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Protracted dendritic growth in the typically developing human amygdala and increased spine density in young ASD brains

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Abstract

The amygdala is a medial temporal lobe structure implicated in social and emotional regulation. In typical development (TD), the amygdala continues to increase volumetrically throughout childhood and into adulthood, while other brain structures are stable or decreasing in volume. In autism spectrum disorder (ASD), the amygdala undergoes rapid early growth, making it volumetrically larger in children with ASD compared to TD children. Here we explore: (a) if dendritic arborization in the amygdala follows the pattern of protracted growth in TD and early overgrowth in ASD and (b), if spine density in the amygdala in ASD cases differs from TD from youth to adulthood. The amygdala from 32 postmortem human brains (7–46 years of age) were stained using a Golgi-Kopsch impregnation. Ten principal neurons per case were selected in the lateral nucleus and traced using Neurolucida software in their entirety. We found that both ASD and TD individuals show a similar pattern of increasing dendritic length with age well into adulthood. However, spine density is (a) greater in young ASD cases compared to age-matched TD controls (<18 years old) and (b) decreases in the amygdala as people with ASD age into adulthood, a phenomenon not found in TD. Therefore, by adulthood, there is no observable difference in spine density in the amygdala between ASD and TD age-matched adults (≥18 years old). Our findings highlight the unique growth trajectory of the amygdala and suggest that spine density may contribute to aberrant development and function of the amygdala in children with ASD.

Keywords

amygdala; autism spectrum disorder; golgi-kopsch; neuromorphology; spines; RRID:SCR_003131

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1 Introduction

The amygdala, a cluster of nuclei in the medial temporal lobe, is implicated in a large number of neuropsychiatric disorders, including schizophrenia, depression, bipolar disorder, anxiety, and autism spectrum disorder (ASD; Schumann, Bauman, & Amaral, 2011; Kennedy & Adolphs, 2012). The amygdala functions as a danger and salience detector and, in turn, plays a key role in social and emotional regulation (Amaral, 2003; Adolphs, 2010). In typical development (TD), the amygdala continues to enlarge throughout childhood and into adolescence, a time period when growth of other brain regions (e.g., the hippocampus) has stabilized or are even decreasing in volume (e.g., the neocortex) (Giedd et al., 1996; Schumann et al., 2004; Ostby et al., 2009; Scott, Schumann, Goodlin-Jones, & Amaral, 2009; Hunsaker, Scott, Bauman, Schumann, & Amaral, 2014; Raznahan et al., 2014). This unique feature of protracted growth could be essential for prolonged social and emotional development throughout the lifespan.

In ASD, a disorder characterized by deficits in social and communication skills, amygdala structure and function is of particular interest. Neuroimaging studies indicate that the amygdala growth trajectory is very different in ASD than in TD. Amygdala volume of children with ASD is significantly larger than in typically developing children (Sparks et al., 2002; Schumann et al., 2004, 2009; Mosconi et al., 2009; Kim et al., 2010; Nordahl et al., 2012). This relative overgrowth correlates with symptom severity, providing further evidence for the structure–function relationship of the amygdala in ASD (Munson et al., 2006; Mosconi et al., 2009; Schumann, Barnes, Lord, & Courchesne, 2009; Iidaka, Miyakoshi, Harada, & Nakai, 2012; Elison et al., 2013; Shen et al., 2016). However, the volume difference in ASD dissipates during adolescence as the amygdala continues to grow in TD (Schumann et al., 2004).

Magnetic resonance imaging (MRI) studies have provided insight into the growth trajectory of the amygdala at the macroscopic level; however, this approach does not have the resolution to investigate changes in individual nuclei or the underlying cellular differences responsible for these observations (Schumann & Nordahl, 2011). To date, few studies have evaluated postmortem human brains across the lifespan to determine the cellular underpinnings of amygdala growth in TD, nor how this is altered in ASD. In a previous study in our laboratory, we found evidence of accelerated cell loss in adult cases of ASD relative to TD cases, especially in the lateral nucleus (Schumann & Amaral, 2006; Morgan, Barger, Amaral, & Schumann, 2014). The lateral nucleus is the largest nucleus of the amygdala and is the main input structure that receives afferent signals from the cortex and hypothalamus. Information flows through the amygdala, either through the dorsal or ventral pathway, and exits via the central nucleus (Schumann, Vargas, & Lee, 2016). The lateral nucleus therefore plays the very important role of ‘gatekeeper’, establishing the initial threshold for which information is attended to via excitatory or inhibitory signaling.

Differences in neuronal morphology are strongly implicated in neurodevelopmental disorders since it is the fundamental feature of neuronal connectivity (Copf, 2016). Dendrites and the spines studded along their length are essential for maintaining neuronal communication, via finely tuned excitatory and inhibitory signaling (E/I balance) (Spruston,

2008; Gao & Penzes, 2015). In the present study, we quantify the morphology of neurons in the lateral nucleus of the amygdala across the lifespan from childhood to adulthood in TD and in ASD. We used a Golgi-Kopsch staining protocol to first quantify morphological measures such as soma size, number of primary dendrites, and total dendritic length in the lateral nucleus of the amygdala. We then conducted a more fine-grained analysis of spine density and maturity as an index of amygdala connectivity. This is the first study to address potential morphological differences in amygdala neurons in ASD.

2 Materials and Methods

2.1 Subjects

Tissue from 32 postmortem human brains (16 TD cases aged 7–44 years and 16 ASD cases aged 7–46 years; Table 1) was obtained from the University of Maryland Brainbank (NIH NeuroBioBank), the BEARS program at UC Davis MIND Institute, Autism Tissue Program (now Autism BrainNet) and the Harvard Brain Tissue Resource Center. ASD cases were selected based on a positive diagnosis confirmed by the Autism Diagnostic Interview-Revised (ADI-R) (Lord, Rutter, & Le Couteur, 1994) administered by a trained clinician or from clinical records. Age-matched TD control cases were defined as having no known medical or psychiatric disorder impacting the brain. Consent from next of kin was obtained for all cases by the collecting institutions.

