed by 24 h in 2% ethanol. Under this condition, the cells were shrinking and the number of cells was decreased.
Lower row: D: Hoechst staining ethanol exposed neuronal cells. Condensed and fragmented nuclei in dying cells (arrows) are characteristic of apoptosis (100 X). E&F: TUNEL assay on sections of neuronal cells (E: control group, cells without treating, F: experimental group that cells exposed to ethanol), showing fragmented DNA (arrows), indicating that these cells die by apoptosis.

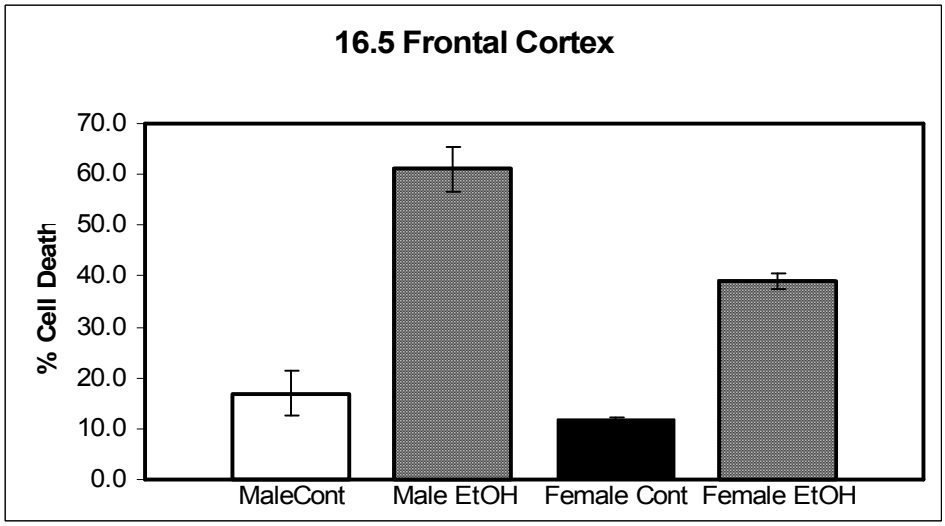

Fig. 2: Male and female frontal cortex cells (ED=16.5) exposed to ethanol (EtoH) as a cell death inducer.

Discussion

Clinical dementia has become one of the most serious and persistent health problems affecting persons over the age of 65. Efforts to find a cure for AD and other causes of dementia are ongoing, while healthcare executives continue to face a number of challenges (10). Understanding the cellular and molecular basis of dementia can help in directing specific treatment that could delay the onset of symptoms which would have a major impact on public health.

As presented in introduction, there appears to be a skewing in the gender differences. Our experiment reported here address the possibility of differential cellular response that may be a reflection of what is seen in the adult situation presented alone.

In our investigation we have tried to eliminate the differential hormonal effect by isolating cells from brain tissue of male and female embryos and placing them in a common environment. In this way the cellular behavior would more specifically reflect the intrinsic factors present in the different cells. Our data indicate that embryonic neuronal cells from male and female embryos seem to be differently sensitive or resistant to environmental challenges. These observations support the findings of Nikezic-Ardolic et al, who proposed genetic factors, most likely governed by X or Y chromosomes (11).

Our data from Hoechst staining and *in situ* DNA fragmentation (TUNEL) confirmed that ethanol triggers an apoptotic neurodegenerative response. The window of vulnerability to ethanol-induced apoptosis (12) differed between the two genders. Apoptosis can be induced via an extrinsic pathway through ligation of death receptors situated in the plasma membrane, or via intrinsic pathways triggered by intracellular organelles sensing disturbances of cellular homeostasis (13). Ethanol-induced apoptotic neurodegeneration involves the intrinsic pathway (6). The male cells showed more sensitivity, suggesting that there are genetic differences in sensitivity between the sexes.

The higher expression of Cdk5 in neuronal cells which are exposed to ethanol(male control 5.1% vs male+ethanol 33.7% and female control 2.2% vs female+ethanol 25.4%) suggests the activity of Cdk5 in neuronal cell death (14, 15) and also the higher expression of Cdk5 in male neuronal cells than female neuronal cells (male+ ethanol 33.7% vs female +ethanol cells 25.4%) suggest that members of cell sur-

vival/death protein families may be different between the two genders, and these differences can alter the vulnerability of each gender to specific diseases, because each agent can trigger specific pathways and activate specific cell survival/death protein pathways.

These data are one of the first demonstrations of sexual dimorphism in neuronal cell signaling. The association of signaling differences with differences in cell survival not only confirms the observation of Luine and McEwen (16) 20 years ago that proposed gender creates a context resulting in a different response to the same biological stimulus, but it also offers a potential mechanistic basis for gender-related differences in susceptibility to cognitive and affective illness, brain disorders in which neuronal and glial death are increasingly believed to play an etiopathologic role (17). At the very least, studies of signal transduction must specify the sex of origin of cells used in *in vitro* studies to avoid spurious conclusions.

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