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Prenatal domoic acid exposure disrupts mouse pro-social behavior and functional connectivity MRI

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Abstract

Domoic acid (DA) is a toxin produced by marine algae and known primarily for its role in isolated outbreaks of Amnesic Shellfish Poisoning and for the damage it inflicts on marine mammals, particularly California sea lions. Lethal effects of DA are often preceded by seizures and coma. Exposure to DA during development can result in subtle and highly persistent effects on brain development and include behavioral changes that resemble diagnostic features of schizophrenia and anomalies in social behavior we believe are relevant to autism spectrum disorder (ASD). To more fully examine this hypothesis, we chose to examine adolescent mice exposed *in utero* to DA for endpoints relevant to ASD, specifically changes in social behavior and network structure, the latter measured by resting state functional connectivity (rsfMRI). We found that male offspring exposed *in utero* to DA expressed reproducible declines in social interaction and atypical patterns of functional connectivity in the anterior cingulate, a region of the default mode network that is critical for social functioning. We also found disruptions in global topology in regions involved in the processing of reward, social, and sensory experiences. Finally, we found that DA exposed males expressed a pattern of local over-connectivity. These anomalies in brain connectivity bear resemblance to connectivity patterns in ASD and help validate DA-exposed mice as a model of this mental disability.

Keywords

Resting state functional connectivity; mouse; social behavior; brain imaging; autism

1. Introduction

Domoic acid (DA) is an algal neurotoxin that accumulates in marine fish and shellfish [1]. Human consumption of highly DA-contaminated seafood can result in Amnesic Shellfish

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Poisoning, with symptoms that include memory loss, seizures, and death [1-3]. Human exposure to DA typically results from consumption of oysters, mussels, and clams in their entirety, including the contaminated viscera. In other cases, commercial processing promotes leaching of DA from the viscera into the consumable muscle meat [4-5]. The regulatory limit for DA harvest, 20 mg of DA/kg tissue, is designed to safeguard from acute Amnesic Shellfish Poisoning [6]. In the Pacific Northwest, Chesapeake Bay and along the coasts of Western Europe, peak annual levels of DA in shellfish can exceed this limit, resulting in intermittent restrictions on shellfish harvests. Lower levels of DA exposure exert more subtle effects that are often observed in pinnepeds stranded along the West Coast of North America. California sea lions exposed to levels of DA that are not acutely lethal can display diminished spatial memory [7], blunted habituation to auditory stimuli [8], heightened aggression [9], highly repetitive behaviors [10], and abnormal locomotion in both water [11] and land [9]. Experimental studies that involve the administration of low doses of DA to adult rodents show that exposed subjects express hypomobility [12], impaired spatial learning [13] (Petrie et al., 1992), aggression [14], and significantly increased stereotypic behaviors [15].

In addition to adult exposures, developmental exposures to low levels of DA can have pernicious and highly persistent effects [16-17]. At ED 13 in rat development, a timepoint corresponding to the onset of neurogenesis and equivalent to the mid-first trimester of human development [18], DA interacts with kainate receptors, depolarizes neurons, and mediates hippocampal damage, with long term effects including a reduction in seizure thresholds [19]. After a single exposure to DA at either ED 14.5 or 17.5, adults express profound decreases in conditioned fear memory and adaptation to novel circumstances [20]. Neonatal rats exposed to DA express, as adults, hypoactivity [21] and impairments in spatial learning [22] and sensory motor gating [23] and, if exposed to DA between 8 and 14 days of age, express as adults lower seizure thresholds [24-26], altered novelty-related behavior [27], altered conditioned responses to drug reward [28], persistent changes in learning and memory [29] and diminished social interaction [30].

This broad timespan of susceptibility, representing the equivalent to a 3-month period extending from the end of the first trimester to the end of the second trimester in humans [18], is not surprising given that DA interacts with kainate receptors 1 and 2 (KA1&2) and glutamate receptors (GluR) 5 and 6 are expressed in the rat hippocampus by E13 and throughout the cortex by E17 [31]. These receptors are also expressed during human fetal brain development [32] in regions including cortical and limbic areas that regulate social behavior. Mechanisms of action for DA likely change over the course of development. DA binds its receptors, promoting gene expression [33-34] and engendering neuroexcitation [16-35], but may also alter the trajectory of development via hippocampal in selective loss of interneurons and oligodendrocytes, or by moderating glutamate tone [19], axon and dendrite morphology [36-37] or myelination [20]. At each stage of fetal and postnatal rodent development, neurons respond to DA exposure in the manner that is relevant for receptor engagement and neuronal development [38]. Effects of DA on early development may be exacerbated by elevated exposures to DA *in utero* [39] and by elevated serum retention during early postnatal development [40].

Early exposure to DA may render the developing fetus at risk for schizophrenia [23, 30]. Autism is also a possibility, as California sea lions exposed to DA present with seizures, diminished social regulation, and repetitive behaviors [9] and these behaviors bear striking resemblance to the clinical features of this disorder.

Rodent models gain greater construct validity when behavioral, anatomical, *and* physiological dimensions of their biology bear resemblance to the human mental disorder they represent. Until recently, imaging techniques commonly used to evaluate the human brain were unavailable for studies of rodent models. Resting state functional connectivity (rs-fcMRI), first described in humans [41] has been used extensively to examine brain network abnormalities in psychiatric disorders [42-45]. Rs-fcMRI offers insight to the default mode network, which has been repeatedly implicated in psychiatric disorders [46]. This network plays a normative role in complex cognitive tasks such as social cognition [47, 48] and may be involved in evaluations of a self-versus other model building of the social environment and self-referential evaluation [48, 49]. Indeed, areas involved in a variety of social tasks largely overlap with areas in the default system [48, 50].

The default network has recently been shown to be conserved in mice [51, 52] and Rs-fcMRI has been adapted to identify robust and biologically plausible functional networks in mice [51-53], a development that helps bridge the gap between human and animal models of disease [52, 54]. The default network spans a distributed network of regions including the anterior cingulate cortex (ACC), retrosplenial cortex, and orbital area [52]. As the ACC plays a critical role in social functioning [55-57] and because DA exposure can target glutamatergic cells which are highly expressed in the cingulate [31], we focused on connectivity of the ACC of the default mode.

