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## Research Report

# Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders

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### ABSTRACT

Multiple types of indirect evidence have been used to support theories of altered cortical connectivity in autism spectrum disorders (ASD). In other developmental disorders reduced spine expression is commonly found, while conditions such as fragile X syndrome show increased spine densities. Despite its relevance to theories of altered cortical connectivity, synaptic spine expression has not been systematically explored in ASD. Here we examine dendritic spines on Golgi-impregnated cortical pyramidal cells in the cortex of ASD subjects and age-matched control cases. Pyramidal cells were studied within both the superficial and deep cortical layers of frontal, temporal, and parietal lobe regions. Relative to controls, spine densities were greater in ASD subjects. In analyses restricted to the apical dendrites of pyramidal cells, greater spine densities were found predominantly within layer II of each cortical location and within layer V of the temporal lobe. High spine densities were associated with decreased brain weights and were most commonly found in ASD subjects with lower levels of cognitive functioning. Greater spine densities in ASD subjects provide structural support for recent suggestions of connective changes within the cerebral cortex that may result in altered cortical computations.

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## 1. Introduction

Autism spectrum disorders (ASD) are a family of developmental disabilities sharing behavioral deficits that include sociobehavioral, cognitive, linguistic, and perceptuomotor abnormalities (Rapin, 1997). ASD has a strong genetic basis (Muhle et al., 2004), and numerous alterations to both the gross and microscopic structure of the brain have been identified (Bailey et al., 1993; Carper et al., 2002; Courchesne et al., 2003; Courchesne et al., 1999; Hutsler et al., 2007; Piven et al., 1996; Sparks et al., 2002).

Although the cerebral cortex in ASD is, in most cases, qualitatively similar to typically developing subjects in its

general organization (Bauman and Kemper, 1985; Guerin et al., 1996; Hutsler et al., 2007), quantitative methods have revealed a variety of alterations to the details of this organization. These include an increased density of cortical columns (Casanova et al., 2002), reports of cortical thickness increases, high neuronal densities, supernumerary neurons in the molecular layer, small areas of neuronal disorganization, poor differentiation of the gray–white matter boundary, and neuronal heterotopias (Bailey et al., 1998; Hutsler et al., 2007).

Recently it has been proposed that the cerebral cortex in ASD may also have profound connective changes and several types of indirect evidence have been cited to support this claim (Belmonte et al., 2004; Courchesne, 2004; Frith, 2004; Hughes,

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2007; Rippon et al., 2007). Increased white matter volumes just under the cortex have been used to suggest an increase in local, short-range connections (Herbert et al., 2003, 2004), while behaviorally, the possibility of local over-connectivity has been linked to hyper-arousal and reduced selectivity (Belmonte and Yurgelun-Todd, 2003) as well as superior abilities in tasks requiring local information processing (Happé, 1999). An associated deficit in long-range connectivity has also been proposed based upon functional imaging studies that show abnormal activity patterns between distant cortical regions (Cherkassky et al., 2006; Coben et al., 2008; Just et al., 2004, 2007), alterations to the gross morphology of the corpus callosum (Vidal et al., 2006), and an absence of top-down modulation of early sensory processing (Frith, 2003). In addition, gene mutations within cohorts of ASD subjects have been identified for SHANK3, a protein found in the postsynaptic density of excitatory glutamatergic synapses in the cerebral cortex (Moessner et al., 2008). Despite these various predictions of connective changes, and the potential importance of such changes to the behavioral symptoms that characterize autism, a direct examination of the structural microcircuitry within the cerebral cortex has not been undertaken.

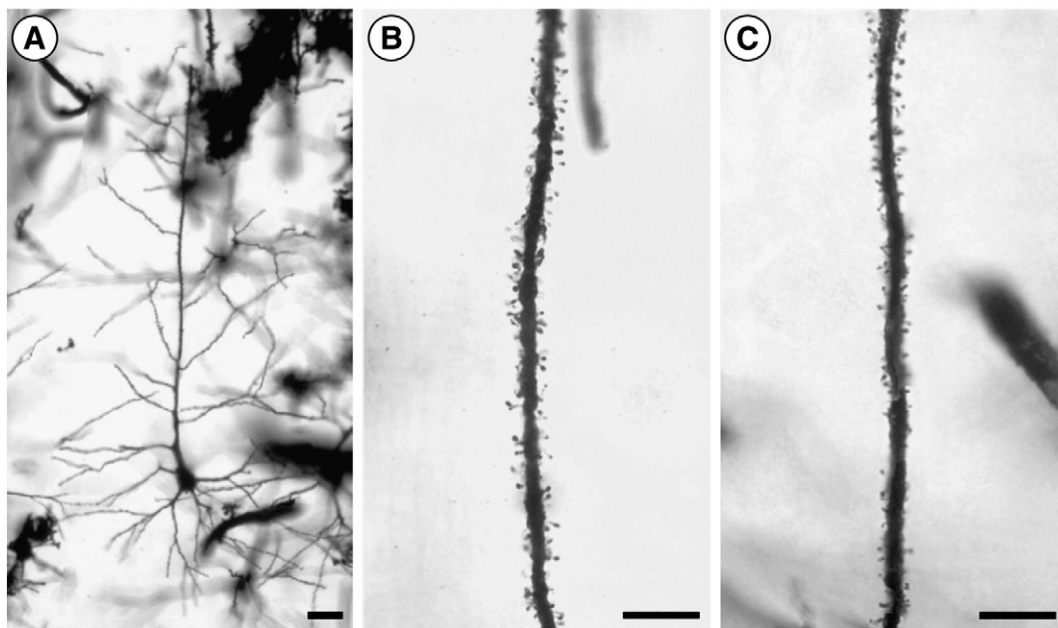
One method of assessing the quantity and distribution of connections onto individual neurons in both animal and human brain tissue has been to examine the expression of dendritic spines (Benitez-Bribiesca et al., 1999; Dietzmann and von Bossanyi, 1994; Ferrer and Galofre, 1987; Fiala et al., 2002; Garey et al., 1998; Huttenlocher and Dabholkar, 1997; Irwin et al., 2000, 2001; Jacobs et al., 1997, 2001; Michel and Garey, 1984; Multani et al., 1994; Suetsugu and Mehraein, 1980; Swann et al., 2000). The spines that cover the dendritic arbors of pyramidal cells in mammals are thought to support excitatory connections and, as such, have been widely used

as an index of excitatory connectivity on to these neurons (Fiala et al., 2002).

