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Decreased synaptic and mitochondrial density in the postmortem anterior cingulate cortex in schizophrenia

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Abstract

Schizophrenia (SZ) is a mental illness characterized by psychosis, negative symptoms, and cognitive deficits. The anterior cingulate cortex (ACC), a structurally and functionally diverse region, is one of several brain regions that is abnormal in SZ. The present study compared synaptic organization and mitochondrial number and morphology in postmortem ACC in SZ versus normal control (NC). Total synaptic density in the combined ACC was decreased in SZ, to 72% of normal controls (NCs), due to selective decreases in axospinous synapses, both asymmetric (excitatory) and symmetric (inhibitory). These changes were present in layers 3 and 5/6. The density of mitochondria in all axon terminals combined in SZ was decreased to 64% of NC. In layer 3, mitochondrial density was decreased only in terminals forming asymmetric synapses with spines, while in layers 5/6 mitochondrial density was decreased in terminals forming symmetric synapses with spines and dendrites. The proportion of terminals making symmetric synapses that contained mitochondria was significantly lower in SZ than in NCs, especially for symmetric axospinous synapses. The number of mitochondria per neuronal somata was decreased in the ACC in SZ compared to NCs; this finding was present in layers 5-6. The size of mitochondria in neuronal somata and throughout the neuropil was similar in SZ and NCs. Our results, though preliminary, are well supported by the literature, and support an anatomical substrate for some of the altered executive functions found in SZ.

Keywords

psychosis; electron microscopy; glutamate; dopamine; neuropathology

1. Introduction

The anterior cingulate cortex (ACC) is part of prefrontal cortex and its neuroanatomy has been well described (DeFelipe et al., 2002; Jones, 1998; Lewis et al., 2002; Peters, 2002). The ACC is composed of distinct anatomical subregions, each with different functional

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properties (Peterson et al., 1999; Vogt et al., 1992). At the microscopic level, each layer of the ACC has characteristic types of neurons, which project to particular targets; each layer is the recipient of inputs from specific regions (DeFelipe et al., 2002; Lewis et al., 2002). Human imaging studies indicate that the dorsal ACC is involved in mediating attention and executive functions, such as task difficulty, remote memory (Koski and Petrides, 2001), conflict (Barch et al., 2001; Kerns et al., 2004), response inhibition and error commission (Braver et al., 2001; Mathalon et al., 2002). The subcallosal ACC is involved in emotional processing (Bush et al., 2000) or internal states (Greicius et al., 2003), the dorsal ACC is involved in cognitive function, and the rostral ACC, located between these two subdivisions, plays an important role in the integration of these functions (Vogt et al., 1992).

The ACC is one of several brain regions that are abnormal in schizophrenia (SZ), as shown in both *in vivo* imaging and postmortem studies (Fornito et al., 2003). *In vivo* imaging of people with SZ has shown abnormalities in multiple transmitter systems (Egerton et al., 2012; Kraguljac et al., 2012a,b; Rowland et al., 2012; Theberge et al., 2003), blood flow (Holcomb et al 2000) and metabolism (Nordal et al., 1996; Tamminga et al., 1992). Functional impairments in cognitive interference (Heckers et al., 2004), error or conflict monitoring (Alain et al., 2002; Carter et al., 1997, 2001) and response monitoring (Kopp and Rist, 1999; Mathalon et al., 2002) have also been demonstrated.

Postmortem studies have shown multiple defects in the ACC in SZ (Eastwood and Harrison, 2001; Fornito et al., 2009). Neurochemical and molecular changes include altered distribution or modulation of dopamine (Benes et al., 1997), abnormalities in multiple aspects of the glutamate system (Barksdale et al., 2014; Bauer et al., 2008, 2010; Drummond et al., 2013; Katsel et al., 2011; Oni-Orisan et al., 2008; Woo et al., 2004, 2007) and intracellular signaling abnormalities (Funk et al., 2012, 2014). Mitochondrial pathology has been implicated repeatedly in schizophrenia (Anglin et al., 2012; Manji et al., 2012) and in the ACC, oxidative stress, a result of mitochondrial production of reactive oxygen species, is increased (Wang et al., 2009). Anatomical abnormalities include layer specific alterations in neuronal distribution and density (Benes and Bird, 1987; Brune et al., 2010; Todtenkopt et al., 2005), and increased numbers of glutamatergic (Benes et al., 1987, 1992) and parvalbumin axons (Kalus et al., 1997, 1999). Of note, previous electron microscopic studies in SZ have shown layer specific abnormalities such as fewer synapses in the ACC (Aganova and Uranova, 1990) and alterations in synapses, mitochondria and oligodendrocytes in other areas of prefrontal cortex (Uranova et al., 2004, 2007, 2011).