Larger scale examinations of global topology offer useful insights into psychiatric disorders. For example, graph theoretical techniques help reveal overall network topology in ASD [45] and schizophrenia [58]. One such metric is “degree”, an index of the overall number of connections of a given region, a useful account of a regions contribution to a network. In addition to node degree, other measures have aimed to distinguish network topology based on the spatial proximity of functional connections. A popular hypothesis in the ASD literature is that children with ASD show an excess of short-range connectivity and a decrease in connectivity between longer-range connections. Multiple studies have demonstrated local over-connectivity in ASD [59-64], but the extent these phenomena are related to early brain insults remains unclear.

We asked whether *in utero* exposure to DA generates anomalous adolescent social behaviors and corresponding changes in functional connectivity. We investigated functional connectivity in both the ACC region of the default mode and in the larger scale network structure. Global network topology was assessed in order to determine whether DA exposure preferentially affects regions associated with social behavior. Local connectivity and longer-range patterns of connectivity were also assessed for similarities to ASD.

2. Materials and Methods

2.1. Behavior Methods

2.1.1 Mouse husbandry—C57BL/6J (B6) mice, purchased from Jackson Laboratories (Bar Harbor, ME, USA), were bred and housed at Oregon Health and Science University (OHSU) under controlled temperature ($21 \pm 1^\circ\text{C}$) and humidity (40–60%). Lighting was maintained on a 12:12 h light/dark (dark period 0900–2100 hours) cycle. Mice were housed in standard polypropylene cages ($290 \times 180 \times 130$ mm) lined with pelleted paper bedding (Ecofresh, Absorption Corp.) and provided ad libitum access to chow (Lab Rodent Diet 5001, Purina Mills) and water. Animal care and experimental protocols were conducted in accordance with the regulations of the institutional care and use committee at the Oregon Health and Science University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Our own laboratory personnel carried out all aspects of the mouse husbandry under strict guidelines to insure gentle and consistent handling of the mice.

2.1.2 Domoic Acid Administration—Pregnant mice were weighed daily. When at a weight of 30 grams ($M = 30.157$ grams, $SD = 1.513$), embryonic day (ED) 16 ($M = 16.2$, $SD = 1.66$), gravid females were injected subcutaneously with either 1.5 mg/kg of DA or saline. We chose this dose of DA based upon results of our own experimental evidence that exposure to this dose causes deficits in adolescent social approach behavior in FVB mice [65] and based upon evidence of effectiveness in comparable doses of DA administered via intraperitoneal injection [20] and below the threshold expected to induce convulsive effects or inducing fetal loss [66]. Weaning occurred on postnatal day (PD) 20–22; pups were separated by sex and placed in separate clean colony cages. Housing groups of siblings were made up of two males and two females. During sexing, mice were randomly assigned as the test or stimulus mouse role for social behavior testing. Test mice were marked on the tail with a black Sharpie and stimulus mice were ear-punched on the top left ear. To avoid euthanizing litters with uneven sex distributions, on rare occasions, two male and female housing groups were combined across litters if both litters were the same age and treatment condition.

2.1.3 Social Investigation (SI)—Juveniles were tested for SI at PD 21, PD 25, and PD 35. All testing was conducted during the dark period of the light dark cycle between 1400 and 1800 under dim red illumination. Prior to testing on $\text{PD}21 \pm 1$, and immediately following weaning, mice were individually isolated into clean new home cages. Cages were returned to a housing rack where they remained for 30 min, then briefly stationed in a laminar flow hood for five minutes. They were then transferred to the adjacent experimental room where they remained for an additional five minutes. The stimulus mouse was then added to the cage of the test mouse, placed in an opposing corner of the cage. Ultrasonic vocalizations (USVs) and social behaviors of the test mouse were recorded for five minutes (particular behaviors are described below). At the end of 5 minutes, mice were removed from the experimental room and returned to their social housing group for subsequent testing.

Juvenile mice were isolated 24 hours prior to SI testing (within one day of PD 25 and 35), into a clean cage containing fresh bedding without nesting material and ad libitum access to food and water. After the 24 hours, the stimulus mouse was introduced to the cage of the test mouse where USVs and social interactions were recorded for 15 minutes. The behaviors were video recorded (Sony, DCR-VX2100, Japan) and stored on a on a desktop computer (Precision T3400, Dell, Round Rock, TX, USA) for additional analysis. All behaviors were scored with the aid of computer assisted analysis software (ButtonBox v.5.0, Behavioral Research Solutions, Madison, WI, USA) by two different observers blind to the age and treatment group of the interacting mice. At the conclusion of behavioral testing on PD35, animals were euthanized by CO₂ in accordance with institutional animal care and use committee (IACUC) protocols.

Individual SI scores were coded for test mouse behavior directed towards the stimulus mouse, including: [i] sniffing or snout contact with the head/ neck/mouth area, [ii] sniffing or snout contact with the flank area, [iii] direct contact with the anogenital area, [iv] social pursuit within one body-length as the stimulus mouse moved continuously throughout the cage and [v] social grooming. These variables were combined into a composite measure of SI (Panksepp et al., 2007). SI testing was conducted during the dark phase (4.5 to 9.5 hours after lights-off.), under dim red illumination, in a sound-dampened room. SI was recoded for each experiment and two independent raters blind to the experimental conditions scored each video until the interrater reliability was greater than $r = .90$ for each observation (up to three times per rater). The presentation of all SI data and statistical outcomes in this study are based on an average of these two independent measurements.

In total, 52 same sex B6 pairs were tested for SI. DA exposed mice included 22 testing pairs (11 male pairs, 11 female pairs), and 30 exposed mice included 30 testing pairs (16 male, 14 female). Two mice exposed to saline and one exposed to DA died before the conclusion of the experiment and four mice were excluded due to climbing on the equipment during SI testing (two DA mice at PD25 and two saline mice at PD35).

2.1.3 Ultrasonic vocalizations—Ultrasonic vocalizations (USVs) were recorded during SI tests with an ultrasound microphone (10–180 kHz flat-frequency range) lowered through a 1/8" thick transparent Plexiglas cage top with a 30-mm diameter hole in its center. USVs were collected with an UltraSoundGate 116 acquisition system (UltraSoundGate model CM16, Avisoft Bioacoustics, Berlin, Germany) and Avisoft-Recorder v.2.97 (Avisoft Bioacoustics), and stored as wav files for subsequent analysis. A 40-kHz band-pass filter was used to minimize background noise during recordings. Using SASLab Pro v.4.39 (Avisoft Bioacoustics), each sonogram was automatically tallied for the total number of USVs produced with during the SI period. However, some wav files contained some 'non-USV' signal that compromised the precision of the automated parameter-measurements. Thus, extraneous noise was manually identified and removed. When a rater encountered a signal that was difficult to interpret, the USV was evaluated by a minimum of one additional trained observer. For the final count, two blind observers manually removed noise from the sonograms, and the total USVs were averaged across the two raters (inter-rater reliability, $r > .9$).