In the present study, dendritic spine densities on cortical pyramidal cells from ASD subjects and age-matched control cases were examined on neurons located within both the superficial and deep cortical layers of frontal (BA 9), temporal (BA 21), and parietal lobe (BA 7) locations in an effort to determine if spine densities on these neurons are altered in individuals with ASD. Although multiple cortical locations are of interest in ASD subjects, these regions were selected so that we could compare eulaminate isocortex (Bailey and von Bonin, 1951) from three cortical lobes. Because cortical locations, such as the temporal lobes, show significant involvement in ASD, we would predict that any differences in density might be regionally specific. Finally, if altered spine densities are present, we expect these changes to be most profound in the superficial layers of the cortex since they are the last to develop and undergo significant pruning during the postnatal period when ASD is typically diagnosed.

## 2. Results

Differences between ASD and typically developing subjects were first evaluated by examining the average spine densities found on individual pyramidal cells (Fig. 1A) according to dendrite type, cortical layer, cortical location, and hemisphere. In subsequent analyses, the distribution of spines was examined on pyramidal cell apical dendrites since these could be reliably followed for long distances. Finally, subject dyads were classified according to whether the ASD member showed a spine density increase or not. Those ASD subjects showing higher densities of synaptic spines were considered



**Fig. 1 – (A)** An example of a Golgi-impregnated pyramidal cell in layer III of the superior frontal gyrus (BA 9) in an ASD case. **(B and C)** Apical dendrite segments showing spine densities of layer V pyramidal cells in BA 9 of ASD **(B)** and control subjects **(C)**. Both examples are approximately 300  $\mu\text{m}$  from the cell soma and show a small difference in spine density (see panel BA 9/layer V in Fig. 4), but little difference in spine morphology. Scale bar in A = 25  $\mu\text{m}$ ; Scale bars in B and C = 20  $\mu\text{m}$ .

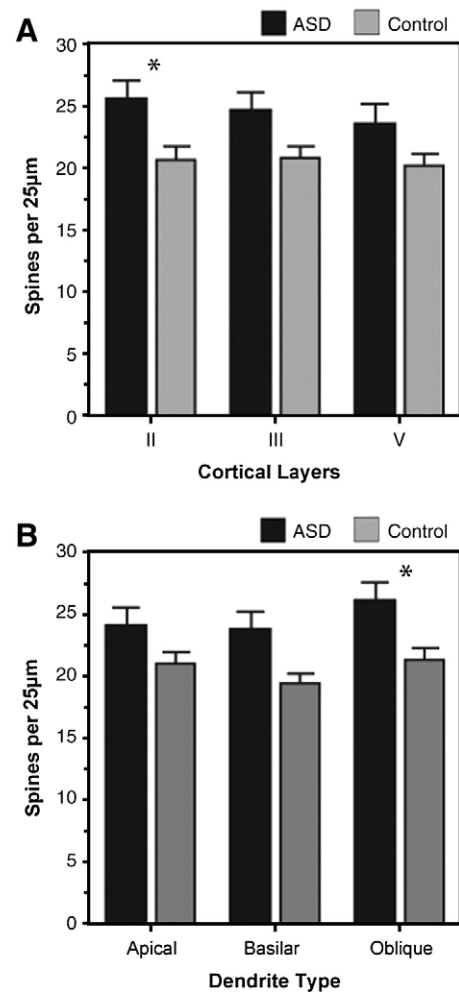
in light of several types of subject characteristics including the presence of epilepsy, the general level of cognitive functioning, and postmortem brain weight at autopsy.

### 2.1. Averaged spine densities

Spine densities were averaged across distances from the cell body to directly compare the three cortical layers (II, III, and V) and the three dendrite types (apical, basilar, and oblique). Using these highly averaged values, ASD subjects showed higher average spine densities ( $\bar{x}=24.60$  per  $25\ \mu\text{m}$ ) when compared to age-matched controls ( $\bar{x}=20.47$  per  $25\ \mu\text{m}$ ;  $F[1,9]=3.798$ ,  $p=.034$ ). Both layer ( $F[2,18]=12.373$ ,  $p<.001$ ; Fig. 2A) and dendrite type ( $F[2,18]=13.284$ ,  $p<.001$ ; Fig. 2B) also had a significant effect on spine density. Pyramidal cells from the superficial layers (II and III) had greater spine densities than those from the deeper layer (Bonferroni: II vs. V,  $p=.002$ ; III vs. V,  $p<.001$ ; II vs. III,  $p=.586$ ; see Fig. 2A), and spine densities were greatest on smaller oblique dendrites as compared to apical and basilar dendrite densities (Bonferroni: Oblique vs. Basilar,  $p<.001$ ; Oblique vs. Apical,  $p=.097$ ; Basilar vs. Apical,  $p=.107$ ). Using these averaged density values there was no main effect for cortical region ( $F[2,18]=.763$ , NS). In addition, layer, dendrite type, and cortical region did not interact with diagnosis (diagnosis $\times$ layer, Fig. 2A:  $F[2,18]=2.154$ , NS; diagnosis $\times$ dendrite type, Fig. 3B:  $F[2,18]=1.706$ , NS; diagnosis $\times$ region:  $F[2,18]=.144$ , NS). Only layer and dendrite type showed marginal, but nonsignificant, interactions with each other ( $F[4,36]=2.37$ ,  $p=.060$ ) and with the region examined (layer $\times$ dendrite type $\times$ region:  $F[8,72]=1.86$ ,  $p=.070$ ). In sum, spine densities were higher on the apical and basilar dendrites of superficially positioned pyramidal cells, while oblique dendrite densities were similar across layers. The remaining two-way and higher-order interactions were all nonsignificant ( $p>.10$ ).