The purpose of the present study is to compare the synaptic organization and mitochondrial number and morphology in SZ versus normal control postmortem ACC. Several synaptic features were quantified, including morphological features which identify excitatory and inhibitory synapses. This work has been presented in preliminary form (Barksdale et al., 2012a,b; Roberts et al., 2013).

2. Methods

Human brain tissue was obtained with IRB approved protocols from the Maryland Brain Collection and the Alabama Brain Collection. Diagnostic criteria have been described

previously (McCollum et al., 2015; Roberts et al., 2008). Demographics are presented in Table I.

Coronal blocks from the dorsal ACC were preserved in fixative and processed for electron microscopy as previously described (McCollum et al., 2015). One series of sections was stained for Kluver-Barrera stain as previously described (Bolding et al., 2013; Roberts et al., 2014) and used to identify the layers of the cortex (Figure 1).

A series of tissue adjacent to the ones used for Kluver-Barrera was processed for electron microscopic analysis using standard techniques (McCollum et al., 2015; Roberts et al., 2008). Two to three samples per case, at least 240 μm apart, were used for quantitative analysis. Each sample was cut into an average of 10-11 serial ultrathin sections (90 nm thickness), mounted on Formvar-coated copper grids, and photographed at 80 kV on a Hitachi transmission electron microscope. To determine the number of synapses in the neuropil and mitochondria in terminals, these serial sections were analyzed using the physical disector technique (Geinisman et al., 1996; Perez-Costas et al., 2007) from layers 3 and 5/6 (Figure 2). In each section, eight pictures were photographed at a magnification of 15,000 and stitched together to form a montage. The images are enlarged by 50% for synapse and mitochondria counting. These methods for identifying and quantifying profiles have been described in detail by us (Roberts et al., 2008; Somerville et al., 2001, 2012). Briefly, neuropil only was quantified; cell bodies were not photographed. For controls (combined layer 3 and layers 5/6), a total of 2,313 synapses were counted in a total volume of 11,559 μm^3 ; the average per case was 386 synapses in a volume of 1,926 μm^3 . For SZ cases (combined layer 3 and layers 5/6), a total of 1,309 synapses were counted in a total volume of 9,248 μm^3 ; the average per case was 327 synapses in a volume of 2,320 μm^3 . A total of 712 and 356 mitochondria were identified in axon terminals in NC and SZ, respectively.

In addition, mitochondria throughout the neuropil in 1-2 randomly selected montages per case per layer were counted and their diameters were measured. For this analysis, an average of 1470 μm^2 per case was analyzed and an average of 289 mitochondria per case were counted and measured. Also, neuronal somata near the center of the neuron were photographed at 5,000-8000 magnification and the number of mitochondria present in a single section of each soma was counted. For this analysis a total number of 174 neurons containing a total of 2,881 mitochondria were counted and their diameters measured.

The individuals counting the synapses and mitochondria and measuring mitochondria were blinded to the identity of the groups. The results were compared with unpaired t-tests.

3. Results

3.1. Demographics

Demographics for the groups were similar for age, PMI, sex and race (Table I).

3.2. Synapses

There was no apparent difference in the integrity of the tissue between the NC (Figure 3) and SZ groups (Figure 4). As expected, the majority of synapses in both groups were asymmetric axospinous synapses (Figures 3A,C,D, 4A,D). Asymmetric axodendritic synapses (Figures 3B, 4A), symmetric axospinous synapses (Figures 3C, 4B), symmetric axodendritic synapses (Figures 3E, 4C) were also present in both groups.

In the ACC (layers 3, 5 and 6 combined) all synapses combined were decreased in density in the SZ cases compared to the NCs (Figure 5A). Both asymmetric and symmetric axospinous synapses were reduced in density in the SZs vs. NCs. Axodendritic synapses, both asymmetric and symmetric types, were similar in density between SZs and NCs. These synaptic density changes were present in layer 3 and layers 5/6 (Figure 5B).

