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Gretchen Neigh, *Emory University*
Christina L. Nemeth, *Emory University*
Sean D. Kelly, *Emory University*
Emily E. Hardy, *Emory University*
Chase Bourke, *Emory University*
Zachary N. Stowe, *University of Arkansas for Medical Sciences*
Michael J Owens, *Emory University*

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Gretchen N. Neigh[1,2,*], **Christina L Nemeth**[2], **Sean D. Kelly**[1], **Emily E. Hardy**[1], **Chase Bourke**[1], **Zachary N. Stowe**[3], and **Michael J. Owens**[1]

[1]Dept. of Psychiatry and Behavioral Sci., Emory University, Atlanta, GA

[2]Dept. of Physiology, Emory University, Atlanta, GA

[3]Department of Psychiatry, University of Arkansas for Medical Sciences, Little Rock, AR

Abstract

Prenatal stress has been linked to deficits in neurological function including deficient social behavior, alterations in learning and memory, impaired stress regulation, and susceptibility to adult disease. In addition, prenatal environment is known to alter cardiovascular health; however, limited information is available regarding the cerebrovascular consequences of prenatal stress exposure. Vascular disturbances late in life may lead to cerebral hypoperfusion which is linked to a variety of neurodegenerative and psychiatric diseases. The known impact of cerebrovascular compromise on neuronal function and behavior highlights the importance of characterizing the impact of stress on not just neurons and glia, but also cerebrovasculature. Von Willebrand factor has previously been shown to be impacted by prenatal stress and is predictive of cerebrovascular health. Here we assess the impact of prenatal stress on von Willebrand factor and related angiogenic factors. Furthermore, we assess the potential protective effects of concurrent anti-depressant treatment during *in utero* stress exposure on the assessed cerebrovascular endpoints. Prenatal stress augmented expression of von Willebrand factor which was prevented by concurrent *in utero* escitalopram treatment. The functional implications of this increase in von Willebrand factor remain elusive, but the presented data demonstrate that although prenatal stress did not independently impact total vascularization, exposure to chronic stress in adulthood decreased blood vessel length. In addition, the current study demonstrates that production of reactive oxygen species in the hippocampus is decreased by prenatal exposure to escitalopram. Collectively, these findings demonstrate that the prenatal experience can cause complex changes in adult cerebral vascular structure and function.

*Corresponding Author Current Contact Information: Gretchen N. Neigh, Associate Professor, Department of Anatomy & Neurobiology, Virginia Commonwealth University, PO Box 980709, Richmond, VA, USA, 23298, gretchen.mccandless@vcuhealth.org, Phone: 804-826-5152.

Keywords

prenatal stress; escitalopram; vascular length; reactive oxygen species; von Willebrand Factor; stress

Introduction

Stress experienced *in utero* is linked to deficits in social behavior, learning deficits, alterations in stress regulation, and susceptibility to adult disease in both humans and rodent models (Schulz et al., 2011; Weinstock, 2008, 2001). Despite the profound importance of vascular integrity in cerebral function, less has been established regarding developmental stress and the cardiovascular system. Vascular disturbances later in life may lead to cerebral hypoperfusion which has been implicated in a variety of neurodegenerative and psychiatric diseases including depression and Alzheimer's disease (de la Torre, 2006, 2012). Indeed, studies have shown that rodents born to stress-treated dams exhibit altered sensitivity of the cardiovascular system in adulthood (Igosheva et al., 2004) and show altered adult resilience to subsequent events (Mastorci et al., 2009; Neigh et al., 2010).

Although our collective understanding of the impact of prenatal stress on the adult cardiovascular system is growing, little is known about the potential protective effects of *in utero* antidepressant treatment in the presence of chronic stress, a common animal model of the clinical state of maternal depression (Bourke et al., 2013; Maccari and Morley-Fletcher, 2007; Newport et al., 2002). For this study we used the selective-serotonin reuptake inhibitor (SSRI) escitalopram which has been purported to have superior efficacy and fewer discontinuations compared to other second-generation antidepressants (Cipriani et al., 2009). In addition, this research was conducted as part of a NIH-funded Translational Research Center in Behavioral Sciences in which human investigation was also undertaken. Escitalopram was the most commonly prescribed SSRI to pregnant women at the Emory Women's Mental Health Program and in order to foster experimental continuity, is was also selected as the SSRI for the rodent research. Particular care was taken during these experiments to ensure that the prenatal escitalopram (Escit) dosing was maintained within a clinically relevant range of serum drug concentrations throughout gestation in order to produce a state as close to the clinical condition as possible in the context of a rodent model (Bourke et al., 2013). Using this model, the current set of experiments were designed to determine the impact of *in utero* experience on the expression of noted markers of vascular health, including von Willebrand factor (vWF), an important marker of vascularization and vascular compromise which has previously been shown to be altered by adverse prenatal environment (Neigh et al., 2010).