2.1.4 Behavioral Statistical analyses—The effect of DA exposure on social behavior and USVs were analyzed using a full factorial repeated measures analysis of variance (ANOVA). Treatment group and sex of each mouse pair were between-group factors and ages at social interaction test (post-natal day (PD) 25 and 35) were repeated measures. Trend level interactions and pre-planned post hoc analyses comparing the effect of DA for males and females, were followed up with t-tests. All statistical analyses were conducted using JMP version 6.0 (SAS Institute Inc.) and $p < 0.05$ was considered statistically significant.

2.2 MRI Methods

2.2.1 Subjects—A total of 16 C57Bl/6J adult male mice (Jackson Laboratory, Bar Harbor, ME), nine control males (prenatal exposure to a saline injection) and seven DA males (prenatal exposure to 1.5 mg/kg DA), ranging in age from PD32-40 were subjected to the MRI portion of the experiment. Mice were maintained on a 12-h light/dark cycle (lights on at 0600 h), room temperature $21 \pm 1^\circ\text{C}$, and allowed food and water ad libitum. All experiments were performed during the animal's light cycle. Protocols were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) "Principles of Laboratory Animal Care."

2.2.2 Animal Preparation—All mice were anesthetized throughout the MR imaging procedure. Anesthesia was induced by 3-4% isoflurane and maintained with 1-1.5% isoflurane. The head was kept stationary using a custom-built head holder designed to fit in the RF coil. Respiration (80-100 bpm) and animal temperature (maintained at 37°C) were monitored and controlled by a small animal monitoring system (Model 1030 Monitoring and Gating System, SA Instruments, Stony Brook, NY).

2.2.3 Imaging Acquisition—Imaging was performed during a single session for each animal on an 11.75T Bruker BioSpec scanner equipped with a Resonance Research, Inc high-bandwidth shim power supply. A 20mm ID RF quadrature volume coil (M2M, Cleveland, OH) was used for all studies. All scans were performed with Paravision 5. Using MAPSHIM, a 3D Fieldmap phase image was acquired; TR=20ms, TE₁=2ms, inter echo time = 4.003ms, FA=20°, FOV=40 mm × 18 mm × 25 mm, matrix = 80 × 90 × 125 (voxel size of $0.5 \times 0.2 \times 0.2 \text{ mm}^3$, matching the EPI voxel size). This was followed by a T2-weighted structural image (RARE, TR = 4590ms, effective TE = 32ms, RARE factor = 8, 30 contiguous slices (0.5 mm thick) with interleaved acquisition, FOV = 18 × 18 mm, matrix = 150 × 150, voxel size $0.12 \times 0.12 \times 0.5 \text{ mm}^3$, 2 repetitions). Global (volume) and local (brain voxel) shimming with MAPSHIM were performed to calculate first and second order shims prior to the functional MRI scan. The resting-state fMRI consisted of a single shot gradient echo-planar imaging (EPI) sequence with the following parameters: 450 repetitions (total scan time = 15min), TR = 2000ms, TE = 10ms, FA = 60°, 30 contiguous slices (0.5 mm thick) with interleaved acquisition, FOV = 25.6 × 16 mm, matrix = 128 × 80, voxel size $0.2 \times 0.2 \times 0.5 \text{ mm}^3$.

2.2.4 General fMRI BOLD preprocessing—Functional images were first processed to reduce artifacts as previously described (Stafford et al., 2014). These steps include: 1)

removal of a central spike caused by MR signal offset; 2) correction of odd vs. even slice intensity differences attributable to interleaved acquisition without gaps; 3) correction of field in homogeneities by applying the fieldmap phase information using FEAT[67]; 4) movement correction; 5) within run intensity normalization to a whole brain mode value of 1000. Processed functional data was transformed to an anatomical atlas for each individual via the T2 scan. Functional data was registered to the rodent atlas supplied by the caret software (map_015 atlas) [68-70]. Each run then was resampled in atlas space on an isotropic 0.2 mm grid combining movement correction and atlas transformation in one interpolation [71].

2.2.5 rs-fcMRI pre-processing—As previously described [52], several additional preprocessing steps were used to reduce spurious variance unlikely to reflect neuronal activity (e.g. heart rate and respiration). These steps included: 1) a temporal band-pass filter ($0.009 \text{ Hz} < f < 0.1 \text{ Hz}$); 2) spatial smoothing (0.4 mm full width at half maximum); 3) regression of six parameters obtained by rigid body head motion correction; 4) regression of the whole brain signal; and 5) regression of the first order derivative of the whole brain and motion parameters.

2.2.6 Regions of Interest (ROI)—168 cortical pre-defined areas, based on the connectonal and architectonic subdivisions in the mouse were used. These areas were obtained by combining a brain atlas as defined by the Allen Institute for Brain Science [72] with that of Paxinos [73], upon which it is based and many regions are identical, including the main olfactory bulb (MOB) and accessory olfactory bulb (AOB). However, as the Allen MOB and AOB regions were directly from histological preparation tissue distortion made it difficult to directly register these ROIs to the mouse MR data (tissue warping from histology at the rostral fringes of the mouse brain are most susceptible to tissue warping). Thus, we chose to use regions directly mapped to the caret atlas as they were 1) MRI based and 2) free of the distortion from histological preparation (Caret, map_015, available at: <http://sumsdb.wustl.edu/sums/mousemore.do>). The remaining 164 ROIs were derived from the ALLEN institute defined cortical areas, for a total of 168 ROIs.

2.2.7 Extraction and computation of regionwise resting state correlations—For each animal, we collected 15 minutes of resting state BOLD data. For each ROI, a resting time series was extracted separately, and correlated region-by-region for each animal to create correlation matrices [68-70]. For statistical analysis, r-values were Fisher Z transformed to improve normality. Finally, for each volume ROI Fisher Z transformed data was averaged across all subjects and used for analysis.