3.3. Mitochondria in the neuropil

The density (Figure 6A) and diameter (Figure 6B) of mitochondria in the neuropil was similar between NCs and the SZ cases in the ACC (layers 3, 5/6 combined) as well as the separate analysis of layer 3 and layers 5/6.

3.4. Mitochondria in axon terminals

Mitochondria specifically located in axon terminals comprise a small proportion of mitochondria found throughout the neuropil. Therefore, we counted the number of mitochondria found just in axon terminals and their various subtypes. In the ACC (layers 3, 5 and 6 combined), the number of mitochondria in axon terminals forming any type of synapse and those forming asymmetric axospinous synapses was lower in SZ as compared to NCs (Figure 7A). There were also fewer mitochondria in terminals forming symmetric synapses, due to a selective loss of mitochondria in symmetric axospinous synapses. The density of mitochondria in terminals forming synapses with dendrites was similar in SZs and NCs. Layer 3 showed a similar pattern to the combined ACC values for asymmetric synapses, but the number of mitochondria in terminals forming symmetric synapses was similar between SZs and NCs (Figure 7B). Layers 5/6 (Figure 7B) showed a pattern of mitochondrial loss from asymmetric synapses, but it did not reach statistical significance. However, the density of mitochondria in terminals forming symmetric synapses, both with spines and dendrites, was lower in SZs than NCs. The density of mitochondria in terminals forming asymmetric axodendritic synapses was similar in SZ and NCs.

A decrease in the density of mitochondria in terminals may be a reflection of fewer terminals rather than fewer mitochondria in existing terminals, so we also examined the proportion of terminals containing at least one mitochondrion (Table 2). The proportion of terminals making symmetric synapses that contained mitochondria was significantly lower in SZ than in NCs. This pattern was significant for symmetric axospinous synapses, with a trend level pattern in symmetric axodendritic synapses. The proportion of terminals in layer 3 (all synapses combined and the various subset of synapses) containing mitochondria was similar between SZs and NCs (Table 2). The proportion of terminals on layers 5/6 containing mitochondria that formed symmetric synapses and symmetric axodendritic

synapses was lower in SZs than NCs; the same pattern was observed in symmetric axospinous synapses, albeit non-significant (Table 2).

3.5. Mitochondria in neuronal somata

In the combined ACC, the number of mitochondria per neuronal somata in the SZ cases was decreased by 43% of that of NCs (Figures 8, 9A,B). This was due to a selective loss in layers 5/6. The size of mitochondria in neuronal somata throughout the ACC was similar in SZ cases compared to NCs (Figures 8, 9C).

4. Discussion

Our main results show reduced overall synaptic density in the ACC in SZ compared to NCs. The synaptic loss was confined to axospinous synapses and was present in layers 3 and 5/6. Both asymmetric and symmetric axospinous synapses were affected, indicating abnormalities in both excitatory and inhibitory transmission, respectively. Overall decreases in synaptic density in the SZ group are consistent with the reduced neuropil hypothesis, which posits fewer cortical synaptic connections (Selemon and Goldman-Rakic, 1999). Fewer axospinous synapses in the ACC in SZ are consistent with previously reported spine loss (Glantz and Lewis, 1999). Mitochondria were reduced in number in specific populations of axon terminals and in the somata of neurons in layers 5/6, suggesting abnormal energy levels. Our results complement and extend previous an electron microscopic study of the ACC in SZ, showing synaptic pathology in layers 1-2 (Aganova and Uranova, 1992).

4.1. Limitations of postmortem studies in schizophrenia

Our small sample size, though a limitation, is not unusual for postmortem EM. Postmortem tissue suitable for electron microscopic analysis is very difficult to procure, and though our cohort is small, it is one of only two studies (Aganova and Uranova, 2004) that have been reported in the ACC. Changes reported herein are consistent with abnormalities shown in previous studies. That said if another larger cohort of subjects become available, replication of this pilot study would be prudent. The fact that all of our subjects were treated with medication could complicate the interpretation of our results. However, antipsychotic drugs administered to animals do not affect cortical levels of synaptophysin (Lidow et al., 2001) a general marker of axon terminals (Calhoun et al., 1996), levels of vGLUT1 (Oni-Orisan et al., 2008) a marker of glutamatergic terminals of cortical origin (Fremeau et al., 2004), or the density of tyrosine hydroxylase labeled processes (Akil et al., 1999) which label dopaminergic terminals (Lewis et al., 1988; Sesack et al., 1995; Smiley et al., 1992). Thus, it appears that antipsychotic drugs do not lower overall synaptic density, glutamatergic or dopaminergic terminals in the ACC and changes reported here are probably not due to medication.