vWF was first noted as a clotting factor, but has since been established to have multiple vascular functions (De Meyer et al., 2012). vWF impacts thrombosis (Wagner and Frenette, 2008), inflammatory processes (Pendu et al., 2006; Petri et al., 2010), and has been associated with functional outcome (Bath et al., 1998) and mortality following stroke (Carter et al., 2007). Furthermore, polymorphisms in vWF increase the risk of cardiovascular disease (van Schie et al., 2011) and stroke (Dai et al., 2001). Due to the diverse roles of vWF

and the potential functional implications of altered *vWF* expression, gene expression findings are expanded upon to include examination of vascularization (vessel length) and vascular compromise (oxidative stress) under the combined conditions of prenatal stress and/or Escit with and without an adult stress challenge.

Methods

Animals

Prenatal stress or Escit-exposed offspring were kept on a 12:12 light:dark cycle (lights on at 7:00 AM) in a humidity (60%) and temperature (20°C-23°C) controlled facility. Rodent Diet 5001 (Purina Mills, Richmond, IN) chow and water were available *ad libitum* throughout the study. Three days after birth, rat pups were sexed and litters were culled. Animals were weaned into same-sex pairs on post-natal day (PND) 21. Only male offspring were used in the current study. No more than two pups were used from each litter in order to prevent litter effects (Holson and Pearce, 1992). Each group was assigned between 8 and 12 pups. Experimental groups included non-stress/saline (Control), non-stress/Escit, stress/saline (Stress), and Stress + Escit. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Emory University and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All groups and sample sizes are assembled in Supplemental Table 1 and described in the text below.

Prenatal Stress Exposure

Rats used in this experiment were bred in house from male Sprague-Dawley experienced breeders and nulliparous females weighing 200-225 grams purchased from Charles River Laboratories (Charles River, Wilmington, MA). Both the stress paradigm and drug administration paradigms have been previously characterized (Bourke et al., 2013; Ehrlich et al., 2015). In addition to the experimental design considerations in selection of Escit that are discussed in the Introduction, Escit was also advantageous because it is soluble in saline. The solubility in saline allows for consistent minipump release without clogging and without irritation to the rat's dermal layers which sometimes occurs with drugs that have to be dissolved in more noxious vehicle solutions. Nulliparous female rats were implanted with Alzet 28-day osmotic minipumps (model 2ML4, Alzet, Cupertino, CA) delivering either 0.9% saline or 12.2 mg/kg/day Escit oxalate in 0.9% NaCl based upon the weight of the pregnant dam on gestational day (G) 21. (Bourke et al., 2013; Ehrlich et al., 2015). The estimated expected weight was based on assessment of G21 weights from 4 previous studies (n=36). For these studies, the actual dose on G21 was 12.2 mg/kg/day. This results in steady-state serum drug concentrations that are always within the clinically observed range even though the dose is slightly higher early in the experiment prior to the weight gain associated with pregnancy (Bourke et al., 2013). This dosing strategy has been shown to mimic clinically relevant serum drug concentrations throughout gestation (Bourke et al., 2013). Escit serum drug concentrations were verified in each pregnant rat by a small blood sample on G15. Three days after minipump implantation, females were bred with retired breeder males. On G9, the chronic unpredictable mild stress model of depression began and consisted of restraint, cage tilt, damp bedding, cage changes, noise, and overnight illumination (Bourke et al., 2013). Prenatal stress began on G9 as it corresponds with

development of the fetal central nervous system (Clancy et al., 2001) and to minimize premature stress-induced termination of the pregnancy. Prenatal stress ended after the session on G20.

Adult Stress

To examine the combined effects of prenatal stress/Escit and adult stress, two additional cohorts of adult prenatal stress- or Escit-exposed rats were subjected to chronic stress or maintained in the home cage beginning on PND 90. Adult stress consisted of 14 consecutive days of immobilization stress in which rats were contained in tapered plastic tubes for two hours (DecapiCones, Braintree Scientific, Braintree, MA).