2.2 Golgi processing

Upon donation brains were immersion fixed in 10% buffered formalin, and either stored as whole hemispheres, or sectioned into 1 cm coronal slabs, depending on the brain-bank of origin. Mean storage period for the TD and ASD groups were significantly different at 171.9 ± 69.2 and 111.6 ± 32.5 months, respectively, yet this did not impact the overall results (see below). Blocks from the lateral nucleus of the amygdala approximately 1.5 × 1.5 × 1.0 cm^3 were excised from the temporal lobe (Figure 1a, b). A modified version of the Golgi-Kopsch staining was selected as the optimal protocol to impregnate neurons for visualization and quantitative analyses from tissue with long fixation periods (Riley, 1979; Jacobs et al., 2003; Rosoklija et al., 2003). Individual tissue blocks were wrapped in gauze and immersed in a 4:1 solution of 5% potassium dichromate and 30% formaldehyde for 5 days, with a change of gauze and solution refreshment on day 3. Blocks were then moved into fresh gauze and placed in new jars containing 3.5% potassium dichromate for 4 days, followed by 2 days in 0.75% silver nitrate solution. Blocks were then cleaned with cotton tipped applicators to remove excess buildup of precipitate, and then placed back into 3.5% potassium dichromate for 4 days followed by 2 more days in 0.75% silver nitrate. After removing precipitate again, dehydration and embedding was conducted at room temperature, in the dark, with slow agitation. Blocks (wrapped in fresh gauze) were dehydrated in increasing alcohol concentrations as follows: 50% (1 hr), 70% (2 hr), 95% (overnight), 100% (24 hr). Blocks were then changed to a 1:1 solution of 100% EtOH and ethyl ether for 4 hr followed by 4% parlodion (VWR, Radnor, PA) overnight. Parlodion was then changed to an 8% solution for another 24-hr period before the blocks were finally embedded in 10% parlodion solution and left to harden for approximately 2 weeks.

2.3 Sectioning and mounting

Once the parlodion had solidified to a firm consistency, the tissue blocks were trimmed of excess parlodion. Blocks were cut on a sliding microtome into 150 μm sections and collected into 70% alcohol. Sections were then further dehydrated in increasing alcohol solutions for 5 min each: 70%, 95%, 100% twice, and twice-through xylene. Sections were mounted rostral side down with DPX mounting medium and ultra-thin coverslips.

2.4 Neuron tracing

The lateral nucleus was delineated in accordance with prior neuroanatomical studies of human amygdala using the temporal lobe white matter tract as the lateral boundary and the fiber tract between the lateral and basal nucleus as the medial boundary (Braak & Braak, 1983; Schumann & Amaral, 2005; Brabec et al., 2010) (Figure 1b). Where feasible, five sections were used closest to the mid-rostrocaudal extent of the amygdala with the average distance between sections being 250 μm. Principal neurons in the ventrolateral region of the lateral nucleus were identified on the basis of a large soma with the presence of densely packed spines. These neurons have previously been referred to as ‘type 1’ or ‘spiny projection’ neurons (Braak & Braak, 1983). Neurons selected for tracing were fully impregnated, central within the z-plane of the slice, and free from obscurities. The full dendritic arbor of 10 neurons per case was traced using Neurolucida 10 (MBF Bioscience, Williston, VT) using 100 × objective lens on a Zeiss (Imager Z2 Vario) microscope. All traces were made with the investigator blind to diagnosis, age and cause of death; thereby eliminating any potential for selection bias.

2.5 Spine analysis

Analysis of spine density was conducted according to methods described in Morgan and Amaral (2014) along the entire length of one dendrite on each of the 10 selected neurons. Spines were marked and classified into one of six morphological categories ascribed in the Neurolucida software: mushroom, thin, filopodial, stubby, detached, and branched. Overall spine density was calculated by dividing the total number of spines by the total length of the dendrite. Finally, the percentage of each spine type was analyzed between diagnostic groups.

2.6 Analysis and statistics

The following morphological data were extracted for each neuron using Neurolucida Explorer (MBF Biosciences, Williston, VT) and examined in accordance with previous studies (Jacobs et al., 2011, 2015): soma size (cross sectional area, μm^2), number of primary dendrites (dendrites projecting from the soma), total dendritic length (summed length of all dendrites, μm), dendritic spine density (DSD; number of spines per μm) and dendritic spine number (DSN) for each separate spine classification. Mean values for each of the above measures were calculated from the ten neurons per case. Statistical analyses were performed in IBM SPSS Statistics (v.23 IBM, Armonk, NY). The data were normally distributed, enabling analysis of covariance (ANCOVA) tests to be run, with ‘diagnosis’ as the between group factor and ‘age’ as a continuous covariate; PMI, storage time, sex and hemisphere were added as co-variates. Significant ‘age’ effects were followed with single linear regression tests as seen in the analysis of total dendritic length. Significant ‘diagnosis’ by

'age' interactions, as in the case of spine density, were further explored. 'Age' was divided into discrete groups [children (<18 years old) vs. adult (≥18 years old)] and a 2-way ANOVA performed to confirm the age by diagnosis interaction, followed by Tukey's HSD post-hoc comparisons.

2.7 Image preparation

Two-dimensional (2-D) composite photomicrographs were created using a stack of through-focus images (separated by 10 μm) taken in Neurolucida and prepared in Adobe Photoshop CS5.1. The image in which the soma was in sharpest focus was used as a background image. Sections of in-focus dendrites from the other images were excised and aligned onto the base image. This resulted in a flattened 2-D representation of the full dendritic arbor. Incidental dendrites from the base image that were not part of the composite neuron were edited out of the image for clarity.

3 Results

The success of Golgi-Kopsch staining in the current set of postmortem human tissue was consistent with previous studies (Jacobs et al., 2003; Rosoklija et al., 2014), allowing quantitative data collection from 27 (13 TD and 14 ASD) of the 32 brains processed, yielding data from a total of 270 traced neurons (Figure 2). Example photomicrographs of lateral nucleus amygdala neurons from typically developing and ASD individuals during childhood and adulthood are depicted in Figure 3.

3.1 Dendritic morphology

The number of primary dendrites projecting from the soma did not differ between diagnostic groups or across age (mean values: TD 4.98 ± 0.13 vs. ASD 4.99 ± 0.17 for all ages, $p = .96$). Total dendritic length significantly correlated with age for both TD and ASD groups (TD: $r = .81$, $p < .01$; ASD: $r = .58$, $p < .05$), yet there was no overall difference in the dendritic growth curve between diagnostic groups ($F_{(1,23)} = 1.05$, $p = .32$; Figure 4a). Soma size did not vary with age ($F_{(1,24)} = 0.01$, $p = .92$) and was not significantly different between diagnostic groups ($F_{(1,23)} = 0.36$, $p = .55$) (Figure 4b). Example tracings of neurons representing the age range studied (7–46 years) are depicted in Figure 5.