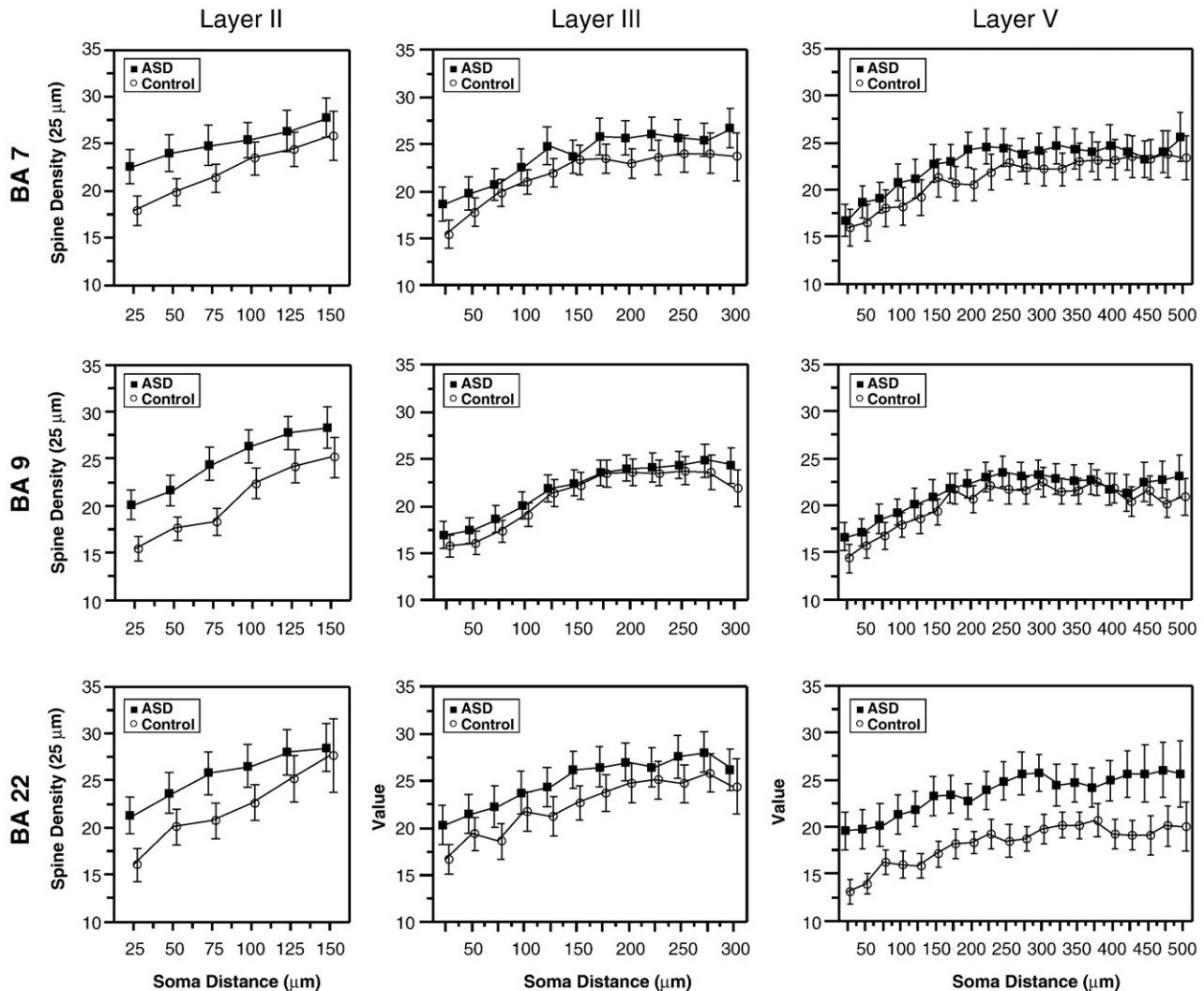
Spine morphology (Figs. 1B and C) varied from stubby to long and thin in both the ASD and control groups. A preliminary study of spine morphology indicates that spine lengths in the ASD group are shorter than those found in typically developing subjects with a difference in length of approximately .6 standard deviations (Glass  $\Delta=.653$ ). When spines were categorized by type, this length difference was primarily attributable to the presence of an increased number of compact spine types (Irwin et al., 2000). A complete description of spine morphology in this group of ASD subjects will be the focus of a subsequent manuscript (Avino and Hutsler, in prep.).

Because spine densities vary along the length of individual dendrites, we looked at whether the length of the dendrites that could be followed in each case differed between the autistic and typically developing groups. As expected, there was an interaction between layer and dendrite type that was driven by the longer apical dendrites on pyramidal cells within the deep layers ( $F[4,28]=145.73$ ,  $p<.001$ ). As expected, both layer ( $F[2,14]=229.576$ ,  $p<.001$ ) and dendrite type ( $F[2,14]=340.172$ ,  $p<.001$ ) showed reliably different sampling lengths. Neither diagnosis, nor region had an effect on the lengths of the sampled dendrites, nor were there any other significant interactions between the four variables (i.e., diagnosis, region, layer, and dendrite type).



**Fig. 2 – (A) Overall mean spine density values and standard error of the mean for ASD and control cases according to the layer of parent soma (spine densities for each subject group were averaged across the three regions examined and across the three dendrite types). Layer II tended to have higher values than III or V, but there was no significant interaction (see text). Asterisks indicate the presence of a simple effect for diagnosis for a specific layer. (B) Average spine density values for ASD and control cases according to the type of dendrite from which the count was made (spine densities were averaged across the three regions examined and across the cortical layers). Spine values were highest on small oblique dendrites. Asterisks indicate the presence of a simple effect ( $p<.05$ ) of diagnosis for a specific dendrite type and error bars indicate the standard error of the mean for each value.**

Finally, because this was a sample of convenience, the hemisphere available from the brain bank in each case could not always be controlled (see Table 1), thus the effect of the hemisphere sampled on averaged spine densities was evaluated along with the variables of diagnosis, region, layer, and dendrite type. The hemisphere sampled did not influence spine densities ( $F[2,14]=1.416$ , NS), nor did hemisphere show any significant interactions with the other variables.



**Fig. 3** – Averaged spine densities of apical dendrites as a function of distance from the cell body, cortical region, and cortical layer for ASD and control subjects. Error bars indicate the standard error of the mean for each value. ASD subjects showed consistently elevated values within superficial layer II of parietal (BA 7), frontal (BA 9) and temporal (BA 21) regions. In layer V, ASD subjects also showed elevated spine densities, but only within the temporal lobe (BA 21).

