Environmental Research (2015) 731–738

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Environmental factors may contribute to autism development and male bias: Effects of fragrances on developing neurons

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ARTICLE INFO

Article history:
Received 24 June 2015
Received in revised form
20 August 2015
Accepted 25 August 2015

Keywords:
Autism spectrum disorders
Fragrance
Neurotoxin
Male bias
Neuroblastoma cell lines
Oxytocin
Arginine vasopressin

ABSTRACT

Background: Autism spectrum disorders (ASDs) are developmental conditions characterized by deficits in social interaction, impairments in verbal and nonverbal communication, and stereotyped patterns of behavior. Previous studies have implicated environmental factors in the development of ASD. Although no reliable neurophysiological network is associated with ASD, low levels of plasma oxytocin (OXY) and arginine vasopressin (AVP) have been reported. The "twin" nonapeptides OXY and AVP are mainly produced in the brain of mammals, and dysregulation of these neuropeptides has been associated with changes in behavior, especially social interactions.
Methods: Previously, we analyzed 91 commonly used fragrances and reported significant mutagenic, neurocytotoxic, and stimulatory effects on fetal neuroblastoma cell lines (NBC). In this study, we analyzed the neuromodifications of three selected fragrances on male and female human fetal brain neurons, utilizing immunohistochemistry.
Results: We show that exposure to femtomolar concentrations of fragrances results in morphological changes by light microscopy in the NBC. Importantly, these fragrances significantly reduced the OXY- and AVP-receptor positive (OXYR+ and AVPR+) neurons in male NBC but not in female NBC, possibly contributing to the development of male bias in ASD.
Conclusion: This study is the first to show a potential link between fragrance exposure, depletion of OXYR+ and AVPR+ neurons, and a male bias in autism.

1. Background

In the United States, it has been estimated that 1:68 children have autism spectrum disorders (ASD), with ratios of approximately 1:42 for boys and 1:189 for girls (Lai et al., 2014; Geschwind, 2009; Lichtenstein et al., 2010; CDC, 2010). Despite intense research on ASD over the last several decades, its underlying etiology remains unidentified (Lai et al., 2014; Geschwind, 2009; Lichtenstein et al., 2010; CDC, 2010). In addition, an inexplicable bias toward males exists in classical autism by a ratio of ~5:1 (Lai et al., 2014; Geschwind, 2009; Lichtenstein et al., 2010; CDC, 2010), and ~10:1 in Asperger's syndrome (AS) (Lai et al., 2014; Geschwind, 2009; Lichtenstein et al., 2010; CDC, 2010; Rutter et al., 2003; De Bruin et al., 2006). Some hypotheses suggest that increased fetal testosterone correlates with increased prevalence of autism and male vulnerability (Baron-Cohen et al., 2004, 2005; Chura et al., 2010; Knickmeyer and Baron-Cohen, 2006). Even so, these hypotheses fail to fully explain a significantly increasing prevalence of autism, from 1:10,000 in the 1960s to around 1:100 today (CDC, 2010).

We investigated whether the development of autism could be related to exposure to fragrances, which are complex mixtures of chemicals, including hormone disruptors (Parlett et al., 2013; Dodson et al., 2012; Braun et al., 2014; NIH, 2010). Previously, we analyzed over 90 fragrances and demonstrated that commonly used fragrances have potential to cause neurological damage to a

Abbreviations: NBC, neuroblastoma cells; ASD, autism spectrum disorders; AVP, arginine vasopressin; AVPR+, arginine vasopressin receptor positive; OXY, oxytocin; OXYR+, oxytocin receptor positive; EMEM, Eagle's Minimum Essential Media; FBS, Fetal Bovine Serum; PBS, phosphate buffer saline; Ab, antibodies; TS, Turner's syndrome

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http://dx.doi.org/10.1016/j.envres.2015.08.025
0013-9351/

developing fetus by introducing mutations and depleting selected neuronal subtypes, which potentially may interfere with normal brain development (NIH, 2010). For this research, we selected three fragrances (A, B, and C) that were previously identified to have significant cytotoxic effects on neural cells and known to impart significant neuromodifications at morphological and immunological bases (Bagasra et al., 2013).

Numerous studies have shown that children with autism have significantly lower levels of OXY and AVP in plasma samples than their typical peers (Meyer-Lindenberg et al., 2011; Insel, 2010; Xu et al., 2013). A recent study also showed that mothers of ASD children have low levels of OXY and AVP, but high levels of testosterone (Xu et al., 2013). In addition, in normal children, lower concentrations of OXY in plasma are associated with lower social and cognitive functioning (Harony and Wagner, 2010; Miller et al., 2013; Insel, 2014; Feldman et al., 2007). Further, AVP, OXY, and their respective receptors, show similar sequences in animal studies, allowing these peptides to activate each other's receptors (Chini and Manning, 2007; Schorcher-Petcu et al., 2010). Animal and human studies have indicated sex differences in the AVP and OXY, such that males appear to have enhanced AVP and OXY functioning compared to females; these differences, however, vary by brain region and species (Sue Carter, 2007).