3.2 Spine density and classification

Spine density (total number of spines/total length of dendrite) was significantly different between diagnostic groups ($F_{(1,24)} = 4.73$, $p = .04$) with a further interaction between age and diagnosis ($F_{(3,36)} = 4.36$, $p = .014$) (Figure 6a). Spine density in childhood cases of ASD was significantly higher compared to age-matched TD cases ($p = .049$) and to adult ASD cases ($p = .013$; Figure 6b). There was an overall significant interaction between group and spine type (young TD, adult TD, young ASD and adult ASD) ($F_{(15,84)} = 2.271$, $p = .002$), allowing for further post-hoc analyses, however when analyzed by individual spine class, there were no significant effects of age or diagnosis (Figure 7). Representative sections of second order dendrites (Figure 8a) from child and adult TD and ASD cases are depicted in Figure 8b. From the limited clinical data we had access to, we had ADI-R scores from three

ASD cases (ages 14, 15, and 29) yet there was no obvious qualitative observation to correlate ADI-R score with spine density.

4 Discussion

4.1 Summary

The present investigation applied the Golgi method to quantify neuronal dendritic morphology in the amygdala of human postmortem brains from typically developing cases and individuals diagnosed with ASD. The dendritic length of neurons in the amygdala lateral nucleus increases with age from childhood to adulthood in both ASD and typically developing individuals. The two groups do not differ on measures of soma volume, total dendritic length, or number of primary dendrites at any age. However, neurons in the lateral nucleus of children with ASD exhibit a higher density of dendritic spines than neurons of age-matched typical children (<18 years of age). As individuals with ASD develop into adulthood, spine density in the amygdala appears to decrease, a phenomenon not found in TD. Therefore, by adulthood, there is no observable difference in spine density in the lateral nucleus of the amygdala between ASD and typical age-matched adults (>18 years of age).

4.2 Protracted dendritic arborization in the amygdala into adulthood

The present study is the first to investigate the dendritic morphology of human amygdala neurons across TD and in ASD from youth to adulthood. Our findings suggest that the protracted growth of the amygdala into adulthood, as observed in MRI studies, could in part be a result of continuous dendritic expansion, further emphasizing the unique developmental trajectory of the human amygdala. Here, we discuss the impact of protracted dendritic arborization on amygdala development at the cellular level.

4.2.1 Typical development—In TD, the human amygdala displays a unique growth trajectory characterized by increasing volume throughout childhood, adolescence, and even into adulthood, despite stabilization of total cerebral volume (Giedd et al., 1996; Schumann et al., 2004, 2016; Ostby et al., 2009). The lateral nucleus, which is the largest of the amygdaloid nuclei, is the primary input nucleus of the amygdala, receiving information from several regions including the primary sensory cortices, association cortices and the prefrontal cortices (McDonald, 1998; Stefanacci & Amaral, 2000; Price, 2003; Schumann et al., 2016). Our findings demonstrate that total dendritic length of lateral nucleus projection neurons continuously increases across development from childhood into adulthood. Since this is the only study of dendritic development from youth to adulthood in human amygdala to date, we can only draw comparisons from other brain regions where neuronal growth trajectories have been mapped across human lifespan, including visual, prefrontal, occipital, temporal, and hippocampal cortices (Scheibel, Lindsay, Tomiyasu, & Scheibel, 1975, 1976; Buell & Coleman, 1981; Becker, Armstrong, Chan, & Wood, 1984; Huttenlocher, 1990; Jacobs, Driscoll, & Schall, 1997; Mavroudis et al., 2015). Although there are brain region and layer specific subtleties, in general, dendrite growth is extremely rapid during late gestation and early childhood in cortical regions (Becker et al., 1984; Huttenlocher, 1990). This early cortical dendritic growth is followed by a slower expansion through childhood into a relatively stable state during adolescence and adulthood (Huttenlocher, 1990; Jacobs

et al., 1997; Travis, Ford, & Jacobs, 2005). In elderly brains, many studies report age-related reduction in cortical dendritic length (Scheibel et al., 1975, 1976; Mavroudis et al., 2015); however, findings of increasing dendritic arborization in the hippocampus between the sixth and eighth decade of life have also been reported (Becker et al., 1984; Flood, Buell, Defiore, Horwitz, & Coleman, 1985). Therefore, our findings suggest that the protracted growth of the amygdala into adulthood, through in part a mechanism of continuous neuronal and dendritic growth, is a unique feature of this brain region and may reflect the importance of amygdala function in modulating adolescent social and emotional development. However we acknowledge that this could be one of several contributing factors for protracted amygdala growth. Other mechanisms of growth, such as neuronal maturation and migration may be co-occurring both in the lateral nucleus and/or other nuclei.

4.2.2 Autism spectrum disorder—The amygdala undergoes a different volumetric growth trajectory in people with ASD compared to TD, such that it is approximately 15% larger in young children with ASD compared to age-matched typically developing children. As the typical amygdala continues to grow in volume into adulthood, the amygdala in people with ASD remains constant, leading MRI studies to find either no observable gross differences (Haznedar et al., 2000; Schumann et al., 2004; Barnea-Goraly et al., 2014), or a slight decrease (Aylward et al., 1999; Pierce, Müller, Ambrose, Allen, & Courchesne, 2001; Nacewicz et al., 2006), in older adolescents and adults with ASD compared to typical controls. We predict that the amygdala first undergoes a period of accelerated growth in individuals with ASD, which plateaus during adolescence. Yet, due to the protracted growth in TD, these early volumetric differences disappear by adulthood. The current analyses suggest a continual increase in dendritic length of lateral nucleus neurons throughout the age range of ASD cases studied is comparable to the growth in TD. Thus, if dendritic length is continuously increasing, yet neuroimaging studies suggest a relative stability in volume into adulthood in ASD, there must be other factors driving this phenomenon. Accelerated cell loss in ASD is one possible explanation, supported by stereological studies showing increased neuron numbers in pediatric cases (T. Avino, personal communication) and decreased numbers of neurons and oligodendrocytes in older cases (Schumann & Amaral, 2006; Morgan et al., 2014). Our finding of a similar pattern of dendritic morphology of amygdala neurons between age-matched typical and ASD cases is in contrast to other brain regions, such as the frontal cortex where a reduction in dendritic number was found in ASD cases (Mukaetova-Ladinska, Arnold, Jaros, Perry, & Perry, 2004) and dendritic arborization of CA4 and CA1 hippocampal neurons was also decreased (Raymond, Bauman, & Kemper, 1996). Finally, it is important to recognize that the oldest case in our cohort was 46 years of age. Therefore, we were not able to evaluate dendritic morphology in older-adult or elderly ASD brains, which are a rarely studied population and an important future direction of research.