## 2.2. Spine distributions on apical dendrites

For each cortical layer (II, III, and V), the distribution of spines was also evaluated along the length of pyramidal cell apical dendrites (see Figs. 1B and C) according to diagnosis (ASD vs. control), cortical region (BA 7, BA 9, and BA 21), and distance from the cell body (Fig. 3). Within layer II (first column of Fig. 3) pyramidal cell spine densities were found to be higher in ASD subjects as compared to controls ( $F[1,20.5]=20.54$ ,  $p=.038$ ), and this effect did not interact with either distance ( $F[1,16.5]=2.88$ , NS) or cortical region ( $F[2,226.2]=.74$ , NS). In addition, there was no interaction between diagnosis, distance, and cortical region ( $F[2,205.8]=.065$ , NS). The three cortical regions did, however, differ from each other in spine density ( $F[2,226.2]=3.77$ ,  $p=.025$ ), and estimates of the fixed effects revealed that the frontal lobe region had marginally lower spine densities than the parietal and temporal lobe regions ( $p=.065$ ). As expected, densities varied according to distance from the cell body ( $F[1,16.5]=143.3$ ,  $p<.001$ ), but there was no interaction between region and

distance along the apical dendrite ( $F[2,205.8]=1.51$ , NS). Thus, within layer II ASD and control subjects differed from each other in spine density, and these differences were consistent across regions and at varying distances from the cell body.

In contrast, layer III apical dendrites did not show reliable spine density differences between ASD and control subjects ( $F[1,18.8]=.475$ , NS; second column of Fig. 3), and diagnosis did not interact with distance ( $F[1,19.4]=.481$ , NS) or with the cortical region ( $F[2,451.9]=1.26$ , NS). Like layer II, the three cortical regions did differ from each other ( $F[2,451.9]=10.64$ ,  $p=.001$ ) with frontal lobe regions showing lower spine densities than either parietal or temporal lobe regions ( $p=.041$ ). Also, spine density varied with distance from the cell body ( $F[1,19.4]=97.03$ ,  $p<.001$ ), but distance from the soma did not interact with cortical region ( $F[2,441.8]=.891$ , NS) nor did diagnosis, distance, and cortical region interact ( $F[2,441.8]=1.028$ , NS). These results indicate a similar distribution of spine densities along the apical dendrites of layer III pyramidal cells despite differences in densities between cortical locations.



**Table 1 – Case information.**

Case ID	Diagnosis	R/L	Age	PMI (h)	Brain weight (g)	Brodmann area			Cause of death
						7	9	21	
001	ASD	R	10	30	900*	X	X	X	Drowning
002	ASD	R	15	3	1390	X	X	X	Pneumonia
003	ASD	R	20	24	1140		X	X	Motor vehicle accident
004	ASD	L	25	26	1220	X	X	X	Seizure
005	ASD	R	27	8	1720		X	X	Drowning
006	ASD	R	27	30	1420	X	X		Neuroleptic malignant syndrome
007	ASD	L	29	24	1340	X	X	X	Cardiac arrhythmia
008	ASD	R	32	21	1710	X		X	Congestive heart failure
009	ASD	L	45	20	1370	X			Pulmonary embolism
010	ASD	R	44	31	1530	X	X	X	Myocardial infarction
Means			27.4	21.6	1347				
011	Control	R	11	20	1420	X	X		Internal bleeding
012	Control	L	17	7	NA	X	X	X	Trauma
013	Control	R	19	19	1555	X	X	X	Pneumonia
014	Control	R	22	21	1360	X	X	X	Unknown
015	Control	R	27	21	1330		X	X	Asphyxiation/Hanging
016	Control	L	27	16	NA	X	X		Arteriosclerosis
017	Control	L	29	24*	1350	X		X	Gunshot wounds
018	Control	R	30	23	1470		X		Myocarditis
019	Control	L	43	24	1450	X	X		Cardiovascular disease
020	Control	R	46	19	1588	X	X	X	Myocardial infarction
Means			27.1	18.8	1440				
021	Control	R	16	21	1130	X			Cardiomyopathy
022	Control	L	22	24	1580	X	X		Motor vehicle accident
023	Control	L	51	24	1600	X	X	X	Myocardial infarction
024	Control	R	56	24	1520	X		X	Cardiac arrest
025	Control	R	59	24	1590	X	X	X	Cardiac arrest
Means			40.8	23.4	1484				

\* = estimated; NA = not available.

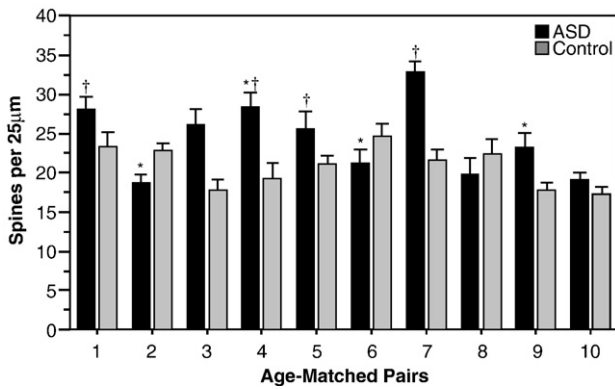
In layer V there was a significant interaction between diagnosis and cortical region ( $F[2,726.8]=3.23$ ,  $p=.040$ ; Fig. 3, column 3), with the temporal lobes showing higher spine densities in ASD subjects. There were, however, no significant main effects for diagnosis ( $F[1,18.1]=.956$ , NS). Like the other layers, cortical regions differed from each other ( $F[2,726.8]=3.053$ ,  $p=.048$ ), and spine densities differed at varying distances from the cell body ( $F[1,17.3]=59.2$ ,  $p<.001$ ). Neither diagnosis ( $F[1,17.3]=.006$ , NS) nor cortical region ( $F[2,699.5]=.919$ , NS) interacted with distance. Finally, there was no interaction between diagnosis, distance, and cortical region ( $F[2,699.5]=1.27$ , NS).