2.3 Analysis methods

2.3.1 Graph Metrics Methods—In order to assess similarities and differences in functional connectomes between DA and saline exposed mice we assessed node degree, which is a measure that identifies the most connected nodes (or ROIs) by counting the number of direct connections to all other nodes. A node with high degree will have strong, direct connections to many other nodes in the network [74-75]. Degree is a binary classifier, for our analysis if a connection was in the top 15% strongest positive functional connections

it was considered functionally connected. The Matlab code was used for degree and can be obtained in the brain connectivity toolbox (see <https://sites.google.com/site/bctnet/measures/list>; [76]). Graph metrics are plotted on the same scale for both groups, with the strongest values in red. To assess the significance of differences between DA and saline exposed mice, a null distribution was built by randomizing the group labels across 10,000 permutations. For each randomized group an average matrix was generated (*i.e.* Schmidt–Hunter method for meta-analyses) and differences between the two groups were calculated. Finally, the percentage of null values that exceeded the true observed value was calculated. A resulting two-tailed p-value for each ROI was then assessed for significance.

2.3.2 Group Comparisons of seed based functional connectivity—Group differences in functional connectivity of the left and right dorsal anterior cingulate were assessed between DA and saline exposed mice. First, each regions resting state time series was computed and a correlation coefficient was computed between the seed region (left or right cingulate) and all other brain regions for each subject. The strength of correlation coefficients from the seed region to all other regions were compared by a t-test and plotted on the brain. Connections with group differences greater than $p < .05$ are plotted on the brain (see figure 3).

2.3.3 Short Range connectivity—For this study, short-range connections were defined as ROI pairs that were directly adjacent and long-range connectivity was defined as interactions between non-adjacent regions. This resulted in a comparison of 1000 short-range connections and 13,028 long-range connections.

To assess differences in short- and long-range connectivity, group connectivity matrices were averaged, connection strengths for adjacent (short-range) and non-adjacent (long-range) connections were computed, and paired two-sample t-tests to test were used to evaluate differences in long- and short- range connectivity strengths.

3. Results

3.1 Social interaction tests

A major aim of this study was to examine whether prenatal exposure to DA reduces social behavior and whether these deficits were exaggerated in male mice. We found a significant time by sex interaction ($F(1,44) = 13.1796$, $p = .0007$) where females tended to increase in SI while males decreased in SI across from PD25 to 35. There was also a trend for lower SI in DA animals at PD25 ($F(1,44) = 3.018$, $p = .0893$). As expected, post hoc analyses showed that at test date PD25, males exposed to DA expressed lower SI responses than saline exposed animals ($p = .0072$). However, this effect was not seen at the PD35 test date. Female SI responses were not sensitive to prenatal DA exposure and there was no main effect of DA across gender and test date ($p = .11$). Results from the repeated measures ANOVA and post hoc t-tests are shown in figure 1. No group or sex differences in SI were found during the shorter, 5-minute SI test at PD21.

3.2 Ultrasonic Vocalizations

Ultrasonic vocalizations were quantified across two 15-minute SI tests. Although correlated with SI ($r = .41$, $p < .0001$) across each of the tests DA exposure did not significantly affect the number of USVs ($F(1,48) = 1.78$, $p = .18$). Males showed a trend towards fewer calls than females ($F(1,44) = 2.925$, $p = .094$), driven by increase in call number for female mice at test date PD35. We also found a significant time by sex interaction ($F(1,44) = 26.45$, $p < .0001$) and effect of time ($F(1,44) = 5.95$, $p < .018$), driven by an increase in USVs in female mice at PD35. As seen in figure 2, there is a trend towards lower USVs in DA mice most clearly in male mice at PD25 ($p = .070$). Means and standard errors are shown for each subgroup.

3.3 Rs-fcMRI Network Results

In order to characterize connectivity change resulting from prenatal DA exposure employed three general analyses. We expected altered connectivity within the default mode, global connectivity alterations would target areas involved in social behavior, and we expected differences in local and long range connectivity between DA and control animals.

3.4 Anterior cingulate functional connectivity

We examined group differences in ACC connectivity because it has been implicated in DA exposure and because this region is a major hub of the default mode network. Unexpectedly, we found a pattern of overconnectivity from the dorsal anterior cingulate to the anterior nodes of the default system, including infralimbic and orbital regions. In contrast, we found that posterior nodes of the default system including the dorsal retrosplenial and CA3 of the hippocampus showed reduced connectivity. Group difference maps for whole brain connectivity are shown in figure 3 for the left dorsal ACC and in figure S1 for the right dorsal ACC.

3.5 Group Differences in Node Degree

Node degree is a central measurement for analyzing network dynamics which quantifies the number of connections each region has to the rest of the brain. Regions that are connected to many other areas (high degree regions) are referred to as “hubs.” We found group differences in hub architecture in a number of regions ($p < .05$, 10,000 permutations). Specifically, we found that mice with prenatal exposure to DA have lower degree in the left ventrolateral orbital area, left nucleus accumbens, left accessory and anterior olfactory area, and the right infralimbic area. Conversely, the DA had higher node degree in the right visual cortex, secondary motor area, the dorsal anterior cingulate, and the left piriform cortex. See figure 4.

3.6 Local vs distributed connectivity

Next, we examined differences in local versus long-range connectivity. For this analysis, adjacent ROIs were considered short range connections and long range connections were non-adjacent ROI pairs. Average connectivity for each pair was assessed and paired sample t-tests were run to assess group differences. No difference in non-adjacent, longer-range, connections were found between groups (DA $M = -.0053$, control = $-.0065$, $p = .34$).

However, DA exposed animals had greater connectivity between adjacent, short range, connections compared to controls (DA $M = .22399$, control $M = .2280$, $t(999) = 2.614$, $p = .0091$). See figure 5.

4. Discussion

Diagnostic assessments of California sea lions and experimental studies with laboratory rodents have shown that DA exposure can result in heightened seizure activity, diminished motor function, aberrant and even maladaptive social behavior, and repetitive behaviors; observations that suggest exposure to DA engenders behaviors resembling features of autism spectrum disorder. To determine whether exposure to DA also results in patterns of brain connectivity that share similarity with ASD, we evaluated resting state functional connectivity in our mouse model.

We exposed mice to DA at ED 16, a timepoint equivalent to ED 18 in rats [18]. This timepoint clearly follows the most frequently targeted period of development for DA studies, ED 13 in rats [77], when the hippocampus undergoes peak neurogenesis [78] and is most sensitive to DA exposure [19]. Critically, DA exposure is neurotoxic throughout development, including demonstrated effects throughout late gestation in mice [20] and extending to PD 14 in rats [23, 24, 28, 29].