4.3 Amygdala neuronal spine density in typical development and ASD from youth to adulthood

The density of dendritic spines has direct implications for the functionality and connectivity of the cell. Synapses tend to be located at the heads of dendritic spines and therefore a higher density of spines would suggest a higher density of synapses, although this was not

measured here. In the present study, neurons of young ASD cases had a greater density of spines in the lateral nucleus of the amygdala compared to age matched typical brains, and older ASD cases. Here we discuss prior observations of spine density in TD and in ASD, and speculate on the underlying neurobiology and implications of aberrant spine density.

4.3.1 Typical development—In typical early postnatal development, neuronal connectivity is shaped by an initial abundance of immature spines and synapses, followed by a period maintaining stronger connections and pruning weaker connections, a process which eliminates roughly 50% of spines (Phillips & Pozzo-Miller, 2015). Synaptogenesis varies by brain region (Hutten-locher & Dabholkar, 1997) and has been extensively studied in the visual cortex (Bourgeois & Rakic, 1993; Missler, Eins, Merker, Rothe, & Wolff, 1993a, 1993b; Elston & Fujita, 2014). Morphometric analysis of pyramidal neurons in the visual cortex demonstrate that spine density increases dramatically during gestation and the first months of life, peaking at 18 months old, then decreases due to the pruning process, and remains relatively stable throughout the lifespan after age 5 (Huttenlocher, 1990). In the amygdala, there is evidence from the rodent literature for a delayed window of enhanced plasticity, as observed by increased axonal boutons from basolateral amygdala neurons in young adult mice compared to juveniles (Johnson et al., 2016). Furthermore, there is an increase in dendritic branch length of rat amygdala neurons between postnatal days 35–90 (the equivalent period of human development from adolescence to adulthood) (Koss, Belden, Hristov, & Juraska, 2014), and delayed pruning of medial prefrontal cortical inputs to the basolateral amygdala (Cressman et al., 2010). Our findings in the human amygdala indicate that spine density in typical individuals is relatively stable from youth to adulthood. Surprisingly, we did not find any difference in the proportion of different spine types between typically developing young and adult cases, where one might hypothesize a shift toward a higher percentage of mushroom spines (as a proxy measure of mature spines) in older cases.

4.3.2 Autism spectrum disorder—We found that spine density on neuronal dendrites in the lateral nucleus of the amygdala is greater in young individuals with ASD compared to TD age-matched controls. This phenomenon is followed by an apparent decrease in spine density as people with ASD age into adulthood that does not occur in TD. Since this is the first ASD related study of spine density in the amygdala, we are not able to make direct comparisons with previous reports. However, there have been a small number of studies on spine density in other brain regions in ASD postmortem tissue. A qualitative observation of neurons in the prefrontal gyrus from three ASD cases suggested a *decrease* in spine density when compared to a single 5-year-old control case (Williams, Hauser, Purpura, DeLong, & Swisher, 1980), whereas increased spine density has been found in the frontal, parietal, and temporal cortices (Hutsler & Zhang, 2010), with the highest density of spines being recorded in the youngest ASD cases in the temporal lobe (Tang et al., 2014). The latter of these studies is consistent with the current finding of increased spine density in young ASD brains. There is a significant decline in spine density from youth to adulthood in ASD cases, leading to no detectable difference between adult typically developing cases. The proportion of each spine class was not statistically different in the ASD cases compared to the typically

developing cases suggesting spines are altered in a quantitative, but not qualitative fashion, which may in part be due to a period of delayed pruning.

4.3.3 Other genetic and environmental factors—Dendritic arborization and neuroplasticity are driven by both genetic and environmental factors (Jan & Jan, 2010; Chaudhury, Sharma, Kumar, Nag, & Wadhwa, 2016). Many genes associated with ASD are related to spine development and stabilization (De Rubeis & Buxbaum, 2015; Copf, 2016; de la Torre-Ubieta, Won, Stein, & Geschwind, 2016; Martínez-Cerdeño, 2016), including synaptic scaffold proteins, receptors, and cell adhesion molecules (Bourgeron, 2015). Genes of particular interest include mammalian target of rapamycin (mTOR) and phosphatase and tensin homolog (PTEN), both of which have roles in regulating cell growth. mTOR is involved in dendritogenesis (Kumar, Zhang, Swank, Kunz, & Wu, 2005; Thomanetz et al., 2013; Skałecka et al., 2016) and mice with constitutively overactive mTOR have greater spine densities, similar to those observed in postmortem ASD cases (Tang et al., 2014). PTEN mutations occur in a subset of the ASD population (Tilot, Frazier, & Eng, 2015) and disruption of the gene in mice leads to increased spine density and morphology in the cerebral cortex, hippocampus, and amygdala (Kwon et al., 2006; Haws et al., 2014). In addition to genetic factors, environmental stressors in rodents can lead to structural changes the brain, including the basolateral amygdala, alterations in spine density and delays in spine maturation in the basolateral amygdala (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005; Mitra & Sapol-sky, 2008; Yasmin, Saxena, McEwen, & Chattarji, 2016). It is possible some of the ASD cases included in our study carried mutations in the aforementioned genes, and we can only surmise about the stress effect of living with ASD experienced by our cohort. However, in addition to ASD, morphological changes of neurons at the dendritic and spine level found in a number of neurodevelopmental disorders including Fragile-X syndrome, Rett syndrome and Tuberous Sclerosis (Phillips & Pozzo-Miller, 2015).