In sum, many ASD subjects showed greater spine densities within layer II for all three cortical regions (Fig. 3, column 1). These differences never interacted with distance from the cell body, suggesting somewhat equivalent distribution profiles despite the higher spine densities. Furthermore, in all three layers spine densities were lowest in frontal lobe locations (BA 9), but diagnosis only interacted with cortical region in layer V (Fig. 3, column 3) where spine density differences between ASD and control cases were largest in the temporal lobe (BA 21), but showed little difference in both the frontal (BA 9) and parietal lobe (BA 7) locations.

### 2.3. Subject characteristics

To further understand spine density changes in the ASD group, we also considered whether individual subjects showed increased spine densities relative to the typically

developing subjects. Spine density differences between ASD subjects and their age- and sex-matched controls were driven largely by a subgroup of cases (see Fig. 4). Only seven of the ten ASD cases showed increased averaged spine densities relative to age-matched control cases, and in one instance the magnitude of this difference was negligible. To assess which factors might be contributing to higher spine density in a subgroup of our larger ASD population, a number of subject-specific measures were considered. These included seizure history, secondary medical conditions, medications, educational history, level of cognitive functioning, and the severity of specific domains of diagnosis as assessed by a postmortem ADI-R (Lord et al., 1994). Four of ten ASD subjects had a history of seizures early in development that were treated with anticonvulsant medications (phenytoin, divalproex sodium, lorazepam, carbamazepine, or phenobarbital). Two of the members of this group showed greater spine densities relative to their age-matched control, while the other two showed spine densities similar to controls (see Fig. 4). The severity of cognitive impairment in our ASD group ranged from very mild to severe. Based upon available patient records, four of the ten cases were described as either not mentally retarded or only mildly mentally retarded ( $IQ>50$ ). Another four cases were described as having moderate to severe mental retardation ( $IQ<50$ ). In the remaining two cases, insufficient information was available to assess the overall level of cognitive functioning. Each of the four lowest-functioning cases showed increased spine densities relative to their age-matched control



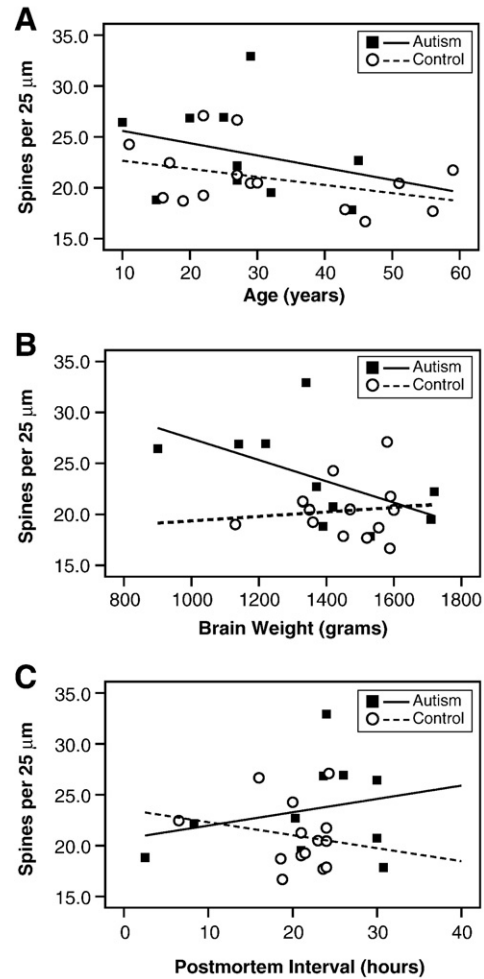
**Fig. 4** – Direct comparison of grand average spine density values, along with standard error of the mean, for each value in age-matched pairs of subjects. Density values for each subject were averaged across the three cortical regions, the three cortical layers and the three dendrite types. In seven of the ten pairs, values were higher in ASD subjects as compared to their age-matched control, but the difference in the oldest pair (10) was quite small. In the remaining three pairs (2, 6 and 8), which were distributed across the range of ages, ASD subjects had smaller spine density values compared to their age-matched controls. Asterisks (\*) indicate the four ASD cases that were known to have a history of seizures, while obelisks (†) indicate the four cases known to have either severe or moderate mental retardation.

(Fig. 4). Of the cases identified as either not retarded or mildly retarded, only one (Case ID 003) had greater spine densities. In the remaining two cases, where the level of cognitive functioning was unknown, one showed higher spine densities and the other did not.

Spine densities decreased with age (all subjects;  $r = -.357$ ,  $p = .08$ ) in both the ASD ( $r = -.287$ ,  $p = .422$ ) and control groups ( $r = -.395$ ,  $p = .145$ ), but this reduction was not significant (see Fig. 5A). Interestingly, higher spine density tended to be associated with the smallest brains in our ASD group ( $r = -.573$ ,  $p = .083$ ) but showed little relationship to brain size in control subjects ( $r = .107$ ,  $p = .727$ ; see Fig. 5B). Spine densities decreased slightly with increasing PMI time in our control group ( $r = -.191$ ,  $p = .495$ ) but increased slightly in our ASD group ( $r = .261$ ,  $p = .466$ ). These relationships were also nonsignificant, which may be due to the tightly controlled range of PMIs in the present group of subjects (see Fig. 5C).

### 3. Discussion

Several specific findings are evident regarding spine density alterations in ASD subjects. First, average spine densities in ASD are higher than those found in control cases and individual ASD cases never showed fewer spines than control cases, as is commonly found in other developmental disorders associated with mental retardation. Second, elevated spine densities were most apparent in superficial layer II of all three cortical regions. This layer-specific effect is of interest due to the protracted maturation of the superficial cortical layers that occurs during the postnatal period and the cortico-cortical



**Fig. 5** – Spine densities as a function of age, brain weight, and postmortem interval. (A) Spine density decreased with age in both the ASD and control groups, and the highest spine values were found in ASD subjects under the age of 30. (B) Spine densities were negatively correlated with brain weight and megalencephalic cases showed spine densities that were closer to the average value found in controls. (C) Postmortem interval was not associated with changes in spine density values, likely because of the restricted range of postmortem times over which the samples were collected.

connectivity that is a characteristic of layer II pyramidal neurons. Third, the temporal lobes showed a region-specific elevation in spine density within layer V indicating that this location may have additional connective changes not found in the frontal and parietal locations that were examined. Fourth, alterations in spine densities within the ASD group did not appear to be associated with a history of epileptic seizure activity, although a weak relationship may exist between high spine densities, the smallest brain sizes, and lower levels of cognitive functioning.