Kainate receptors are expressed during late-gestation in the rodent [31] and human [32] brain, which corresponds with E20 in the rat [79] and the first trimester of human fetal development [79]. To engage these receptors in regions outside the time and place of hippocampal neurogenesis, we injected a single dose to DA (1.5 mg/kg) to pregnant B6 dams on ED16. Exposure at this dose and developmental timepoint diminishes social investigation in adolescent FVB mice and results in an increase in parvalbumin immunoreactivity, an anatomical finding that suggests DA exposure dysregulates excitatory/inhibitory tone [65]. We do not know how DA disrupts mouse social behavior because prenatal exposure to straddling timepoints, ED14.5 and ED17.5, which also causes profound changes in conditioned fear memory and adaptation to novel circumstances, effects myelination and growth of neuronal processes [20]. One likely possibility is that domoic acid exposure during development has pleiotropic effects on brain functioning because glutamate receptors are fundamental to many aspects of neuronal development.

Adolescent B6 mice prenatally exposed to DA expressed diminished social interaction, as indicated by our measure of social investigation. We also found a trend toward lower USV production, suggesting a relationship between social investigation and USV production that we have noted previously [80, 81]. Effects of DA on adolescent social investigation by B6 mice are modest in the current report and are similar to our previous studies with FVB mice [65]. Our finding of DA effects on *adolescent* social behavior is consistent with the juvenile onset of social deficits diagnosed in autism [82]. Our finding that *prenatal* exposure of mice to DA more robustly diminished adolescent male social interactions is consistent with a previous report that *postnatal* exposure of rats to DA more effectively diminishes social interactions among adult males [30]. Our results are also consistent with evidence that human males are more sensitive to DA exposure than females [83] and consistent with the

sex ratio associated with autism [84]. We urge caution in placing stock in the greater sensitivity of males to both DA exposure and autism risk because developing males are generally more sensitive to developmental insults than are females.

Like other animal models of human disease and disability, our model of ASD may be challenged for lack of construct validity. Common to all laboratory models, housing of rodents in standard caging denies them the spatial and temporal variation they require for robust anatomical [85], or social-emotional development [86]. In this view, our model benefits from parallel assessments of California sea lions, what we might consider a free-range sentinel species. In general, experimental models also gain construct validity is when they express multiple features of the disorder they are designed to simulate. We might have improved upon our construct validity by studying more behavioral phenotypes, such as deficits in empathy [87] or social reward [88], other behavioral phenotypes relevant to ASD, rather than just SI [89-91]. In this study, we chose instead to assess construct validity by examining functional connectivity.

The human default mode network is comprised of brain regions that support social cognition, including processing related to self versus other [47-48]. With recent success identifying an analogous default network in mice [52], and anomalies in the human default mode associated with autism and schizophrenia [46], we chose to examine this network in DA-exposed mice. We focused on the ACC as it plays a critical role in the functionality of the default network and in social interaction [55-57], including perception of distress in others [92], an ability is diminished in autism [93-94]. Indeed, the cingulate cortex can have impaired function in autism [95-97]. The ACC is a likely target of DA exposure, as it binds with high affinity to the glutamate receptor GluR6 [98], which is expressed in the developing cingulate cortex [31]. The ACC of our DA-exposed mice expressed a complex and atypical patterns of connectivity, with over-connectivity between the ACC and frontal regions, such as the orbital and infralimbic cortex, and under-connectivity in posterior regions, including the retrosplenial cortex, and the CA3 region of the hippocampus.

Though the exact nature of connectivity within the default mode remains incompletely characterized in ASD [99] and schizophrenia [100-101], adults with ASD can express weak connectivity between the anterior and posterior cingulate [102] and over-connectivity between default mode components [45]. As ASD represents a spectrum of social disability, we expect connectivity to vary across the disorder. For instance, if one child has difficulties with empathy and another with emotional expression, we might assume these discrete social deficits are associated with distinct patterns of functional connectivity. To further explore these relationships, future studies might incorporate caging that offers mice temporal and spatial complexity for more healthy social development, and with these mice identify variations in a range of social behaviors (social approach, social reward, empathy, etc) and in default mode connectivity, and then consider within-subject relationships between discrete social behaviors (social approach, social reward, empathy, etc) and default mode connectivity.

Node degree is a metric of global network structure that characterizes connectivity between each region and the rest of the brain. We hypothesized that regions most impacted would be

involved in social behavior. Accordingly, a number of regions relevant to the social brain distinguished DA from saline exposed mice. DA-exposed mice expressed a lower node degree (i.e. fewer connections to the rest of the brain) in the infralimbic and ventral lateral orbital areas, both default network regions critical for higher cognitive processing and sensory integration. Additionally, we found underconnectivity of the nucleus accumbens in DA-exposed mice, which could potentially relate to reduced reward seeking associated with social interaction [88-103]. DA-exposed mice also expressed increased node degree in sensory regions including the visual cortex, secondary motor cortex, and piriform cortex. Incidentally, children with ASD show similar increases in node degree in sensory systems such as the visual cortex and decreases in degree in regions of the frontal cortex [104], where node degree is predominately decreased in schizophrenia [105]. Although we primarily find a pattern of under-connectivity of higher order regions and over-connectivity in sensory regions of DA-exposed animals, this pattern was not globally observed. DA-exposed mice also express under-connectivity in the sensory related anterior olfactory nucleus, and over-connectivity in the right dorsal anterior cingulate of the default mode. Overall, these results suggest that DA exposure leads to a complex reorganization of network topology affecting both higher order as well as sensory networks and share similarities with network abnormalities associated with ASD.

Mice exposed to *in utero* to DA also expressed an increase in local connectivity measured by higher connectivity between adjacent brain regions. Local over-connectivity and long-range under-connectivity has been associated with ASD [59-61-64]. Multiple studies have demonstrated decreased long-range connectivity (between more distant regions) in children with ASD. These variations in connectivity may promote atypical reliance on local, rather than distributed processing, and an inefficient integration between brain systems. Although, we found increased local connectivity, we did not observe a difference in long-range connectivity in DA exposed mice (measured by connectivity between non-adjacent regions). However, a comprehensive analysis of how connectivity changes as a function of each regions relative distance may yet identify group differences as a function of longer-range connections. Nevertheless, our findings suggest that prenatal DA exposure results in a brain organization characterized by a higher local connectivity.