4.4 Methodological considerations

Several factors need to be considered when interpreting data from human postmortem brains, including small sample sizes, cause of death, postmortem interval (period between time of death and tissue fixation), tissue storage period, and the idiosyncratic nature of the Golgi method. These factors have been addressed elsewhere in depth (de Ruiter, 1983; Jacobs et al., 2001; Jacobs et al., 1997; Jacobs, Schall, & Scheibel, 1993). Here, we note that the interpretation of our finding of continual increasing dendritic length in both TD and in ASD is limited by the fact that postmortem studies are cross-sectional, as opposed to longitudinal. Second, the Golgi impregnation method, although employed for nearly 150 years, is not without limitations. Spines parallel to the z-axis are masked by the girth of the dendrite, and therefore reported spine densities are likely an underestimation (Feldman & Peters, 1979). All cases were subject to the same measurement of spines along the entire length of a dendrite, mitigating the impact of dendritic width. More modern techniques exist, such as filling cells by injecting fluorescent dye; however, these methods have proven successful primarily for studying dendrite morphology in cultured cells, non-human fresh post-mortem tissue, or tissue from surgical resections in humans, rather than human postmortem tissue that tends to be stored for longer periods in formalin (Aliashkevich et al.,

2003; Staffend & Meisel, 2011; Cheng, Trzcinski, & Doering, 2014). Dye-leakage can occur in human postmortem tissue immersed in fixative for a prolonged period of time (Ohm & Diekmann, 1994), and thus the Golgi method remains the most reliable method for visualizing neurons in this cohort of samples (Jacobs et al., 1997, 2003).

Additionally, storage time and postmortem interval may influence visualization of spine density in brain samples (Williams et al., 1978; de Ruiter, 1983), although neither factor appeared to impact our primary finding of increased spine density in children with ASD. For example, there is some evidence from rodent literature that the appearance of spines may decrease with increasing PMI at 1.5 hr (de Ruiter, 1983); however, the present findings indicate that there was no relationship between spine density and PMI in our sample, which had a PMI range of 8–45 hr, consistent with the findings of Hutsler and Zhang (2010). There are many other potentially confounding variables, including, but not limited to, the heterogeneous symptom severity in ASD, medication use, and cause of death. Therefore, to draw meaningful conclusions about the impact these variables, more extensive clinical information is required on larger sample sizes, further highlighting the importance of human brain donations for research. One noteworthy exclusion criterion for our tissue was seizure as the primary cause of death. Seizures are a co-morbid feature of ASD (Keller, Basta, Salerno, & Elia, 2017), often originating in the temporal lobe (de Moura et al., 2012) and have been shown to impact dendritic morphology and spine density (Yilmazer-Hanke et al., 2000; Aliashkevich et al., 2003). In the present study, we received tissue from multiple brain banks and only had clinical records for a handful of ASD cases (3 of 16), therefore not enough to analyze our findings in terms of correlating spine density with symptom severity, although we hope to include this in future studies.

4.5 Conclusions and functional implications

In summary, the primary finding in this study is an increase in spine density on amygdala lateral nucleus neurons in young ASD brains. In addition, both TD and ASD show a similar pattern of increasing dendritic arborization with age in our cohort from 7 to 46 years. Both findings support the emerging story of age related changes in the amygdala, both in TD and in ASD from youth to adulthood. Although more extensive studies are required, the emerging hypothesis of amygdala development in ASD appears to be one of excess (of volume, neurons, spines, etc.) in childhood followed by loss (of neurons, spines, oligodendrocytes, etc.) in adulthood.

Although it is beyond the scope of measurement in human postmortem studies, it is interesting to consider the downstream implications of aberrant amygdala spine density and connectivity, especially in ASD, given the functional role of the amygdala in social behavior, anxiety, and sensory input processing (Kliemann, Dziobek, Hatri, Baudewig, & Heekeren, 2012; Tottenham et al., 2014). Functional imaging (fMRI) studies find “hyperconnectivity” of the amygdala in ASD (Welchew et al., 2005; Kleinhans et al., 2008; Monk et al., 2010; Weng et al., 2011; Murphy, Foss-Feig, Kenworthy, Gaillard, & Vaidya, 2012; Carpenter et al., 2015), which correlates with the severity of increased anxiety and/or social impairments (Dalton et al., 2005). In addition to acutely affecting neuronal connectivity, increased spine density could have long-term effects on cellular function including excitotoxic degeneration

(Wang & Qin, 2010), which could potentially explain findings of neuronal loss in adult ASD cases (Schumann & Amaral, 2006).

Finally, longer dendrites and a greater number of spines increase a neuron's receptive surface area and are therefore important factors in the regulation of neuronal activity, formation of neural circuits, and maintaining the finely tuned balance of excitation and inhibition. Imbalances may arise from problems in initial circuit formation or maintenance, yielding downstream effects on the structure and function of glutamatergic excitatory and GABAergic inhibitory circuits in individuals with ASD (Gao & Penzes, 2015). Disruption of the E/I balance in the ASD amygdala, via early aberrant synaptic formation and connectivity, could have profound effects on its function and the role it plays in modulating social communication and anxiety, making this an important focus for future studies.

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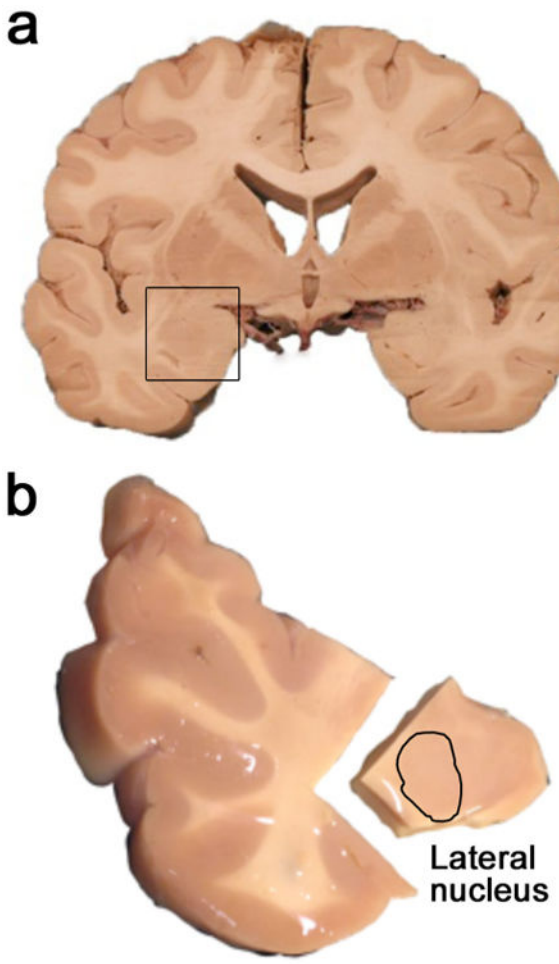

Figure 1.

