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Full Length Article

Impact of selective serotonin reuptake inhibitors on neural crest stem cell formation

Cecilia Vichier-Guerre, Margaret Parker, Yael Pomerantz, Richard H. Finnell, Robert M. Cabrera*

Dell Pediatric Research Institute, The University of Texas at Austin, Austin, TX 78712, United States

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ABSTRACT

The use of antidepressants in pregnant women is rising, with rates up to 7.5% in the United States. Selective serotonin reuptake inhibitors (SSRIs) are currently the most common antidepressant prescribed to pregnant women. The teratogenic effects of SSRI exposure are debated because of discrepancies in epidemiological studies. As an alternative to epidemiological and animal studies, human embryonic stem cell research (hESC) provides a human-based experimental model to examine the risks of prenatal SSRI exposure. Neural crest stem cells (NCSCs) play an important role in craniofacial and cardiac development as precursors to craniofacial bones and heart septa. This study examines the effects of paroxetine (Paxil) and sertraline (Zoloft) exposure on proliferation, migration, and AP-2α protein expression of NCSC *in vitro*. hESCs were exposed to paroxetine and sertraline at three concentrations while undergoing directed differentiation into NCSCs. Our results indicate exposure to paroxetine significantly increased proliferation, migration, and AP-2α protein expression in NCSCs. Exposure to sertraline significantly decreased proliferation and significantly increased AP-2α protein expression in NCSC. This evidence suggests paroxetine and sertraline alter normal NCSC behavior and may thereby disrupt cardiac and craniofacial development.

1. Introduction

It has been reported that 10–15% of pregnant women have symptoms of depression during pregnancy (Andrade et al., 2008; Evans et al., 2001). Antidepressants are used by up to 7.5% of pregnant women in the United States, with selective serotonin reuptake inhibitors (SSRIs) being the most commonly prescribed (Huybrechts et al., 2013). Epidemiological studies have reported that SSRI use during pregnancy increases the risk of low birth weight (Huang et al., 2014), preterm birth (Huang et al., 2014), persistent pulmonary hypertension (Chambers et al., 2006; Huybrechts et al., 2015), and congenital malformations (Furu et al., 2015), including congenital heart defects (Bérard et al., 2015; Wemakor et al., 2015), and craniofacial defects (Bérard et al., 2015).

The majority of epidemiological studies of prenatal SSRI exposure are unable to control for the effects of maternal depression and confounding by indication. Live-birth studies also risk Type II errors, due to spontaneous abortions often associated with embryo toxicity or medical termination of pregnancy. The diversity and limitations of epidemiological studies have resulted in controversy regarding the risks associated with prenatal SSRI exposure (Yonkers et al., 2009; Holmes 2010). For example, one study determined a significant increased risk of cardiac defects with paroxetine or sertraline use during pregnancy (Louik et al., 2007), whereas a more recent study reported the relative risk of any cardiac malformation was 1.25 (95% CI 1.13–1.38) with any SSRI, but depression-restricted analysis indicated an OR of 1.12 (95% CI 1.00–1.26) for SSRI exposure and cardiac malformation, and the study ultimately reported no significant associations between any SSRI [OR 1.06 (95% CI: 0.93–1.22)] after additional adjustment using Propensity-Score Stratification (Huybrechts et al., 2014).

Human embryonic stem cell (hESC) research provides an alternative human-based model to elucidate potential teratogenic effects of prenatal SSRI exposure (Ebert and Svendsen, 2010). By differentiating into all three embryonic germ layers and their derivatives, hESCs can be used to identify potential teratogens and their effects on the formation of many different tissue types (Thomson et al., 1998). Testing using hESC has a number of distinct advantages compared to observational and epidemiological studies. Specifically, hESC research can be conducted with experimental designs as opposed to descriptive and correlational designs; it can also provide predictive results that indicate the underlying mechanisms of toxicity or teratogenicity.

One important cell type that can be derived from hESCs are the neural crest stem cells (NCSCs), which are often referred to as the fourth embryonic germ layer by developmental biologists due to their

* Corresponding author.
E-mail address: rcabrera@austin.utexas.edu (R.M. Cabrera).