Gene Expression

In a third identical cohort of adult rats, control (Control n = 9; Escit n = 10; Stress n = 10; Stress + Escit n = 11) and adult stressed (Control n = 10; Escit n = 10; Stress n = 10; Stress + Escit n = 10) rats were killed by rapid decapitation. The brains were removed and flash frozen on dry ice. To quantify changes in gene expression following prenatal exposure to stress and/or antidepressants and their interaction with subsequent adult stress, brains were dissected frozen under RNAse-free conditions and brain regions were extracted for later analysis. Hippocampal tissue was homogenized, and RNA was extracted using the Qiagen RNeasy Mini Kit (Valencia, CA) following manufacturer's instructions. RNA was then reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Waltham, MA). cDNA was quantified and normalized using the PicoGreen method (Invitrogen, Grand Island, NY). Samples were prepared in triplicate using 1 μg of sample and completed plates were run on an Applied Biosystems HT7900 Fast Real-Time PCR system (Carlsbad, CA). Measured primers include *Angiopoietin I (Angpt I*, sense: AACAGGAGGTTGGTGGTTTG; antisense: TCAGTTTTCCATGGTTTTGC), *Angiopoietin II (Angpt II*, sense: AGTGGCTGATGAAGCTGGAG; antisense: TGGTTCTGCACCACATTCTG), *Claudin* (sense: GCACATGCAATGCAAAGTGTA; antisense: GCCGGTCAAGGTAACAAAGA), *endothelial nitric oxide synthase* (eNOS, sense: CAATCTTCGTTCAGCCATCA; antisense: AGCTGGCTGTTCCAGATCC), and *von Willebrand Factor (vWF*, sense: CAATTCTCGGGAACTTCCAA; antisense: GGCAAACAAATGGATGTCAA). All mRNA measurements were standardized to *hypoxanthine-guanine phosphoribosyltransferase (Hprt*, sense: GTCCCAGCGTCGTGATTAGT; antisense: GCAAGTCTTTCAGTCCTGTCC) and *hydroxymethylbilane synthase (Hmbs*, sense: GAAATCATTGCTATGTCCACCA; antisense: AACAGGCTCTTCTCTCCAATCTT). We calculated fold change using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and presented fold changes for each experimental group as a value normalized to the Control group.

Vascular Length

Immediately following chronic stress in adulthood, one cohort of rats (n = 6 for each group) were transcardially perfused with 4% paraformaldehyde (PFA), brains were removed and stored at 4 °C in 4% PFA until cryoprotected (30% sucrose, 10% glycerol in 0.01 M PBS) and sectioned at 50 μm. Free floating sections (ssf = 4) were pretreated with H_2O_2 for 20 minutes at 4°C. After rinsing, sections were incubated for one hour in blocking buffer (3%

normal goat serum [Vector Laboratories, Burlingame, CA], 0.5% Triton X-100) followed by a rinse and incubation with primary antibody (mouse anti-RECA; 1:200; Abd Serotec, Raleigh, NC) overnight at 4°C. On Day 2, tissue was rinsed and incubated with secondary antibody (biotinylated horse anti-mouse; 1:200; Vector Labs) for two hours at room temperature. Sections were again rinsed and incubated for 90 minutes in streptavidin-peroxidase-HRP (Sigma; St. Louis, MO). Following another rinse, sections were exposed to DAB (Sigma, St. Louis, MO) for two minutes. Sections were rinsed, mounted, and allowed to dry overnight. On Day 3, slides were counterstained with cresyl violet.

Sections were analyzed using a Nikon Eclipse 90i microscope (Melville, NY) fitted with Micro Bright Field Stereo Investigator version 9.14.1 (Williston, VT). Using the Spaceballs probe (16 μm hemisphere thickness; 2 μm guard zone; 150 μm frame size) vessel length was assessed in the prefrontal cortex (PFC; grid size: 190×428 y), hippocampus (550×500 y), and amygdala (380×365 y).

Reactive Oxygen Species

In the first cohort of adult rats (Control n = 6; Escit n = 8; Stress n = 4; Stress + Escit n = 7), dyhydroethidium (dHEt; EMD Millipore, Darmstadt, Germany) was prepared (1 mg/mL in 1% DMSO) and injected (1 mg/kg) into the tail vein. Thirty minutes after the injection, rats were restrained in flat bottom plastic rat restraints (Braintree Scientific, Braintree, MA) for 60 minutes. Immediately thereafter, rats were overdosed with 50 mg/kg sodium pentobarbital and transcardially perfused with 4% PFA. Brains were removed and stored at -80 °C until sectioning.

Brains were sectioned on a microtome (HM 450, Thermo Scientific, Waltham, MA) at a thickness of 60 μm and stored in cold 0.01 M PBS. Sections were mounted on Permafrost slides (Fisher Scientific, Pittsburgh, PA) and counterstained at room temperature for five minutes with Hoechst stain (1:1000, Life Technologies, Grand Island, NY). Sections were then coverslipped using Vectashield mounting media (Vector Labs, Burlingame, CA).

Imaging was performed on an Olympus FlouView 1200 Laser Scanning confocal microscope (Center Valley, PA). In neuronal cell bodies and axons, ethidium bromide was excited at 488 nm and emitted red at 605 nm. In the nucleus, Hoechst stain was excited at 352 nm and emitted blue at 449 nm. Z-Stacks were collected from the CA1, CA3, and dentate gyrus of the hippocampus. Each Z-stack was captured at 0.4 μm increments out of an entire 60 μm cross-section. This allowed for 150 images throughout each Z-stack. To see a large representation of the hippocampus, a frequency of three Z-stacks were captured on three different cross-sections for each animal and for each different region. Each cross-section was 720 μm apart, which allowed for a representation of 2.16 mm of hippocampus.