4.2. Decreased density of asymmetric axospinous synapses in SZ

Asymmetric axospinous synapses, characteristic of glutamatergic inputs, were decreased in density in layers 3 and 5/6. In layer 3 of prefrontal cortex of non-human primates, asymmetric axospinous synapses are formed on the spines of pyramidal cells by afferents

from the medial dorsal thalamus (Jones and Hendry, 1989; Jones 1998; Giguere and Goldman-Rakic, 1988), contralateral ACC (Vogt and Pandya, 1987; Vogt et al., 1987) and intracortical connections (Melchitzky et al., 1998). The loss of axospinous synapses may involve any of these projections and is consistent with reduced spine density of layer 3 pyramidal neurons (Glantz and Lewis, 1999), as the number of spines and axospinous synapse change in tandem (Hensch, 2005). Our results are probably not due to fewer pyramidal neurons, as no difference in pyramidal neuron number has been found in the layers we examined (Benes et al., 2001; Bouras et al., 2001). The thalamocortical projection from mediodorsal nucleus could be involved as it has fewer neurons in SZ (Pakkenberg, 1990; Young et al., 2000) and this may translate to fewer projecting axons.

In the deep layers of prefrontal cortex of non-human primates, asymmetric axospinous synapses are formed mainly by cortical association inputs from the contralateral hemisphere, local collaterals of excitatory neurons (Bannister, 2005; Morecraft et al., 2012; Vogt and Pandya, 1987), and contacts from calretinin-labeled cortical interneurons (del Rio and DeFelipe, 1997; Melchitzky et al., 2005). Thalamic afferents other than the medial dorsal nucleus terminate in the deep layers of the cortex in rat (Shibata, 1993) and in monkey (DeFelipe and Jones 1991) and may also innervate the ACC in human. Another source of excitatory inputs onto spines is from the amygdala (Cunningham et al., 2002) another pathological region in SZ (Benes 2010). Pyramidal neurons in layers 5/6 project to the striatum, brainstem or thalamus (Bannister, 2005; Goldman and Nauta, 1977). Taken together, the ACC in SZ has a decrease in excitatory synaptic connections, possibly from multiple sources, which may have effects on several downstream pathways. Decreased excitatory axospinous synapses have also been reported in the hippocampus of subjects with SZ (Kolomeets et al., 2005, 2007).

4.3. Decreased density of symmetric axospinous synapses in SZ

Symmetric axospinous synapses, characteristic of dopamine inputs and inhibitory interneuron connections, were decreased in density in layers 3 and 5/6. Layers 1, 3 and 6 contain the highest densities of dopaminergic axons in primates (Lewis et al., 1988; Williams and Goldman-Rakic, 1993), including humans (Gaspar et al, 1989; Smiley et al., 1992). Dopaminergic afferents form symmetric synapses with pyramidal cell spines and the dendritic shafts of GABAergic interneurons (Sesack et al., 1995). Thus, the symmetric axospinous synapses that are reduced in density in SZ in both layers 3 and 5/6 may reflect a loss of dopamine input to spiny pyramidal neurons, consistent with the hypodopaminergic cortical tone (Howes and Kapur, 2009). In support of this are previous studies showing a decrease in density of dopaminergic fibers in deep cortical layers in the ACC (Benes et al., 1997) as well as other regions of prefrontal cortex (Akil et al., 1999). Other candidate connections that may be altered include the terminals of local interneurons, which form symmetric synapses onto both spines and dendritic shafts (del Rio and DeFelipe, 1995, 1997; Hendry et al., 1984; Kisvárdy et al., 1990). Taken together, the ACC in SZ has a decrease in inhibitory synaptic connections probably from multiple sources.