#### 3.1. Layer II spine densities

Dendritic spines first appear on cells within the deep cortical layers of humans in the late prenatal period (Huttenlocher and

Dabholkar, 1997; Michel and Garey, 1984), while neurons in the superficial layers do not express spines until several months postnatal (Koenderink and Uylings, 1995; Koenderink et al., 1994). In the present study, the most superficial layer (layer II) showed greater spine densities in ASD subjects for all three cortical locations examined. The consistency of greater spine density in the superficial layers is perhaps most interesting given that these late maturing layers establish synaptic connections during the postnatal period. In addition, the pyramidal cells in layer II are predominately involved in interconnectivity between cortical regions within a hemisphere. Recent interest in an increase in connectivity in ASD subjects (Courchesne and Pierce, 2005) is partially supported by increased volumes in white matter compartments just below the cortical sheet (Herbert et al., 2003, 2004). In addition, functional imaging studies have shown a disconnection between distant cortical regions with increased activity found in regions associated with early cortical processing (Just et al., 2004; Koshino et al., 2005). These types of results have been used to propose an inability to integrate information processed in distant cortical locations (Frith, 2004; Just et al., 2004, 2007; Geschwind and Levitt, 2007).

In typically developing humans, spine densities reach a maximum between 12 and 36 months postnatal depending upon the cortical location (Huttenlocher and Dabholkar, 1997; Michel and Garey, 1984). This maximum is followed by a gradual decline in spine numbers, which is believed to be associated with the “culling” of unused connections and the establishment of mature cortical networks. Since spine culling occurs postnatally, this process is thought to be experience dependent and guided by environmental interactions (Dawson et al., 2000; Huttenlocher and Dabholkar, 1997; Jacobs et al., 2001; McAllister, 2007). It has been proposed elsewhere that individuals with ASD may have impaired synapse elimination attributable to their inability to fully utilize this environmental input (Courchesne, 2004; Frith, 2004; Mundy and Neal, 2001). This hypothesis is similar to one proposed for fragile X syndrome, another pervasive developmental disorder that shares several behavioral characteristics with autism (Demark et al., 2003; Rogers et al., 2001). In fragile X, over-expression of synaptic spines has also been demonstrated using Golgi methods in postmortem human tissue (Irwin et al., 2000, 2001; Sabaratnam, 2000).

### 3.2. Temporal lobe spine densities

Alterations in spine densities within the deep layers of the temporal lobe suggest that this region may have additional impairments in ASD subjects, while the other two cortical locations showed only minor, nonsignificant increases in layer V spine densities. Although it is now clear that no single neural structure is associated with ASD (Müller, 2007), both limbic areas and mesial temporal lobe structures have been identified as having significant neuroanatomical abnormalities in ASD groups (Bauman and Kemper, 1985, 2005). These regions are heavily interconnected with adjacent temporal regions (Amaral and Price, 1984), and it has been suggested that abnormalities in temporal-limbic circuits might be associated with deficits in facial and emotional recognition, as well as associated social-emotional functioning (Baron-

Cohen et al., 1999; Critchley et al., 2000; Lee et al., 2007; Schultz et al., 2000). Functional neuroimaging studies have also targeted the temporal lobes as a specific site of bilateral hypoperfusion at rest in ASD (Meresse et al., 2005). These regions also show abnormal activation in auditory studies, as well as the processing of simple (Gage et al., 2003) and complex speech-related sounds (Gervais et al., 2004; Boddaert et al., 2004; Just et al., 2004). Finally, volumetric and morphometric alterations to temporal lobe structures have also been reported (Bailey et al., 1998; Bigler et al., 2003; Boddaert et al., 2004). In aggregate, these findings point to substantial deficits in temporal lobe circuits that underlie some of the behavioral symptoms that characterize ASD patients.

Cortical maturation proceeds in a gradient across cortical locations with the temporal and frontal lobes lagging behind parietal locations (Shaw et al., 2008). Regionally specific density changes within layer V could be the product of developmental timing, since spine generation and elimination occur according to different timetables depending upon the cortical region examined. Spine densities in the temporal lobes reach a peak much later (approximately 3 to 4 years postnatal) than they do in visual cortex (approximately 18 months postnatal), but at about the same time as the frontal lobes. Spine elimination within the temporal lobes, although more protracted than in the visual cortex, occurs earlier than in the frontal lobes, where it can extend into adolescence (Huttenlocher and Dabholkar, 1997). If higher spine densities are due to a deficit in spine reduction, then the period between four and ten, when spines are eliminated from the temporal lobes in typically developing humans, may be of particular importance in ASD subjects. Such a model suggests that behavioral impairments could interfere with the environmentally dependent process of spine elimination during specific periods of cortical development in ASD subjects (Glasson et al., 2004; Ijichi and Ijichi, 2004).

### 3.3. Subject characteristics

Although this is the first quantitative examination of spine densities in ASD, one previous qualitative study described decreases in pyramidal cell spine density along the midportion of the apical dendritic shafts in two cases, one of which had uncontrolled seizures (Williams et al., 1980). In other studies examining the cortex of ASD individuals for neuropathological changes (Bailey et al., 1998; Koenderink and Uylings, 1995), spine densities have not been directly examined. In mice exposed to valproic acid, a teratogen that increases the risk of developmental delays and autism-like behaviors, increased spine numbers associated with local connectivity and weaker connectivity have also been found (Rinaldi et al., 2008). The lack of a previous report of higher spine densities in ASD is not surprising, given the small number of subjects studied utilizing Golgi methods in the cortex, the difficulties associated with nonquantitative evaluations of spine density, and the layer and region-specific nature of elevated spine densities within our ASD group.

Several relevant factors in autism could influence spine counts within the cerebral cortex. Most notable among these are the presence of seizure activity, whether an individual is taking anticonvulsant medications, overall level of cognitive

functioning, and total brain size. Spine density values can potentially be reduced by the presence of seizure activity (Multani et al., 1994; Swann et al., 2000) as well as by neuroleptics used in the treatment of epilepsy (Benes et al., 1985; Garey et al., 1998). Although it is unclear whether a relationship exists between spine density, seizure frequency, seizure type, or the medications utilized to maintain seizure control, in the present group of subjects half of cases with epilepsy did not show higher spine densities. Whether medications or seizure activity masked an effect in these cases is unknown.