The number of neuronal cells was quantified by viewing every frame in each Z-stack and counting the number of fluoresced neurons. The counting frame (1024 μm × 1024 μm) included a 150 μm vertical Z-stack. To show the neuronal area of the Z-stack, these three areas of the counting frame were multiplied, resulting in a hippocampal area of 0.1573 mm^3.

Data Analysis

Gene expression data were analyzed as described above, averaged by group, and compared using a three-way ANOVA with prenatal stress (control, stress), prenatal drug (saline, Escit), and adult stress (control, stress) as factors. Data for vascular length were averaged by group and compared using a three-way ANOVA. For assessment of reactive oxygen species, the number of fluoresced cells was converted to neuronal area by dividing the number of cells by 0.1573 mm^3. Neuronal areas were averaged by group and assessed via a two-way ANOVA. Graphing and statistics were completed using GraphPad Prism 6 (La Jolla, CA) and IBM SPSS for Windows, Version 21.0 (Armonk, NY). In all cases, group means were considered significantly different when p　0.05. All data are expressed as mean ± the standard error of the mean (S.E.M.). *Post-hoc* differences were assessed with Tukey's multiple comparisons test.

Results

Hippocampal vWF expression is increased by prenatal stress, prevented by Escit

To determine the effect of the prenatal experience and adult stress on vascular health, we examined hippocampal expression of vasoactive factors including growth factors, markers of endothelial cell health and tight junction integrity, and endothelial nitric oxide synthase. vWF, an endothelial cell marker important for hemostasis, was altered as a result of both prenatal exposure to Escit ($F_{1,71} = 4.921$, $p < 0.05$) and exposure to adult chronic stress ($F_{1,71} = 7.172$, $p < 0.05$; Figure 1A). Furthermore, an interaction between prenatal exposure to stress and prenatal exposure to Escit demonstrates that the prenatal experience has disparate effects on hippocampal expression of *vWF* ($F_{1,71} = 6.915$, $p < 0.05$). Specifically, *post-hoc* analysis reveals a significant increase in *vWF* expression in prenatally stressed rats exposed to subsequent adult stress as compared to control rats ($p < 0.05$). Importantly, this effect of adult stress is prevented in rats that experienced the combination of stress and Escit *in utero* ($p < 0.05$).

Expression of angiogenic factors in the hippocampus is altered by prenatal stress and/or Escit

Together, claudin and occludin comprise the main components of tight junctions formed between cells of the vascular epithelium. A three-way ANOVA of *occludin* showed a trend towards increased expression of *occludin* as a result of exposure to chronic stress in adulthood ($F_{1,61} = 2.809$, $p = 0.099$; data not shown). Similarly, *claudin* expression was increased by exposure to chronic stress in adulthood. However, a three-way interaction of prenatal stress, prenatal Escit, and adult stress illustrates that the effects of chronic stress in adulthood on *claudin* expression vary as a function of both Escit and stress experienced *in utero* ($F_{1,51} = 3.683$, $p < 0.05$; Figure 1B).

Angiopoietins (Angpt) I and II, growth factors that contribute to cerebral angiogenesis, were differentially affected by exposure to stress. While levels of *Angpt I* were unchanged in the hippocampus as a function of *in utero* exposure to stress and/or Escit (Figure 1C), levels of *Angpt II* in the hippocampus were significantly altered by *in utero* exposure to stress ($F_{1,73} = 5.280$, $p < 0.05$; Figure 1D). Furthermore, a significant interaction of prenatal exposure to

Escit and chronic stress in adulthood suggested that exposure to Escit during gestation alters the expression of *Angpt II* following chronic stress experienced in adulthood ($F_{1,73} = 5.953$, $p < 0.05$).

Vascular endothelial growth factors (Vegf) participate in angiogenesis and maintain endothelial cell health. Expression levels of *VegfC*, similar to *Angpt II*, showed a significant interaction between prenatal exposure to Escit and adult exposure to chronic stress which demonstrated differential expression of the growth factor to adult stress as a function of prenatal history ($F_{1,72} = 5.095$, $p < 0.05$; Figure 1F). In contrast, no differences were detected in the expression levels of *VegfA* ($p > 0.05$; Figure 1E).

Lastly, neither prenatal stress/Escit exposure nor adult stress altered the expression of endothelial nitric oxide synthase (*eNOS)* in the hippocampus ($p > 0.05$; data not shown).