4.4. Decreased mitochondrial density in SZ

Mitochondria are crucial for cellular functions including energy (Wong-Riley, 1989), calcium buffering (Duchen et al., 2008; Gunter et al., 1994), production of reactive oxygen species (Dugan et al., 1995), regulation of apoptosis (Susin et al., 1999) and modulation of synaptic activity (Duchen et al., 2008; Li et al., 2004; Miller and Sheetz, 2004). The size of mitochondria throughout the neuropil and in neuronal somata was similar in SZ and NCs. This is consistent with our recent finding that levels of Mitofusin 2, a protein responsible for mitochondrial fusion, are normal in the ACC in SZ (Barksdale et al., 2014). However, the density of mitochondria in certain populations of axon terminals and in the somata of neurons in layers 5/6 was decreased in SZ. The proportion of terminals containing mitochondria was lower in inhibitory synapses in layers 5/6. The reduction in number of mitochondria per terminals forming symmetric synapses suggests compromised metabolism in inhibitory synapses. Pyramidal neurons in layers 5/6 project to the striatum or brainstem, or thalamus (Goldman and Nauta, 1977). The reduction of mitochondria per soma in these layers suggests that neurons that project to these subcortical regions may have compromised metabolism. The loss of mitochondria may reflect their death, or abnormalities in mitochondrial trafficking such that mitochondria are transported out of the soma and/or axon terminal at a rate faster than they return (Chang and Reynolds, 2006; Ligon and Steward, 2000). Oxidative stress, an index of mitochondrial dysfunction, is increased in the ACC in SZ (Wang et al., 2009). Thus, remaining mitochondria in the ACC may be functionally compromised.

4.5. Integrating postmortem and imaging studies

Several magnetic resonance spectroscopy studies in SZ have reported decreased N-acetyl-aspartate (NAA) levels in multiple brain regions (Fannon, et al., 2003; Steen et al., 2005), including the ACC (Jessen et al., 2013; Kraguljac et al., 2012; Reid et al., 2010). NAA is an amino acid that is primarily synthesized in neuronal mitochondria (Bates et al., 1996) and is metabolized in oligodendrocytes to acetate, a precursor of myelin lipid synthesis (Chakraborty et al., 2001). Thus the amount of NAA as seen in imaging studies may reflect neuronal integrity, the number of axon terminals and synapses, myelin integrity and/or mitochondrial function (Bates et al., 1996; Chakraborty et al., 2001; Ledeen et al., 2006; Lentz et al., 2005; Moffett et al., 2007; Stork and Renshaw, 2005). Thus, the results of the present study and those of Aganova and Uranova (1992) support that any of these mechanisms could underlie decreased NAA as seen in *in vivo* imaging studies. The decrease in density of axospinous synapses together with a lower proportion of remaining terminals that contain mitochondria and lower somatic mitochondrial counts, suggests a decrease in cortical synaptic efficiency. Overall, our changes suggest alterations in multiple cortical connections that may impact on cognitive functions subserved by the dorsal ACC.

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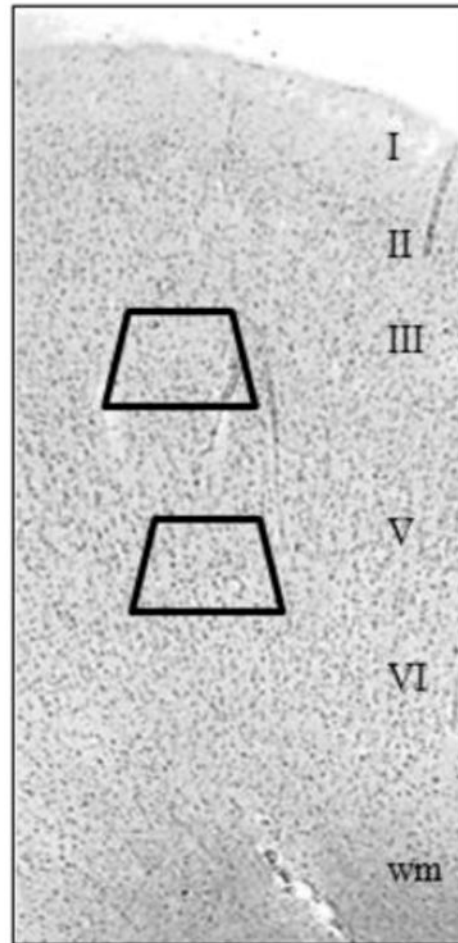


Figure 1. Kluver-Barrera stained section of dorsal ACC. Cortical layers and subcortical white matter (wm) are indicated. Trapezoids show the typical locations of samples taken from layers 3 and 5/6.