In response to an outbreak of Amnesic Shellfish Poisoning in 1988, Canada promulgated a regulatory action limit for DA in mussels of 20 mg of DA/kg bivalve tissue designed to protect against the acute effects of DA exposure [83-106-107]. The US and the European Union have adopted this regulatory limit, though studies conducted since promulgation of this regulatory action limit suggest that developmental exposure to low levels of DA can have pernicious and long-term behavioral effects [40-108]. Findings that the mammalian fetal brain expresses receptors with high affinity to DA over a prolonged window of development, that DA concentrates in amniotic fluid, and that juveniles are more sensitive to DA than adults, also suggest that low concentrations of DA in the environment could have adverse effects on a developing fetus [1-39-109-110]. These developmental sensitivities may have real world implications. Prenatal exposures to DA may render juvenile California sea lions with subtle neurological disabilities and less capable of survival [111]. Some reports suggest humans may confront analogous risks. A high prevalence of ASD has been associated with coastal communities adjacent to marine environments with high levels of

DA [84, 112–114]. Taken together, developmental exposures to domoic acid and other natural and synthetic ligands of glutamate receptors deserve attention for their potential contributions to ASD susceptibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Prenatal domoic acid (DA) exposure results in behavioral and neural phenotypes associated with developmental disorders
- Prenatal DA exposure leads to reduced social behavior
- Functional connectivity MRI abnormalities include atypical default mode connectivity, disrupted network structure in social, reward, and sensory areas, and local over-connectivity.

Social Behavior

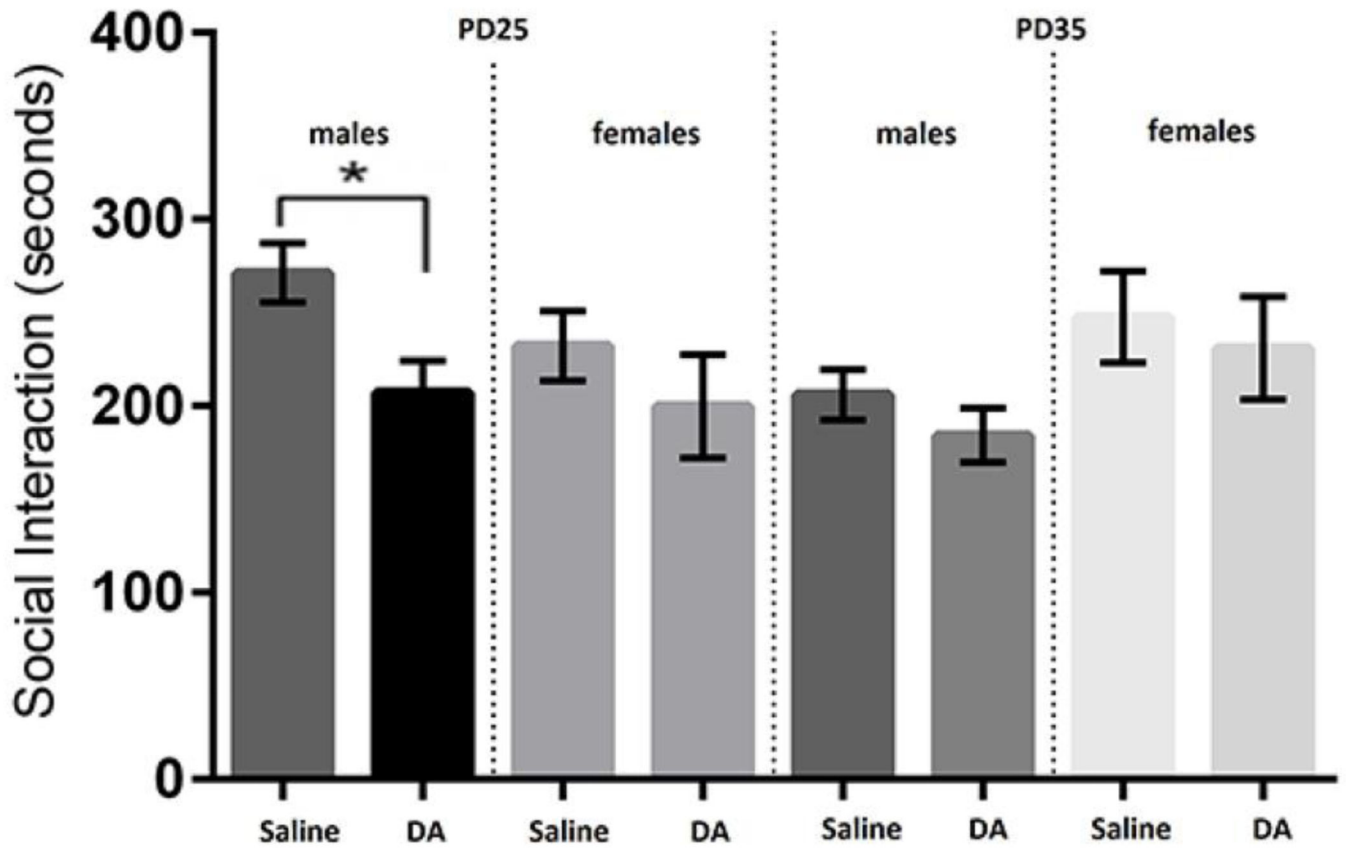


Figure 1.

DA exposed males show reduced time spent in social interaction which is mainly driven by observations at PD25 (* $p = .0072$). We also found that males tended to decrease their SI across time, where females increased their SI behavior ($p = .0007$). Means and standard errors are shown.