In contrast, greater spine densities were loosely associated with smaller brain sizes, while in our control subjects there was no relationship between average spine density and brain size. Average brain sizes in ASD populations are consistently enlarged in younger individuals between the ages of 1 and 7 years (Courchesne et al., 2003). The adult ASD population is characterized by high variance in brain size, which includes a 20% rate of megencephaly (Bailey et al., 1993; Filipek et al., 1992; Piven et al., 1995, 1992) and average values that do not differ significantly from control groups (Courchesne et al., 1999). As ASD subjects age there are significant reductions in total brain volume (Aylward et al., 2002; Lewis and Elman, 2008) and accelerated morphometric changes (Hardan et al., 2004). Although growth dysregulation (Akshoomoff et al., 2002) may continue to influence cortical organization throughout the lifespan (Bauman and Kemper, 2005), there was little evidence in the present study of an increased rate of spine loss in older ASD subjects.

Higher spine densities were found consistently in each of the four cases that were known to have moderate to severe mental retardation, but in the present work small brain size, a low level of cognitive functioning, and higher spine density tended to be associated with each other. In general, subjects with small head circumference are characterized by a higher incidence of low IQ values (Miles et al., 2000) as well as discrepancies between verbal and nonverbal IQ subscales (Deutsch and Joseph, 2003). Thus, although intriguing, the strength of the relationships between general measures of mental aptitude, brain size and spine densities require further study.

Using the same methodology employed here, many developmental disorders, such as fetal alcohol syndrome (Ferrer and Galofre, 1987), severe infant protein-calorie malnutrition (Benitez-Bribiesca et al., 1999), infant brain damage (Dietzmann and von Bossanyi, 1994), and Down syndrome (Suetsugu and Mehraein, 1980), show reductions in the number of synaptic spines. These spine reductions are presumed to be associated with connective loss and abnormal cortical circuits (Fiala et al., 2002; Halpain et al., 2005). Only a few conditions associated with mental retardation have shown an increase in spine densities, including fragile X syndrome (Irwin et al., 2001) and hemi-megalencephaly (Takashima et al., 1991). Like ASD, fragile X is more common in males than females (Demark et al., 2003), and it shares several cognitive and behavioral traits (Kaufmann et al., 2004). Interestingly, overall brain size is also larger in individuals with fragile X (Sabaratnam, 2000). Estimates of the co-occurrence of fragile X in ASD subjects range from 1.6% to 16% (Demark et al., 2003), but none of the ASD subjects in the present study were diagnosed with fragile X. Conversely, as many as a third of individuals

diagnosed with fragile X carry a diagnosis of ASD, although there is significant variability in the prevalence rates across studies (Bailey et al., 1998; Cohen et al., 1991; Rogers et al., 2001). Unlike ASD, fragile X is diagnosed by genetic rather than behavioral markers, and several authors have argued that the conditions are only similar at a superficial level with nonhomologous behavioral phenotypes (Demark et al., 2003; Rogers et al., 2001). Although we did find greater spine densities in ASD subjects, we did not observe an increased preponderance of long-skinny dendritic spines as has been reported in fragile X (Irwin et al., 2000). Even if fragile X and ASD do not share similar causative antecedents, it is still intriguing that two developmental disorders sharing greater spine densities also share similar behavioral phenotypes and can coexist in the same individual (Demark et al., 2003).

### 3.4. Neuroanatomical context

Golgi methods are currently the best approach for revealing individual cell morphologies and the density of synaptic spines in postmortem human tissue samples. These methods have long been criticized for their capricious nature, their apparently pseudorandom staining of individual neurons (Glaser and Van Der Loos, 1981; Spacek, 1989), and their inability to guarantee complete staining of the dendritic arbor (Spacek, 1989, 1992). In the present work, we utilized a modified Golgi-Kopsch technique that is ideally suited to well-fixed samples (Riley, 1979). To minimize the impact of incomplete staining and the preferential staining of certain cell types, examples of one specific cell class—the cortical pyramidal cell—were selected based on the quality of staining. Since this is not a random sample of pyramidal cells, the present work assumes that the method does not systematically stain different subgroups of pyramidal cells in ASD and control groups.

Neuronal morphology is critical to a full interpretation of spine density changes since increases could reflect crowding due to reduced dendritic trees. For example, hippocampal neurons in ASD individuals are reduced in size, have smaller dendritic trees, and have less complicated branching patterns (Raymond et al., 1996). In the cortex of ASD individuals, neuronal morphology has not been quantitatively examined, but in our cases we did not observe obvious between-group differences in the lengths of the dendritic arbors as was evident in the lengths of three dendrite types upon which spines were assessed. Case reports also indicate that cortical dendritic arbors are relatively normal when compared to control subjects (Bailey et al., 1998; Williams et al., 1980). Individual cortical neuron morphology remains to be quantitatively evaluated in ASD, but the present results demonstrate a clear difference in the morphology of pyramidal cells in a majority of ASD subjects regardless of whether this difference is specific to dendritic spines or is ultimately the result of altered dendritic trees.

### 3.5. Conclusions

Synaptic spine densities on cortical pyramidal cells have direct implications for several recently proposed hypotheses of connective changes in the cerebral cortex of ASD individuals (Courchesne, 2004; Frith, 2004; Rippon et al.,



2007). In aggregate, these hypotheses have proposed a deficiency in the quality of long-range cortico-cortical connections in ASD (Hughes, 2007) and an increase in short-range connections, as well as connections between subcortical areas and the cortex (Mizuno et al., 2006). As has been previously suggested, alterations in spine densities could be the result of improper synaptic culling during the postnatal period that results from an impairment in the experience-dependent strengthening and weakening of neuronal interconnections. The present results are the first to indicate a structural basis for such connective changes in ASD. Furthermore, these changes suggest an alteration to the density of excitatory synapses on dendritic segments within the cortex. These results do not, however address the presynaptic origin of these connections, which could be local, intermediate or long range. Nor do they address whether inhibitory synapse distribution on the cell surface is modified in these cases. ASD is, therefore, among a small group of developmental disabilities involving mental retardation where there is no apparent loss of dendritic spines. Instead, a substantial subgroup of ASD individuals shows increased spine densities which may indicate important alterations to cortical circuitry.