Adult chronic stress reduced vascularization irrespective of prenatal environment

The observed changes in gene expression, in particular *vWF*, suggest that Escit may modify the vascular impact of chronic prenatal stress. Given our previous documentation of altered vascular length following prenatal glucocorticoid exposure as measured by vWF immunohistochemistry combined with stereology (Neigh et al., 2010), we determined the extent to which Escit and prenatal stress could modify vascular length. Stress, administered chronically in adulthood, reduced vessel length in the hippocampus ($F_{1,40} = 6.036$, p < 0.05; Figure 2; photomicrographs in Supplemental Material as Figure S1). Although the focus of the current study was the hippocampus, we also assessed vascularization in two additional stress-sensitive brain regions: the PFC and amygdala. Similar to the hippocampus, adult stress decreased vessel length, regardless of prenatal exposure, in, PFC ($F_{1,40} = 11.60$, p < 0.05), and amygdala ($F_{1,40} = 4.513$, p < 0.05; Figure 2). In the amygdala, an interaction of prenatal stress and adult stress approached significance, suggesting that adult stress may differentially alter vessel length in the amygdala as a function of prenatal exposure to stress ($F_{1,40} = 3.155$, p = 0.08).

Prenatal exposure to Escit decreases a marker of reactive oxygen in CA1 and CA3

An alternate indication of altered *vWF* is one of compromised vascular integrity (De Meyer et al., 2012). In order to test the hypothesis that vascular compromise was present in the hippocampus of rats with a history of prenatal stress, and that this would be prevented by prenatal Escit, we assessed ROS in rats from both prenatal backgrounds. Dihydroethidium was examined via its oxidized product, ethidium bromide, within the CA1, CA3, and dentate gyrus of the hippocampus. When assessed in adulthood, prenatal exposure to Escit, regardless of stress experience, reduced ROS expression in the CA1 of the hippocampus ($F_{1,12} = 6.530$, p < 0.05; Figure 3A). Similarly, in the CA3, Escit exposure decreased ROS detection ($F_{1,12} = 5.137$, p < 0.05; Figure 3B); however, an interaction between prenatal stress and Escit suggests prenatal stress differentially influenced ROS in the presence of Escit ($F_{1,12} = 14.63$, p < 0.05). Post hoc assessments of ROS in the CA3 revealed decreased ROS expression in both Stress rats and Escit rats compared to Control, but not in rats receiving the combination of prenatal stress and prenatal Escit (p < 0.05). Finally, and in contrast to data from CA1 and CA3, the combination of prenatal Escit and stress led to

increased expression of ROS with no effect of prenatal stress in the dentate gyrus ($F_{1,12}$ = 6.072, $p < 0.05$; Figure 3C).

Discussion

Previous reports have established that the prenatal experience alters the adult response to stress and susceptibility to cardiovascular disease states in adulthood. The studies reported here expand upon the potential mechanisms underlying these observed changes and demonstrate that the impact of prenatal experience on adult cerebrovasculature is complex, multifaceted, and not easily summarized with a single conclusion. We first demonstrated a robust increase in expression of *vWF* following prenatal stress which is further augmented if adult stress is also experienced (Figure 1A). Concurrent Escit appeared to be profoundly protective against the impact of stress on this particular endpoint; however, when other vascular factors are considered (Figures 1B-F), determining which prenatal experiences are protective and which are harmful, is less clear. Because *vWF* expression could indicate increased vascularization (Neigh et al., 2010) or vascular compromise (De Meyer et al., 2012), we expanded our data set to consider two additional hypotheses. The data presented in Figure 2 demonstrate a clear impact of adult stress on vascularization, but these data do not support that hypothesis that the increase in *vWF* noted in Figure 1A is due to increased vascularization of the hippocampus following prenatal stress. The third experiment tested the hypothesis that the increase in *vWF* was due to vascular compromise which could be reflected by increased ROS in response to an acute stressor. Again, our hypothesis did not appear to be supported, such that prenatal stress did not increase ROS (Figure 3); however, prenatal Escit does appear to reduce ROS in the CA1 and CA3 regions of the hippocampus. Although these findings do not collectively convey a succinct unidimensional theme, they are important because they demonstrate that the prenatal experience can alter adult cerebral vascular structure and indicators of vascular integrity. Such changes may underlie susceptibility to later life disease and indicate that additional inquiry regarding the individual contributions of prenatal stress, prenatal antidepressants, and adult stress to cerebrovascular alterations are warranted. Below we discuss the potential implications of the described findings and suggest areas of future research.

Vasoactive factors triggered by prenatal or adult experience may mediate damage to neurons and vasculature. Furthermore, psychological stress impairs endothelial function which may be partly mediated by increases in cortisol and prolonged activation of the sympathetic nervous system (Poitras and Pyke, 2013). vWF, an important coagulation factor produced by endothelial cells and a risk factor for stroke (Dai et al., 2001), *vWF* is increased by the combination of prenatal and adult stress, but this impact is prevented in rats that also experienced concurrent *in utero* Escit (Figure 1A). Although previous studies have failed to show antidepressant effects on *vWF* expression (Geiser et al., 2011; Halperin and Reber, 2007) current findings suggest that *in utero* exposure to Escit provides protection from concurrent and delayed stress exposure. Although the current experiments did not ascertain the functional consequences of increased expression of *vWF* following prenatal stress, given the existing knowledge of implications for *vWF* in prediction of cardiovascular disease (van Schie et al., 2011) and pathogenesis following stroke (Bath et al., 1998; Carter et al., 2007;

De Meyer et al., 2012), this finding may have implications for cerebrovascular insults in individuals with a history of prenatal stress exposure.