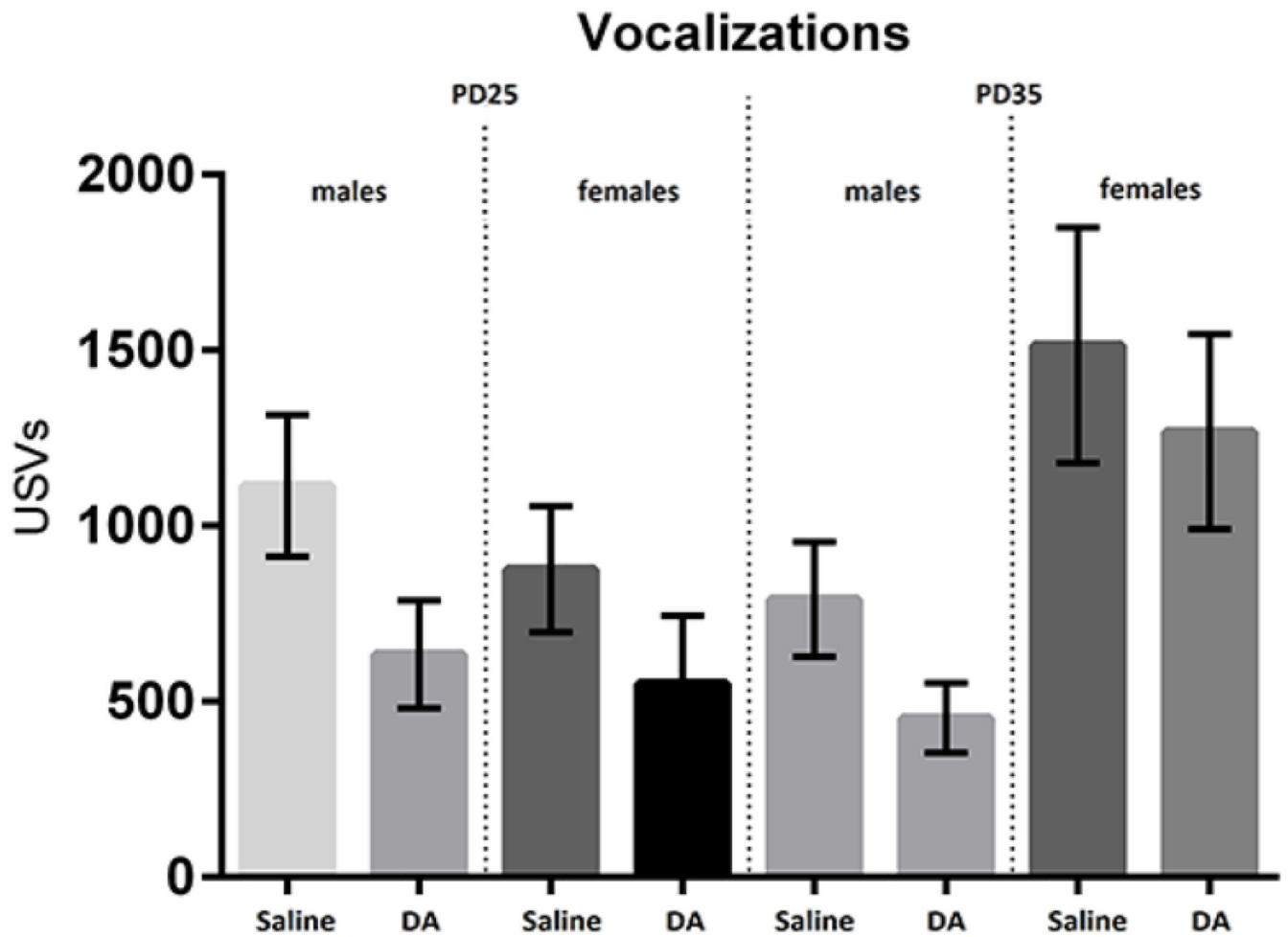


Figure 2. Number of USVs in saline and DA treated rats at PD25 and PD35. Overall, the effect of DA did not reach significance ($p = .17$), however, there was a trend towards fewer USVs in male mice on PD25 ($p = .07$). Means and standard errors are shown.

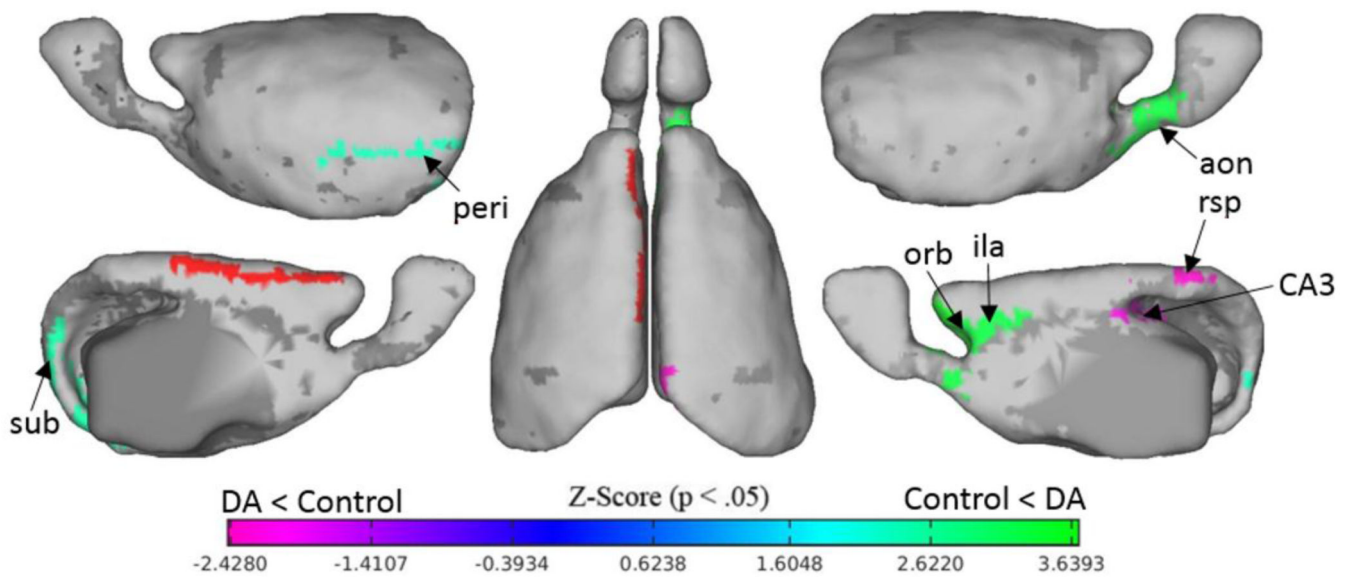


Figure 3. Group differences in left anterior cingulate functional connectivity. The red region is the left dorsal anterior cingulate (seed region), green colors correspond to connections which are stronger in DA exposed animals and pink colors represent connections with weaker connectivity in DA exposed animals. All regions shown represent connections with group differences of $p < .05$, and a z-score of above or below 1.98, uncorrected. Noteworthy differences include decreased connectivity to posterior nodes of the default system (rsp and CA3) and overconnectivity to anterior regions (orb, ila). Regions shown are the perirhinal area (peri), orbital (orb), infralimbic area (ila), hippocampal CA3 (CA3), the rsp (retrosplenial cortex), and the subiculum (sub).