(a) Whole brain coronal section with the amygdala demarcated by a black box. (b) Section of temporal lobe and example excision of amygdala tissue block for staining, with the lateral nucleus outlined. Blocks were approximately 1.5 × 1.5 × 1.0 cm^3 [Color figure can be viewed at wileyonlinelibrary.com]

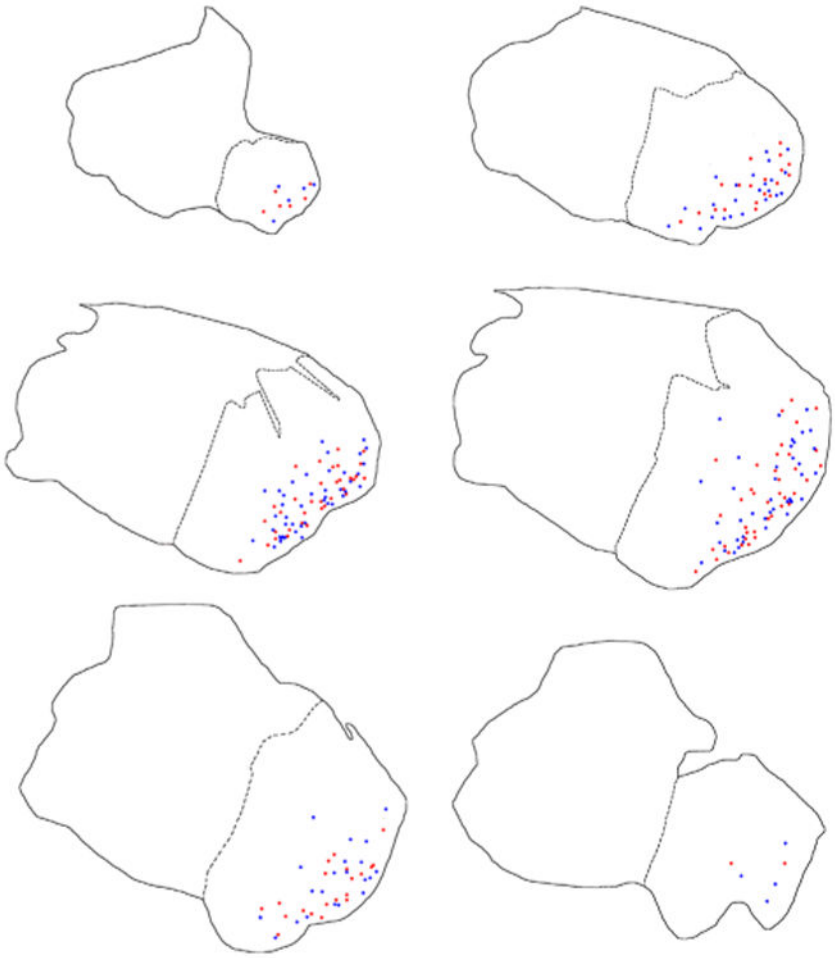

Figure 2.

Representation of the position of each traced neuron. Outline drawings of the entire amygdala with the lateral nucleus demarcated by a dashed line (blue - TD, red – ASD). Scale bar 400 μm [Color figure can be viewed at wileyonlinelibrary.com]

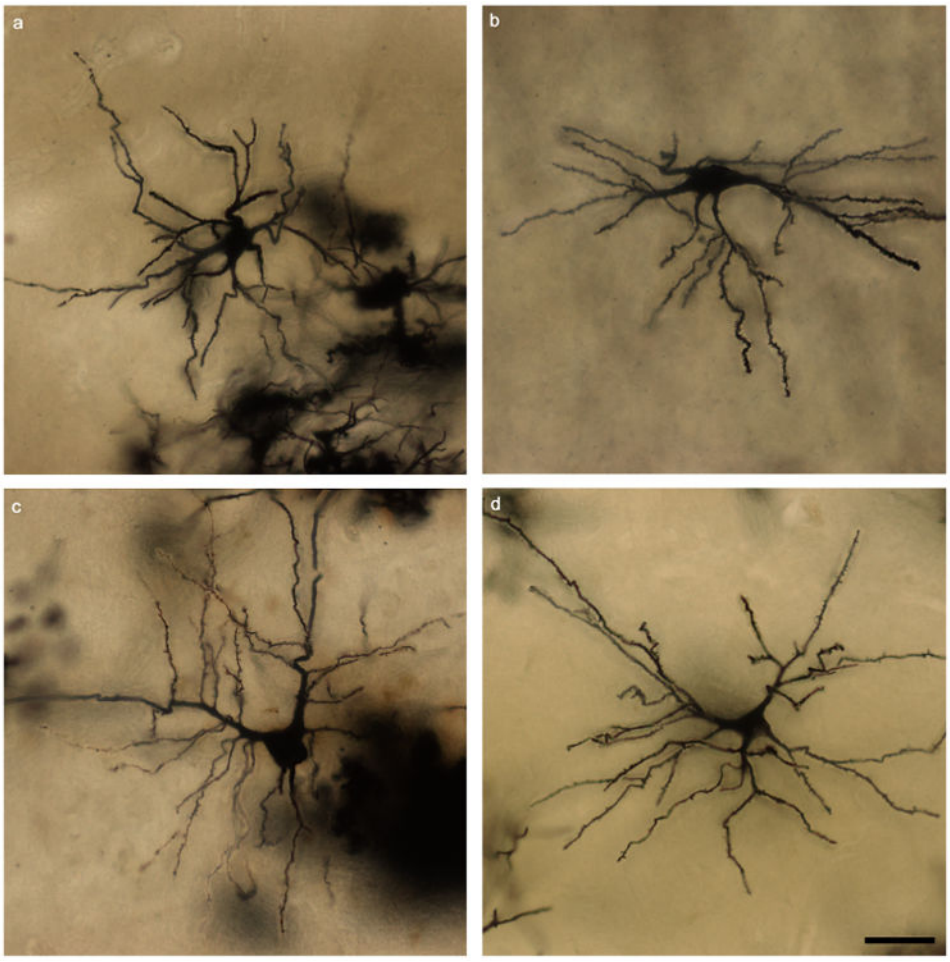

Figure 3.