Other factors including claudins, and Angpt I and II are critical during development and are subject to influences during this time. While claudins regulate tight junction efficiency (i.e. increased claudin ~tightened junctions), Angpt I and II have opposing roles in the endothelium (Jeansson et al., 2011; Kratzer et al., 2012). Claudins are responsive to infection and stress and are often down-regulated in high inflammatory states (Kratzer et al., 2012) and the current data set confirms the claudin response to be quite complex and dependent upon both prenatal and adult environments. Similarly, the responses of Angpt I and II are complex, as Angpt I is critical for development, promotes cell stability and endothelial cell health, and may serve as a protective factor following injury or disease (Jeansson et al., 2011). In contrast, Angpt II triggers inflammation, destabilization, and endothelial cell activation and in the presence of VegfA, promotes angiogenesis (Jeansson et al., 2011). In the current study, effects of adult stress on levels of *Angpt II* were differentially affected by a history of *in utero* exposure to stress, suggesting long-lasting effects of the prenatal experience. Because Angpt I can act as an inhibitor of Angpt II activity, increased expression of Angpt II over Angpt I may indicate an adverse endothelial cell state. Furthermore, Angpt II, in the absence of both VegfA and Angpt I, may lead to cell death and accelerated disease pathology (Jeansson et al., 2011).

Stress during development or in adulthood alters cerebral vasculature, including vessel density, rates of angiogenesis, and perivascular cell health (Neigh et al., 2010). In the current study, exposure to chronic adult stress decreased vessel length, irrespective of prenatal history, though this decrease is largely driven by increased vessel length detected in rats receiving prenatal treatments in the absence of an adult stress. Regardless, decreased vessel length is consistent with previous reports of stress-induced vascular hypotrophy in the hippocampus (Neigh et al., 2010) and has implications for perfusion of brain tissue (Faraci, 2011). Similarly, offspring born to dexamethasone treated dams exhibit decreased vasculature in a region specific manner, including in the paraventricular nucleus of the hypothalamus, further supporting the sensitivity of brain vasculature to the prenatal environment (Frahm and Tobet, 2014).

Although not specifically assessed in this study, we have previously reported that the prenatal stress paradigm used here results in increased glucocorticoid secretion (Bourke et al., 2013). Stress and exposure to glucocorticoids lead to reductions in neurogenesis, suppression of angiogenesis, and delayed maturation of vasculature, thereby altering both cerebral blood flow and neuronal function (Heine et al., 2005; Huang et al., 2001). Reduced perfusion of brain tissue as a result of decreased angiogenesis can affect synaptic plasticity (Font et al., 2010) and reductions in blood flow are noted in disease states such as major depressive disorder (Nobler et al., 1999) and Alzheimer's disease (Aliev et al., 2003). Patients with depressive disorders experience global brain hypoperfusion (Fountoulakis et al., 2004) and chronic antidepressant exposure reverses this effect (Ishizaki et al., 2008; Passero et al., 1995), also in a region and response specific manner.

Although it is unclear if hypoperfusion related to depressive disorders is a result of reduced brain vasculature, chronic hypoperfusion of brain tissue is a detrimental consequence, resulting in oxidative stress and damage to both neurons and vasculature (Aliev et al., 2003). Given this knowledge and the increased expression of *vWF* noted, we assessed ROS as a marker of oxidative stress. Detection of ROS was decreased by prenatal exposure to Escit (Figure 3A and 3B). A previous report in adult rats demonstrated SSRI-induced protection against lipid peroxidation in the brain (Abdel Salam et al., 2013), but lipid peroxidation was not measured in the current study and is a necessary future course of assessment. In addition, the interpretation of ROS finding is limited by the absence of a total cell count for the hippocampus. We cannot rule out the possibility that the reduction in labeled cells is a reflection of an overall reduced cell count as opposed to a reduction in ROS-positive cells specifically. ROS are important cell signaling markers and participate in the maintenance of cerebral vascular tone. Although high levels of ROS are most commonly associated with disease states, low production of ROS is associated with severity of immune-related disease states (Bengtsson et al., 2014) and with accelerated cellular aging (Remacle et al., 1995). Given the complex role of ROS in vascular health, low ROS formation may be an important element or risk factor of neuronal vulnerability to disease (Bengtsson et al., 2014). The functional consequences of the alterations in ROS noted here cannot be ascertained from the current study; however, these findings highlight an important area for further investigation given the critical role of ROS in both health and disease within the brain.