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multipotency (Menendez et al., 2013). In early human development, at approximately 4-weeks post-fertilization, NCSCs migrate from the ectoderm of the neural tube to differing regions of the body, where they contribute to a diverse range of cell types including craniofacial bones, sensory nerves, autonomic nerves, Schwann cells, and heart septa (Kirby et al., 1983; Graham et al., 1996; Joseph et al., 2004; Shakhova and Sommer, 2010).

Loss or ablation of NCSC produces cardiac defects (Kirby et al., 1983). Specifically, the neural crest cell population influences outflow tract septation, and the cardiac phenotypes produced by neural crest ablation include outflow tract or conotruncal defects, such as persistent truncus arteriosus (Nishibatake et al., 1987). Developmentally, the head, face, and heart can be considered as a single morphogenetic field or a cardiocraniofacial developmental module. A morphogenetic field or module is a spatial domain in which every part has a stage determined by the state of neighboring parts so that the whole has a specific relational structure (Kirby et al., 2003; Keyte and Hutson, 2012).

The function of serotonin as a neurotransmitter and influence on the CNS is the basis of SSRI activity. However, the naming and function of serotonin (5-HT) is actually based on the activity of 5-HT as a serum vasoconstrictor (Rapport et al., 1948). These initial studies observed a biphasic response, with initial dilation followed by constriction of the vasculature due to 5-HT (Page and McCubbin, 1953). The influence of 5-HT and serotonergic compounds on human arteries is well-documented (Haugen and Rognerud, 2001; Haugen, 2008; Delaney et al., 2013). The biphasic nature of this response is reported to function by signaling at different 5-HT receptors at different 5-HT concentrations (Saxena, 1989; Ullmer et al., 1995). Serotonin also plays a key role in regulating NCSC migration, which implicated serotonin-disruption as a possible mechanism of SSRI-linked defects (Moiseiwitsch and Lauder, 1995). Experiments have also indicated that physiological concentrations of serotonin influence migration and proliferation of mouse neural crest cells (Lauder et al., 1988; Narboux-Nême et al., 2008; Kawakami et al., 2011). Other animal models have shown some SSRIs, including sertraline, promote neurogenesis by protecting rat neural stem cells against lipopolysaccharide-induced cellular damage (Chiou et al., 2006; Peng et al., 2012), but there remains a lack of research regarding potential effects of SSRIs on human NCSC formation.

Various cellular markers can be used to follow the induction, migration, and differentiation of NCSC. Slug is a zinc finger transcription factor and is one of the earliest markers expressed by neural crest cells (Kirby et al., 2003). Other transcription factor families expressed by neural crest cells in the early differentiation include Sox, Pax and AP-2 (PMID: Werner et al., 2007). In this paper, we report altered AP-2 alpha (AP-2a), a transcription factor important for neural crest development. Mutations in AP-2a can produce Branchiooculofacial syndrome (BOFS) in humans and cranioabdominoschisis with facial dysmorphogenesis in mice (Zhang et al., 1996). Brewer et al. reported AP-2a is also required for normal outflow tract formation (Brewer et al., 2002). They also observed DORV and a low frequency of PTA, and a higher incidence of aortic arch artery defects were found in AP-2a mutant mice.

This study examines the potential effects of sertraline (Zoloft) and paroxetine (Paxil) exposure on neural crest stem cell function *in vitro*. Specifically, hESCs were exposed to sertraline and paroxetine while undergoing directed differentiation into NCSCs. Three NCSC behaviors were assessed: proliferation, migration, and AP-2α protein expression; AP-2α is a transcription factor vital to craniofacial development (Zhang et al., 1996). Since NCSCs contribute to craniofacial bones and heart septa, the data test the capacity for paroxetine and sertraline exposure to alter human NCSC behavior. We hypothesize that abhorrent change to NCSC behavior is expected to increase the risk of cardiac and craniofacial birth defects.