Representative photomicrographs of lateral nucleus amygdala principal neurons from childhood and adulthood cases of typical development and ASD. Scale bar = 50μm [Color figure can be viewed at wileyonlinelibrary.com]

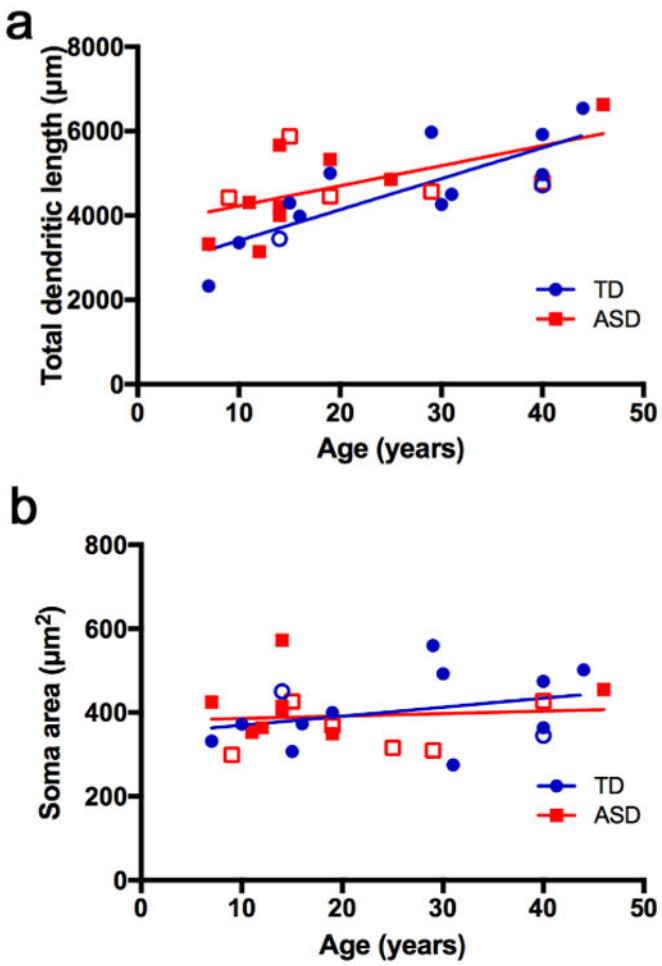

Figure 4.

(a) Total dendritic length (TDL) of lateral nucleus neurons in typical development (blue circles) and autism spectrum disorder (red squares) cases across age. Although both TD and ASD groups show an increase in TDL with increasing age, there was no significant difference between diagnostic groups. (b) Soma area remained stable across the lifespan of both diagnostic groups. Open data points denote female cases [Color figure can be viewed at wileyonlinelibrary.com]

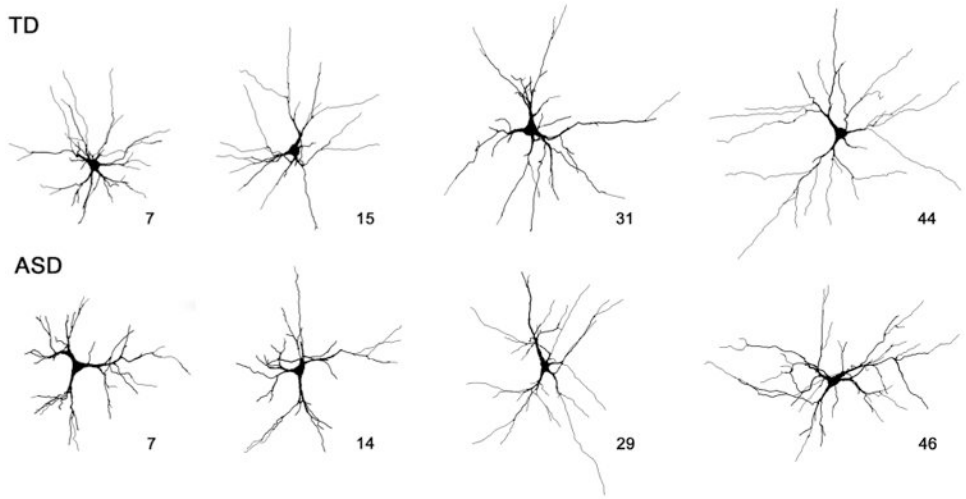

Figure 5.

Example neuronal tracings in the lateral nucleus of the amygdala in typical development (TD, top row) and autism spectrum disorder (ASD, bottom row) cases from ages 7 through mid-40s (age of subject depicted lower right hand side of each tracing). No significant differences were observed in the dendritic morphology between diagnoses. Scale bar = 100 μm

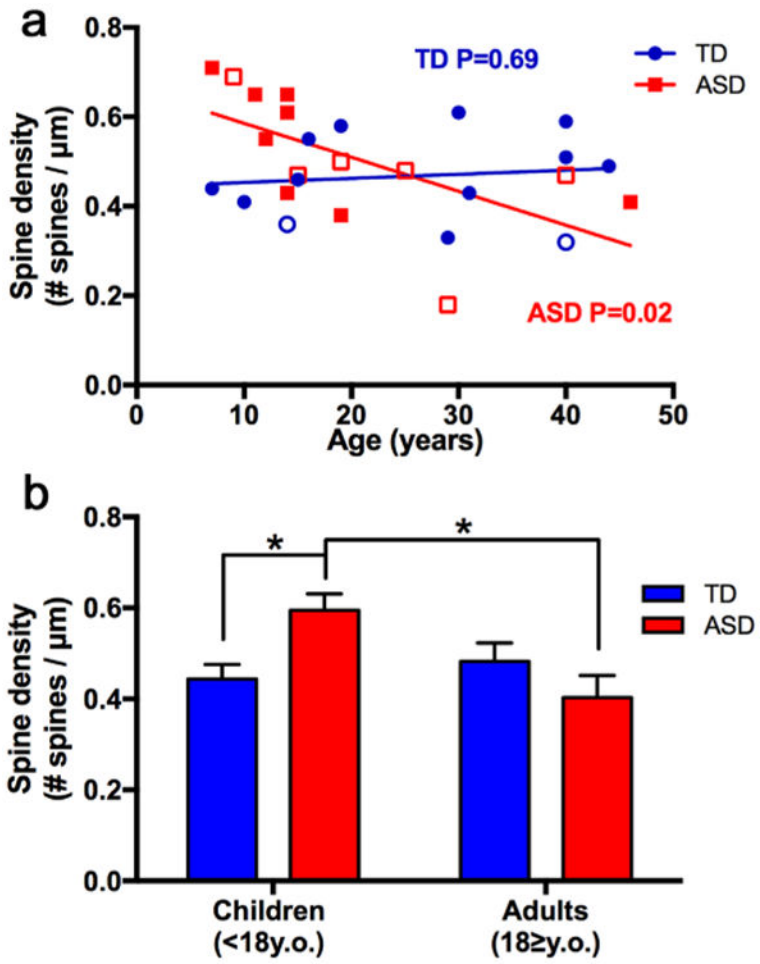

Figure 6.