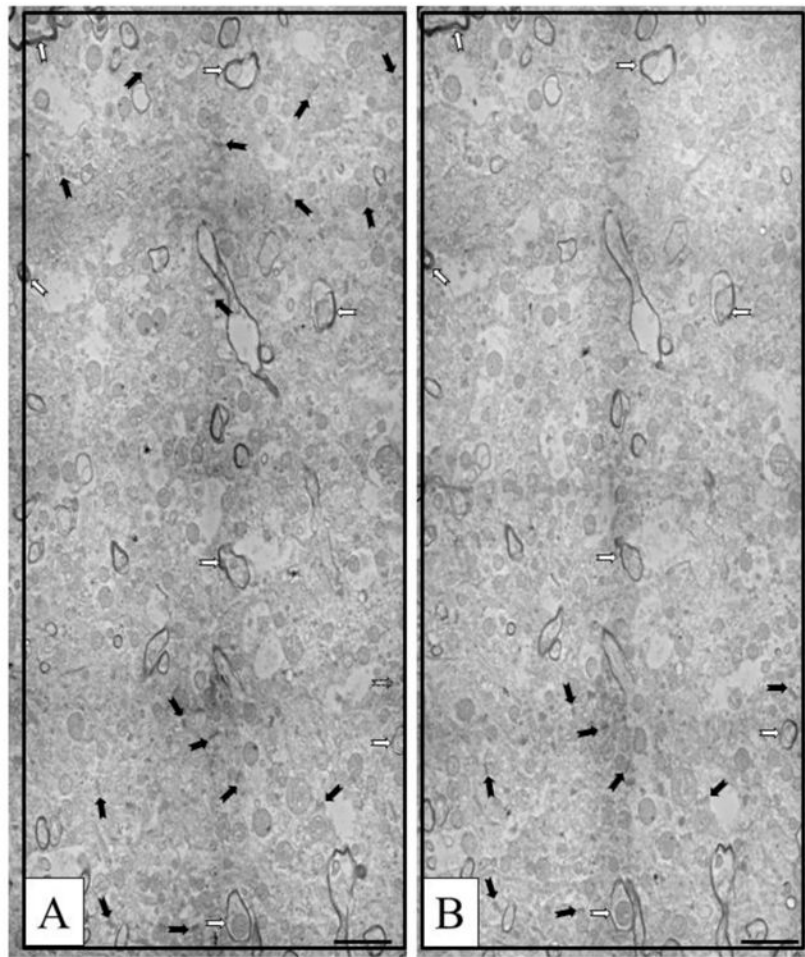


Figure 2. Representative working images used for counting. A and B are taken from serial sections. Black arrows indicate synapses (identified at higher magnification). White arrows with black outlines indicate myelinated axons used as landmarks. Scale bars =2 μ m.