#### 4. Experimental procedures

All subject material utilized in these studies was acquired with the assistance of the Autism Tissue Program (ATP; Princeton, NJ), and the procedures for tissue collection, case identification, and processing were reviewed by the Medical Internal Review Board at the University of Michigan and the Biomedical Internal Review Board at the University of Nevada, Reno. Cortical tissue samples fixed in formalin were acquired from ten postmortem ASD males and fifteen male control subjects from the Harvard Brain Tissue Resource Center and the Brain and Tissue Bank for Developmental Disorders at the University of Miami. Identification of the ASD cases was made based upon available medical and psychological records, and the diagnosis was subsequently confirmed with a Revised Autism Diagnostic Inventory (ADI-R; Lord et al., 1994) administered to the parents or primary caregiver following tissue donation. In the present group, all cases met the criteria for a diagnosis of autism except for case 009 (see Table 1), who did not meet the cutoff in the area of communication and was classified as pervasive developmental disorder, not otherwise specified (PDD-NOS). For comparison to the ASD cases, ten of the fifteen control cases available were selected blindly based upon age, postmortem interval (PMI) and tissue block availability to obtain a final set of ten age- and sex-matched pairs (cases 1–20; Table 1). Control cases had no history of neurological disorders. Brain weights for both groups were collected at autopsy prior to fixation.

For each subject, tissue blocks were obtained from three cortical regions: the superior parietal lobule (BA 7; superior parietal lobule, posterior, but not adjacent to the primary sensory cortex and lateral from the longitudinal fissure by 5 cm), the lateral prefrontal cortex (BA 9; anterior portion of the first frontal convolution, anterior to the premotor cortex and lateral from the longitudinal fissure by 5 cm), and the middle temporal gyrus (BA 21; posterior middle temporal gyrus,

ventral to the termination of the Sylvian fissure). Tissue block locations were provided to the brain banks using these location descriptors as well as a photomicrograph indicating the location of the block. Block location was later confirmed using the detailed cytoarchitectonic descriptions provided by von Economo and Koskinas (1925). In several cases, one or more of these regions was unavailable for analysis, and thus, a total of 47 blocks were used in the final study. Tissue blocks were recut to a thickness of approximately 3–4 mm. All blocks were cut vertically to the pial surface and perpendicular to the long axis of the gyrus. Prior to processing the blocks in a modified Golgi-Kopsch technique (Riley, 1979), the tissue was postfixed in a 10% formaldehyde solution for 2 weeks. Each tissue block was then immersed for 9 days in 50 ml of a potassium dichromate solution (100 ml distilled water, 3.5 g potassium dichromate, 15 g sucrose, and 1.65 ml of 37% formaldehyde) and stored in the dark at room temperature. Blocks were washed in 0.75% silver nitrate in dH<sub>2</sub>O and immersed in a fresh 0.75% silver nitrate solution in the dark at room temperature for an additional 3 days. The resulting blocks were cut serially into 120  $\mu$ m thick sections in a 50% alcohol solution using a Vibratome. Sections were mounted on gelatin-coated slides, dehydrated in a graded series of alcohol solutions, cleared in xylene, and coverslipped with Permount (Sigma). All slides were assigned a numerical code to conceal both the case number and cortical area.

##### 4.1. Spine analysis

Spine densities were collected both blind to diagnosis and cortical region. For each tissue block, ten well-stained Golgi-impregnated pyramidal neurons were selected from cortical layers II, III, and V, resulting in 30 neurons being analyzed from each cortical region. Cortical layers were identified based upon pyramidal cell size, morphology, and position (Jacobs et al., 1997, 2001) and confirmed with Nissl sections taken from the same region. Well-stained neurons exhibited good dendritic filling and well-stained spines along the surface of the dendritic shafts. When all three cortical locations were available for analysis from a single case (see Table 1), a total of 90 neurons were examined from each subject. For each neuron, spines were counted along 25  $\mu$ m lengths of an apical, basilar, and an oblique dendrite (Feldman and Peters, 1979). In the case of apical and basilar dendrites, counts started at the soma and continued to the end of the dendrite within the 120  $\mu$ m section. For oblique dendrites, counts started at the origin of the process with the apical dendritic shaft and continued to the end of the dendrite. The distance of the origin of these oblique dendrites from the cell body was also recorded. In addition to the spine counts, the diameter of the dendritic segment was recorded, as well as the cortical thickness at the location of the parent neuron and the distance of the parent neuron from the pial surface. All measurements and counts were collected using a Leica microscope equipped with a motorized stage at a final magnification of 1000 $\times$ .

##### 4.2. Data analysis

Spine counts were analyzed according to diagnosis, dendrite type (apical, basilar, or oblique), cortical layer (II, III, or V), and

cortical region (BA 7, BA 9, or BA 21) using a repeated-measures analysis of variance that averaged spine densities over the length of the dendrite. Because spine density varies as a function of distance to the soma we used the same statistical approach to compare the lengths of the sampled dendrites from the two subject groups. Spine counts were also analyzed according to hemisphere of origin in conjunction with the repeated measures of dendrite type, cortical layer and cortical region. Because apical dendrites were the easiest to follow over long distances, they were used exclusively in analyses that considered distance from the cell body and the density distribution along the dendrite. In addition, since pyramidal cell depth constrains apical dendrite length, when distance from the soma was a factor the three layers were evaluated separately. A mixed model was constructed with diagnosis, cortical region, and distance from the soma as factors predicting spine density (SPSS, Inc.). Cortical region and distance to the soma were treated as repeated factors with an unstructured covariance matrix. The intercepts and slopes of distance to the soma were treated as random factors with a first order auto regressive covariance matrix.

The results of these analyses were further evaluated within the context of the available individual case information, which included seizure history, secondary medical conditions, medications, educational history, level of cognitive functioning, and the severity of specific domains of diagnosis. Standardized intelligence scores were only available in a few cases, however level of cognitive functioning could typically be assessed from medical evaluations. Each case was classified as co-occurring with either severe to moderate mental retardation (IQ<50) or mild to no mental retardation (IQ>50). The relationships between age, brain weight, PMI, and spine density were explored with bivariate correlations.

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