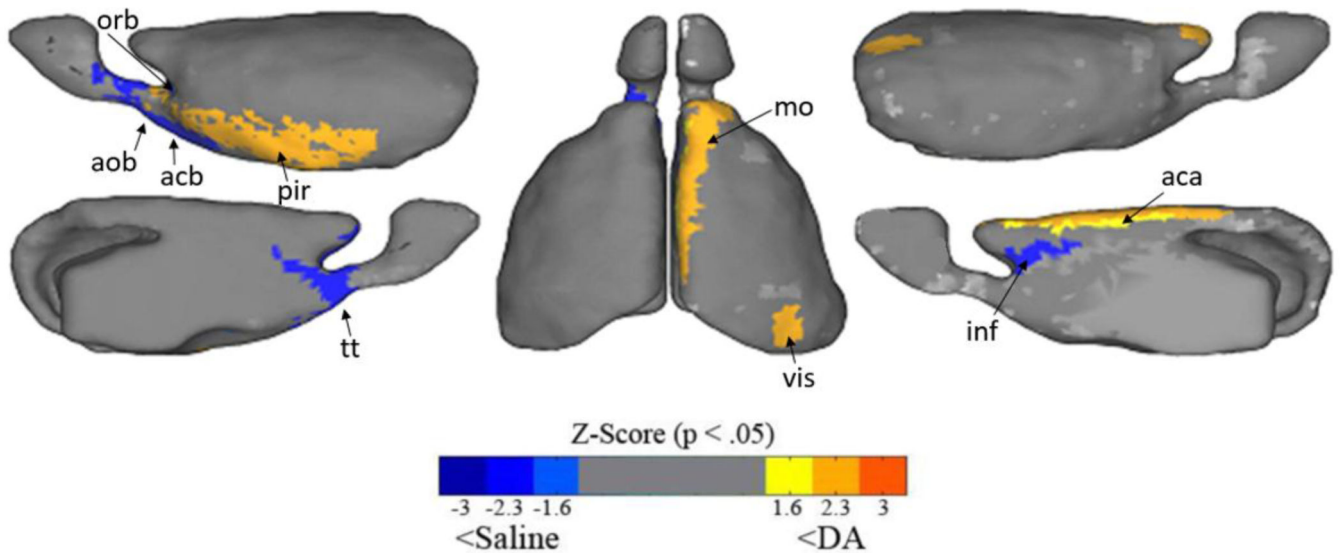


Figure 4. Group differences in node degree. The total number of functional connections from a region to all other regions, are plotted on the mouse brain. Warm colors are regions with higher node degree in DA exposed males and cool colors are areas with higher node degree in saline exposed males. DA exposed animals have global connectivity, that is, more connections (higher degree) to the piriform cortex (pir), secondary motor area (mo), visual (vis), and dorsal anterior cingulate (aca). DA exposed animals have lower degree to the infralimbic cortex (inf), taenia tecta (tt), nucleus accumbens (acb), anterior olfactory (aob), and ventral lateral orbital area (orb). All z-scores visualized are $p < .05$ based on 10,000 random permutations.

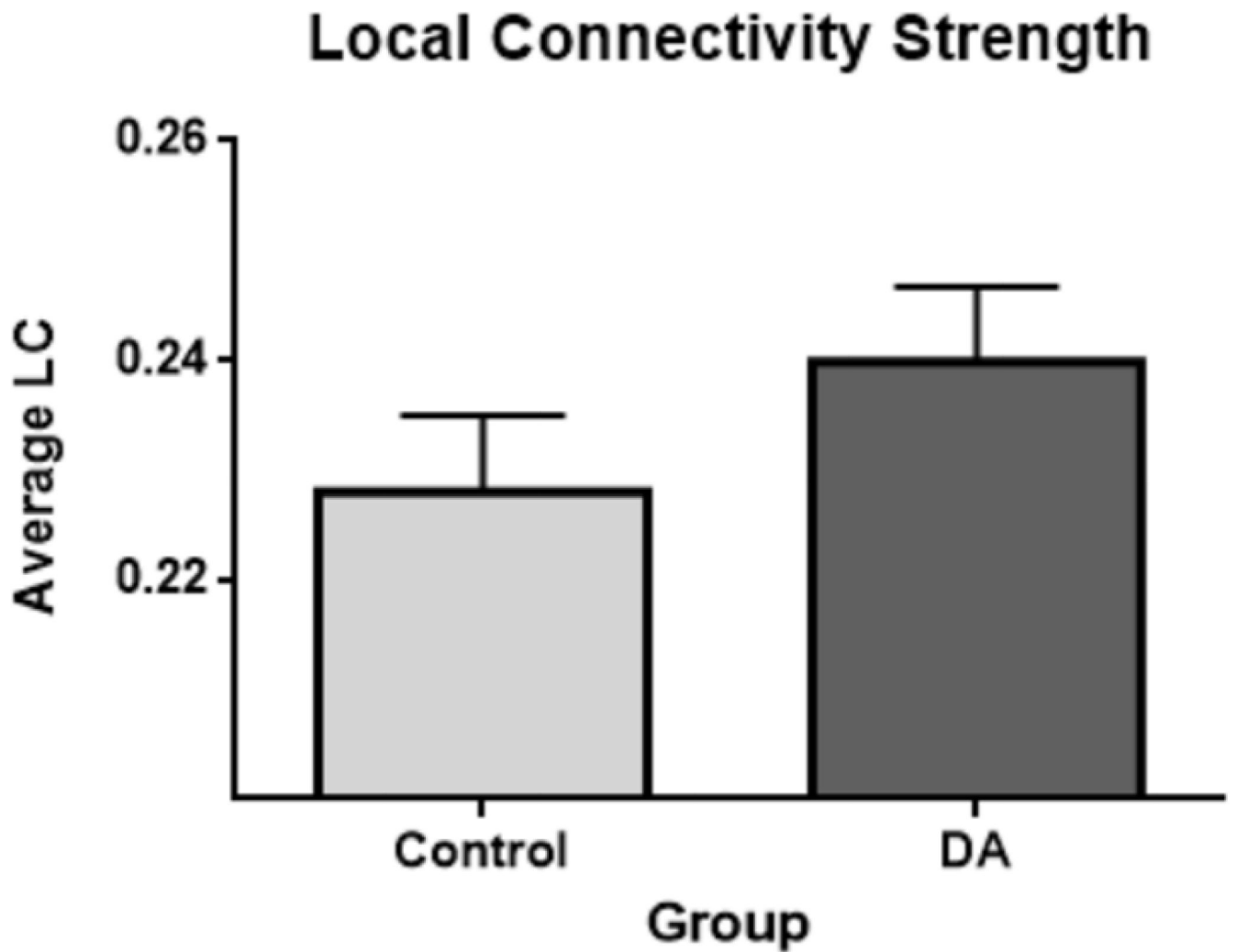


Figure 5. DA exposed animals have greater local connectivity compared to controls ($p = .0091$). Local connectivity (LC) is the group averaged connectivity strength (r) between all adjacent ROI pairs. Error bars represent the standard error of measurement for each group.