The complex and multifaceted changes in cerebrovascular structure and reactivity noted here have potential functional implications as such changes have been linked to cardiovascular disease (Thornburg et al., 2010). Although Escit administration during gestation in conjunction with, or prior to, prenatal or adult stressors, respectively, was not globally protective against changes to vasculature, prenatal exposure to antidepressants may have some beneficial effects as observed in expression levels of *vWF*. These findings are important, as cardiovascular disease is intimately linked with the manifestation of psychiatric disorders such as depression (Mavrides and Nemeroff, 2013; Nemeroff and Goldschmidt-Clermont, 2012; Plante, 2005), and the permanent vascular changes facilitated by an adverse *in utero* environment may yield changes in brain and behavior. Given the possible neurological and psychiatric implications of altered blood flow to the brain, well defined effects of prenatal history on brain vasculature may help to better elucidate disease susceptibility following early life exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Prenatal stress augmented expression of von Willebrand factor.

- Effects of prenatal stress on vWF were prevented by *in utero* escitalopram treatment.

- Chronic stress in adulthood decreased blood vessel length.

- Escitalopram reduced reactive oxygen in the hippocampus.

- Prenatal experience causes complex changes in adult cerebrovascular endpoints.
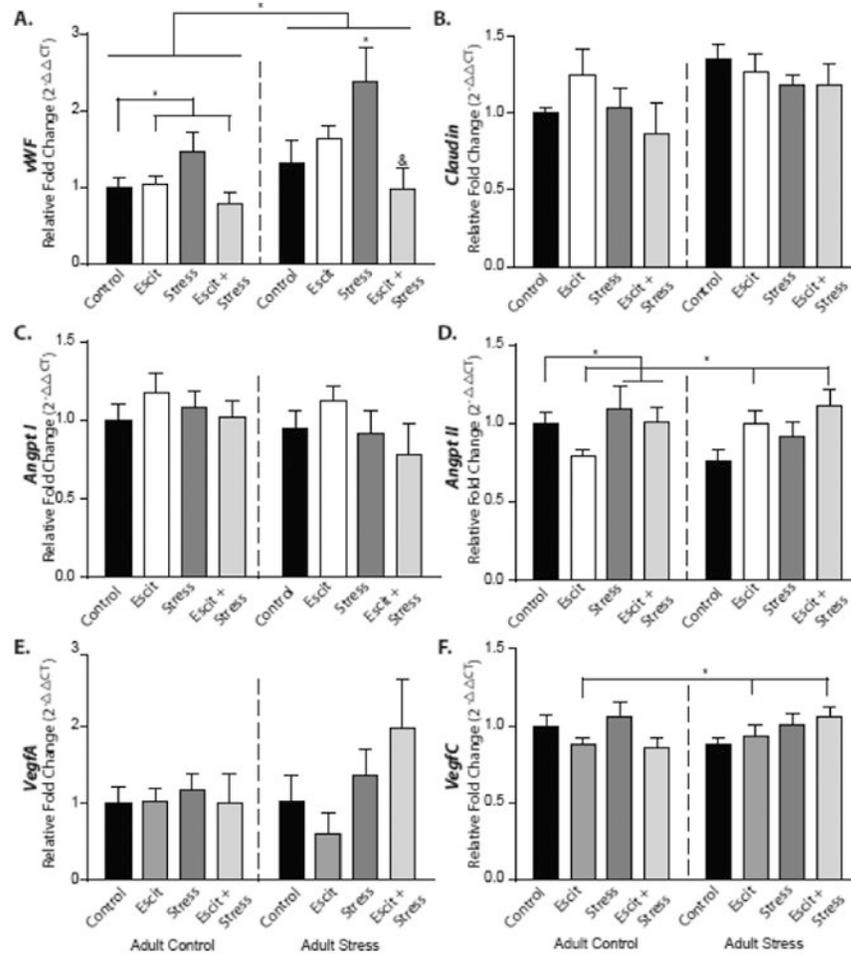
Figure 1.
Angiogenic factors were measured in the hippocampus of adult rats with a history of prenatal exposure to chronic unpredictable mild stress and/or Escit. The same factors were measured in adult rats with the same *in utero* experience with an additional adult chronic stress. **(A)** von Willebrand Factor (vWF), a protein involved in hemostasis, was altered as a result of prenatal exposure to Escit ($F_{1,71} = 4.921$, $p < 0.05$) and increased as a result of adult chronic stress ($F_{1,71} = 7.172$, $p < 0.05$). *Post-hoc* tests reveal that a history of prenatal stress in combination with adult chronic stress significantly increased *vWF* compared to controls ($p < 0.05$; denoted with "*") and that this effect was attenuated by concurrent exposure to Escit and stress during gestation ($p < 0.05$; denoted with "&"). **(B)** Tight junction components *occludin* and *claudin* were differentially affected by