2. Methods and materials

2.1. NCSC differentiation

Human ESCs (H9) were obtained from WiCell Research Institute (UW-Madison). Cells were individualized and passaged (passage number 29) with StemPro Accutase Cell Dissociation Reagent (Life Technologies, Grand Island, NY) and seeded at a concentration of approximately 92,000cells/cm^2 on Matrigel matrix (Fisher Scientific, Pittsburg, PA) in hESC maintenance medium [DMEM/F12 (Life Technologies, Grand Island, NY), bovine serum albumin (BSA, 20%, Life Technologies, Grand Island, NY), penicillin-streptomycin (1%, Life Technologies, Grand Island, NY), Glutamax (1%, Life Technologies, Grand Island, NY), NEAA (1%, Life Technologies, Grand Island, NY), human transferrin (10 μg/mL, Life Technologies, Grand Island, NY), trace elements A (0.1%, Corning, Tewksbury, MA), trace elements B (0.1%, Corning, Tewksbury, MA), trace elements C (0.1%, Corning, Tewksbury, MA), ascorbic acid (200 μM, Fisher Scientific, Pittsburg, PA), heregulin β-1 (10 ng/mL, Peprotech, Rocky Hill, NJ), activin A (10 ng/mL, Peprotech, Rocky Hill, NJ), LONGR3 IGF-1 (200 ng/mL, Peprotech, Rocky Hill, NJ), FGF2 (8 ng/mL, Stemgent, Cambridge, MA)]. The day after passaging cells, the media was replaced with NCSC media [DMEM/F12, 20% BSA, 1% penicillin-streptomycin, 1% Glutamax, 1% NEAA, 0.1% trace elements A, 0.1% trace elements B, 0.1% trace elements C, 10 μg/mL human transferrin, 200 μM ascorbic acid, 10 ng/mL heregulin β-1, 200 ng/mL LONGR3 IGF-1, 8 ng/mL FGF2, SB431542 (20 μM, Tocris, Bristol, UK), and GSK3 inhibitor IX (4 μM, Stemgent, Cambridge, MA)]. The media was replaced daily. Cells were passaged with Accutase four days after the previous passage and seeded at a concentration of approximately 92,000cells/cm^2 on Matrigel matrix with rock inhibitor (Y-27632, 10 μM, STEMCELL Technologies, Vancouver, BC, Canada). The NCSC differentiation ended fifteen days after cells had been exposed to NCSC media.

During cell culture and assay development, NCSC were identified phenotypically and were confirmed by flow cytometry. The cell markers were consistent with the cellular phenotypes and confirmed stem cell (Pax-2) to NCSC differentiation (Sox10, AP-2a) (data not shown). AP-2a was selected as the NCSC differentiation marker for this study.

2.2. Paroxetine and sertraline exposure

The experimental layout consisted of six experimental treatments and a control. Paroxetine and sertraline were added to the NCSC media at three concentrations: a low-dose of 30 nM, a medium-dose of 300 nM, and a high-dose of 3000 nM. The control contained NCSC media without paroxetine or sertraline. Control cells were treated with 0.1% of the vehicle, dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO). NCSC differentiation was conducted on 6-well plates. Paroxetine and sertraline exposure lasted for 15 days, the total amount of time for NCSC differentiation.

2.3. NCSC proliferation measurement

Cells were counted during each passaging. Passaging occurred before cells approached 70–80% confluent, which occurred every three to four days throughout the fifteen-day differentiation process. Cells were washed with 1× PBS and released with Accutase as indicated by the manufacturer. The cells were counted manually on a Neubauer hemocytometer with the aid of a tally counter. The total number of cells was calculated from the number of counted cells. The frequency of cell cycles per day (f) was calculated from the total number of cells at the time of passaging (N_t), the initial number of cells was 920,000 cells (N_o), and the time between passages (t) with the equation: $N_t = N_o 2^{tf}$ (Sherley et al., 1995). Each time the cells were passaged, the cells were counted. In total, four proliferation assays were measured, including the final fifteenth day. The presented data is an average of percent