(a) Dendritic spine density across age in typical development and cases of autism spectrum disorder. In typical development, spine density is relatively stable across the age range we examined, whereas ASD cases show a significant decrease with age. (b) Spine density in children (<18 years) is significantly higher compared to age matched TD cases and older (>18 years) ASD cases. *$p < 0.05$. Open data points denote female cases [Color figure can be viewed at wileyonlinelibrary.com]

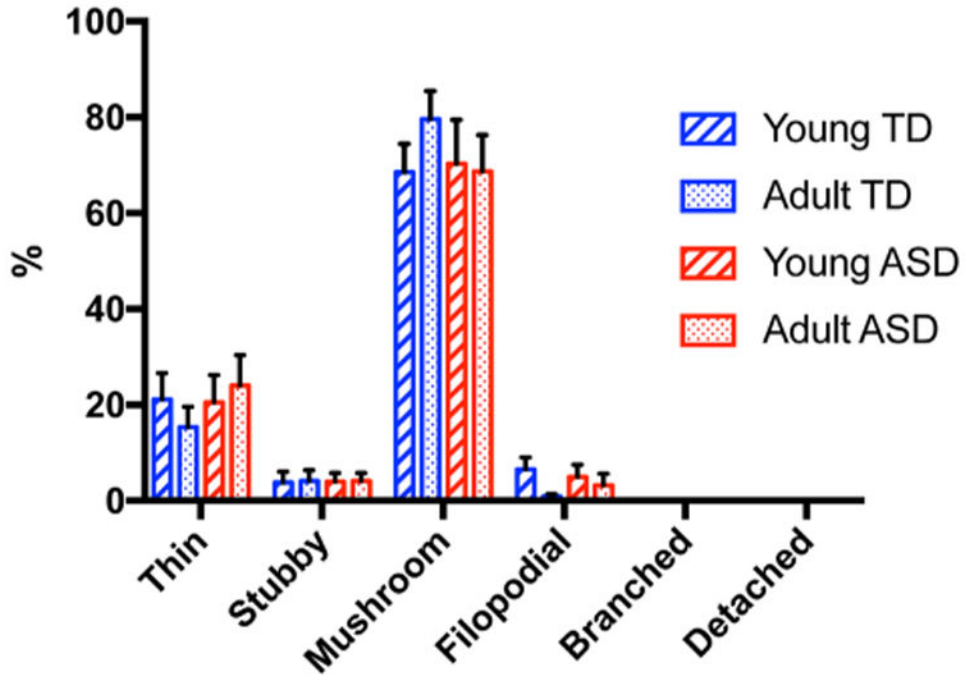

Figure 7.

Percentage of each spine classification in young and adult typical development and ASD cases. There were no significant differences between diagnostic groups or age groups [Color figure can be viewed at wileyonlinelibrary.com]

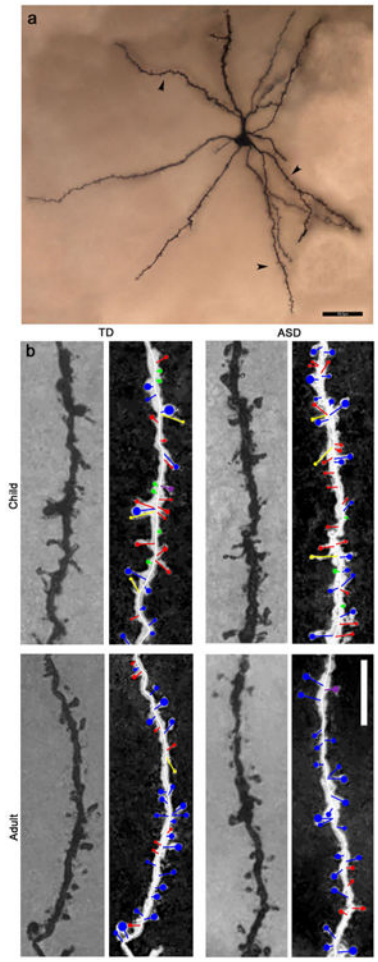

Figure 8.

(a) Representative 2D photomicrograph of a lateral nucleus amygdala principal neuron. Arrowheads depict examples of 2nd order dendrites. (b) Representative high magnification images of 2nd order dendrites and their spines of amygdala lateral nucleus neurons. Young ASD cases have a greater number of spines that tend to be more immature than in TD cases. Each picture is accompanied by a representative trace image. Blue – mushroom spine; Red – thin spine; Yellow – filopodial spine; Green – stubby spine; Purple –branched spine. Scale bar in a = 50 μm, in b = 10 μm [Color figure can be viewed at wileyonlinelibrary.com]

Table 1

Case information for all 32 subjects, split into typically developing (TD) and autism spectrum disorder (ASD) groups

Case ID	Diagnosis	R/L	Age	Sex	PMI	Storage (mo.)
4203	TD	R	7	M	24	165
4337[a]	TD	R	8	M	16	86
210	TD	R	10	M	24	265
5309	TD	R	14	F	8	93
2843	TD	R	15	M	20	226
388[a]	TD	R	15	M	34	255
1158[a]	TD	R	16	M	15	192
4669	TD	R	16	M	16	143
630	TD	R	19	M	25	241
4929	TD	R	29	M	18	126
1879	TD	R	30	M	21	233
5539	TD	R	31	M	24	43
7333	TD	R	40	M	25	84
2832	TD	R	40	F	14	227
1375	TD	R	40	M	23	191
1901	TD	R	44	M	26	180
6403	ASD	L	7	M	25	124
1182	ASD	R	9	F	24	192
4334	ASD	R	11	M	27	87
4305	ASD	L	12	M	13	108
6477	ASD	L	14	M	35	120
4315	ASD	R	14	M	22	115
4899	ASD	R	14	M	9	129
5278	ASD	R	15	F	13	86
5136	ASD	R	19	M	22	97
5561	ASD	R	19	F	34	40

Case ID	Diagnosis	R/L	Age	Sex	PMI	Storage (mo.)
4269[a]	ASD	R	19	M	45	125
6931	ASD	R	25	F	24	102
6640	ASD	R	29	F	18	113
6232	ASD	R	40	F	33	?
1575[a]	ASD	R	40	F	24	138
5115	ASD	R	46	M	29	99

Note. R/L, right/left hemisphere; PMI, postmortem interval

[a]Case did stain satisfactorily and was not included in the final analysis