prenatal and adult experience. While a trend towards increased expression of *occludin* was apparent following chronic stress in adulthood (data not shown), the effects of chronic stress in adulthood on *claudin* vary as a function of both *in utero* Escit and stress exposure (three way interaction, $p < 0.05$). **(C, D)** Growth factor angiopoietin *(Angpt) I* was unchanged by prenatal or adult experience; in contrast, *Angpt II* was increased by prenatal exposure to stress (*Angpt1*, p = 0.095; *Angpt II*, p < 0.05). Furthermore, prenatal exposure to Escit alters expression of *Angpt II* when stress is experienced again in adulthood ($p < 0.05$). **(E, F)** While prenatal
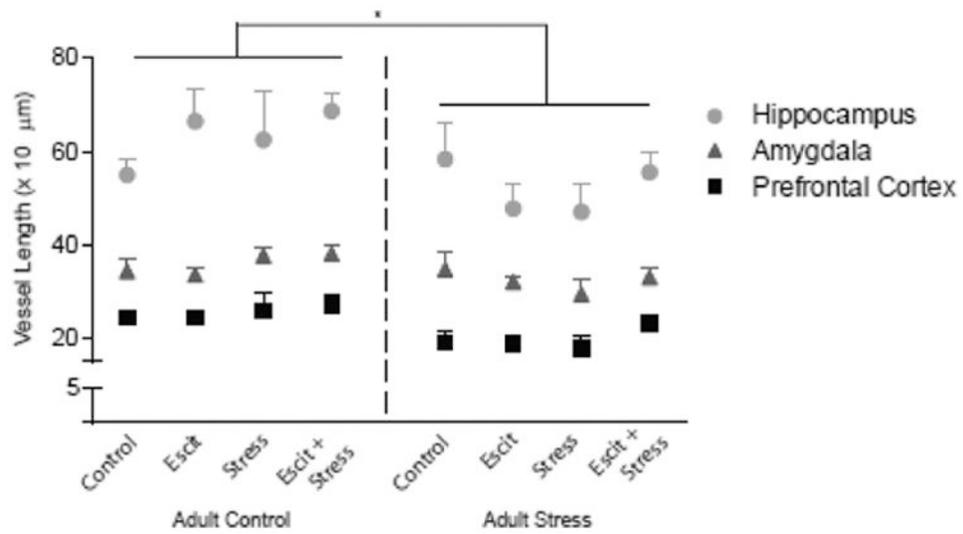
experience or adult stress had no effect on the expression of vascular endothelial growth factor *(Vegf) A, VegfC* was altered in animals prenatally exposed to Escit and chronically stressed as adults. For all, error bars indicate S.E.M. and * indicates p < 0.05.
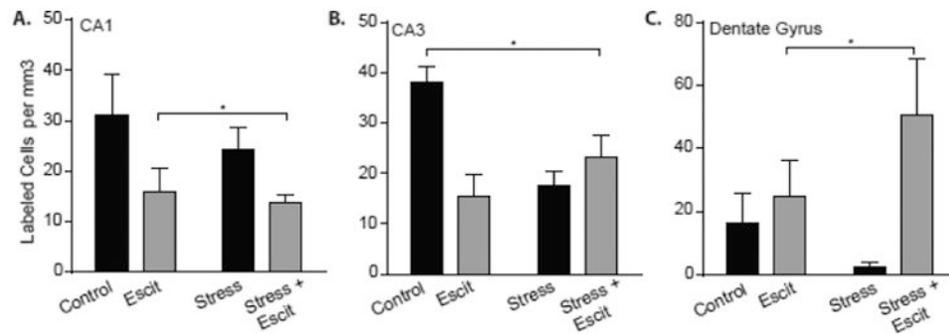
Figure 2.
Vessel length was assessed in the hippocampus, amygdala, and prefrontal cortex (PFC) of adult stressed and non-stress prenatally exposed rats. Vessel length was measured to determine the effect of prenatal experience on adult vasculature and the response to subsequent stress. Regardless of prenatal exposure to stress and/or Escit, adult stress reduced vessel length in each of the regions assessed ($p < 0.05$). Additionally, in the amygdala, prenatal exposure to stress differentially affected vessel length in rats exposed to subsequent stress in adulthood (interaction of prenatal and adult stress, $p = 0.08$). Markers indicate mean vessel length and error bars indicate S.E.M.; * indicates $p < 0.05$.

Figure 3.
Ethidium bromide was assessed as a marker of reactive oxidative species (ROS) within the hippocampus of adult animals exposed to stress and/or Escit *in utero.* **A)** Regardless of stress experience, prenatal Escit reduced ROS in the CA1 ($p < 0.05$). **B)** In the CA3, ROS are differentially affected by prenatal stress in the presence of Escit; decreased ROS expression was detected in both Stress and Escit rats, as compared to controls, but not in rats exposed to both Escit and stress *in utero* ($p < 0.05$). **C)** Lastly, in the dentate gyrus, Escit increased ROS expression ($p < 0.05$), with no apparent effect of stress. For all, error bars indicate standard error of the mean (S.E.M.) and * indicates p < 0.05.