We hypothesize that fragrances, which are complex chemical mixtures, may have differential effects on the human neural development and differentiation. To investigate these effects, we utilized human neuroblastoma cell lines (NBC) from male and female origins as an *in vitro* developing fetal brain model. These cell lines represent a suitable model since they possess biochemical properties of human neurons *in vivo* (Sherer et al., 2001). Furthermore, these cell lines are acquired from human tumors, which encourage continuous division and provide required quantities of cells for investigations without significant changes in variability (Biedler et al., 1978). Here we find that male NBC, but not female NBC, exposed to femtomolar concentrations of certain fragrances results in significant reductions in both OXY and AVP receptor positive neurons. Interestingly, exposure to femtomolar concentrations of fragrances results in neuromodifications by light microscopy in both male and female NBC, but more significantly in male NBC. Our studies are the first to reveal a direct connection between fragrance exposures and neuromodifications that may contribute to autism and provide insight into gender bias.

2. Experimental procedures

2.1. Reagents and cell lines

All NBCs were purchased from ATCC (New York, NY). SH-SY5Y CRL 2266 (designated 2266) of female origin and M17 CRL 2267 (designated 2267) of male origin were cultured in Eagle's Minimum Essential Media (EMEM) (ATCC, Manassas, VA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), and L-glutamine–penicillin–streptomycin solution (Sigma, St. Louis, MO) at 37 °C, 5% CO_2. Cells were exempted from consideration as human subject research by Claflin University Institutional Review Board. The stock cell cultures were grown in 25 mL or 75 mL flask (Thermo-Scientific, Nunc, Rochester, NY). A total of 91 commercially available fragrances were initially used to carry out mutagenic assay by the Ames method and screening on NBCs for neuromodifying effects of each of the fragrances (Bagasra et al., 2013). Three fragrances with high mutagenic and neurocytotoxic effects were selected to carry out the detailed analyses described below.

2.2. Immunostaining

For immunocytochemistry studies, each cell line was grown in 8-well chamber slides (Thermo Scientific, Nunc, Rochester, NY) with $\sim 1 \times 10^4$ cells in 0.5 μL of media for 4–7 days. For seeding the NBC, the stock cell cultures grown in the flask were gently washed once with 1 × PBS followed by trypsinization until single cell suspension and inactivation of trypsin with 1 mL FBS. The cells were counted on a Hemocytometer chamber and adjusted to 1×10^5 cell/mL. The 8-well chamber slides were labeled and 100 μL of cells were then added to each well of the respective 8-well chamber slides, and 1 μL of fresh media was added containing diluted concentrations ($1{:}10^6$) of fragrances. For the present study, three fragrances were selected (A, B, C) that were previously shown to be highly mutagenic and caused neuromodifications among 91 fragrances tested (Bagasra et al., 2013). Two wells of the chamber were used as a control with EMEM without fragrances. Slides were then observed under the microscope. Between 4 and 5 days after culture, the media was removed from the slides and the cells were fixed by adding 1 mL of Streck Tissue Fixative (STF, Streck Laboratories Inc, LA Vista, NE), a non-ionic fixative, in each well and allowed to set overnight at 4 °C. The wells were then washed gently three times using 1 × sterile Phosphate Buffered Saline (PBS) (Fisher Scientific, Fair Lawn, NJ) and then soaked with the blocking agent (containing 2% Bovine Serum Albumin (BSA) in 1 × PBS) for 10 min. All antibody dilutions were carried out in 2% BSA in 1 × PBS.

2.3. Differential effects of fragrances on female and male NBC

Both female and male NBC were seeded into separate Nunc 8-well chamber glass slides and cultured without fragrances (controls) or with $1{:}10^6$ dilution fragrances A, B, and C. After 4–5 days, cells were fixed with STF, overnight, and stained by the immunohistochemistry (IHC) method to observe the intensity and distributions of AVPR or OXYR, and by H&E staining to analyze the neuronal morphology and characteristics.

The primary antibodies (Ab) used included polyclonal OXY (rabbit) (ABCAM, Cambridge, MA) and polyclonal antibodies; vasopressin polyclonal (rabbit), AVPRIα (goat) and AVPRIβ (goat), (Thermo Scientific, Rockfield, IL). The primary Abs were diluted in blocking buffer according to the manufacturers protocols. In each chamber, 100 μL of diluted primary Ab (pAb) was added to each well. The slides were incubated at 4 °C overnight and then washed three times in 1 × PBS. The appropriate fluorescein conjugated anti-goat (Rockland Immunochemicals, Gilbertsville, PA), or anti-rabbit (Chemicon International, Temecula, CA) secondary antibodies used were at a working dilution of 1:20 with 100 mL in each well. The slides were incubated for 1 h at 37 °C in humidified chambers and then washed three times with PBS with 10 min of incubations each time. The tops of the 8-well chamber were carefully removed without disturbing the fixed cells. The cells were washed in PBS three times and then mounted with glass coverslips, using a drop of mounting solution containing 50% glycerol and 50% PBS. The slides were observed at resolutions 10 ×, 40 ×, and 1000 × (with oil) using an Olympus BX51 fluorescent microscope. Each experiment was repeated at least 10 times. The slides were analyzed independently in a blinded fashion by four different observers from two different locations. The agreement between observers was approximately 87%. The immunological studies included determination of up or down expressions of OXYR+ or AVPR+ antigens, percentage of reduction of the receptor positive or negative neurons, and the detailed morphologic and morphometric analyses.