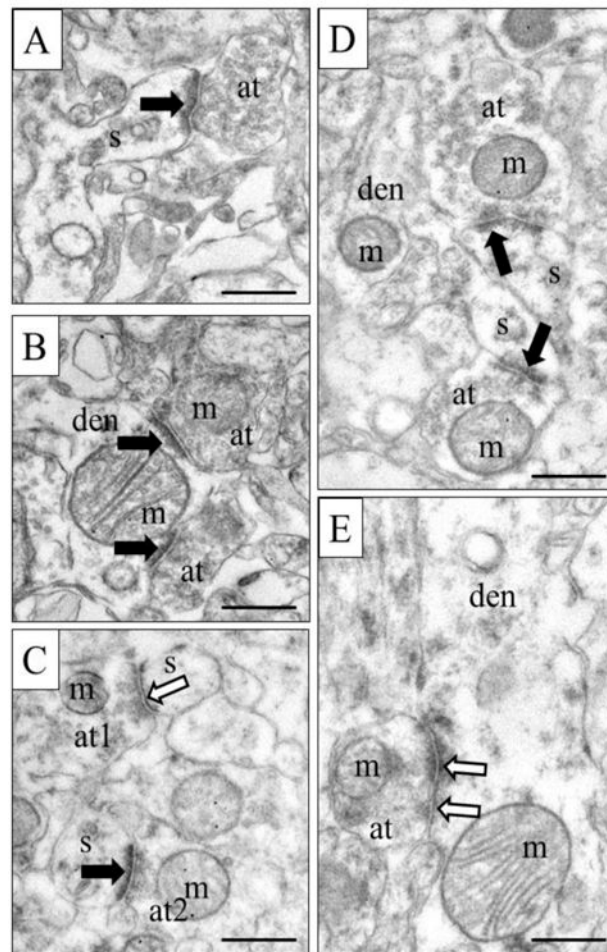


Figure 3. Representative electron micrographs from control tissue. Asymmetric synapses (excitatory) are marked with black arrows, while symmetric synapses (inhibitory) are marked by black-lined white arrows. A) An asymmetric axospinous synapse. B) Two asymmetric axodendritic synapses. C) Two axospinous synapses, one asymmetric and one symmetric. D) Two asymmetric axospinous synapses. E) Symmetric axodendritic synapse. Note that mitochondria are present in all axon terminals except the one in A. S, spine; den, dendrite; m, mitochondrion; at, axon terminal. Scale bars = 500nm.

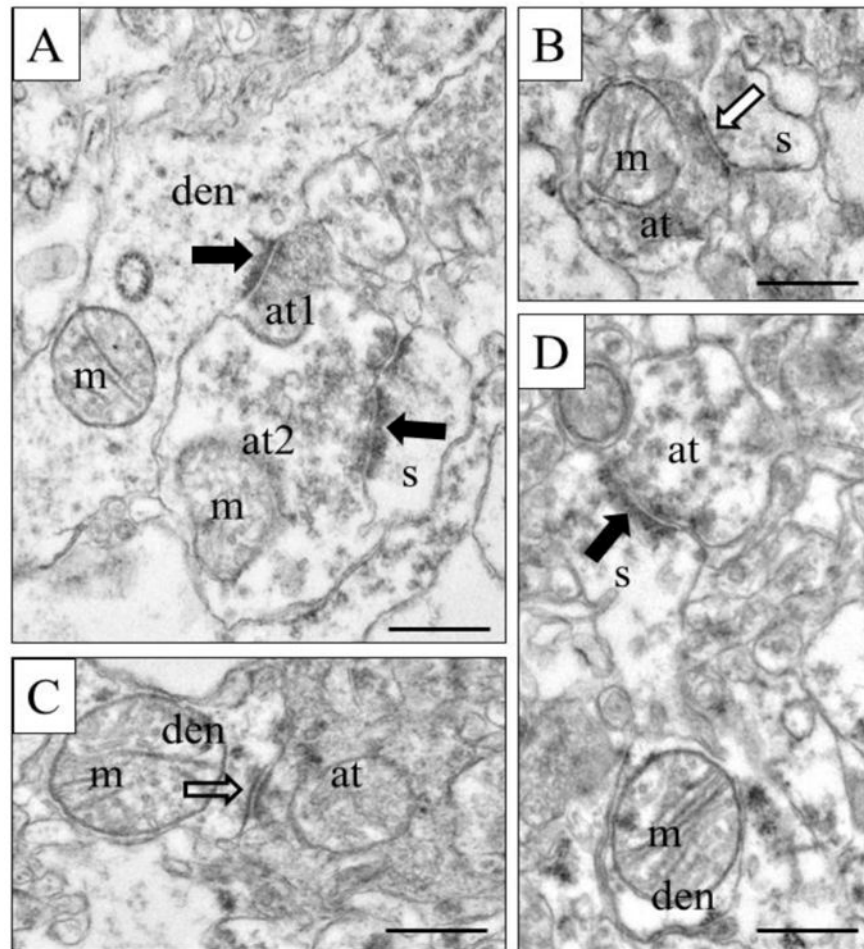


Figure 4. Representative electron micrographs from SZ tissue. Asymmetric synapses are marked with black arrows, while symmetric synapses are marked by black-lined white arrows. A) Two axon terminals form asymmetric synapses, at1 with a dendrite and at2 with a spine. B) A symmetric axospinous synapse. C) A symmetric axodendritic synapse. D) A spine emerges from a dendrite and receives an asymmetric synapse. S, spine; den, dendrite; m, mitochondrion; at, axon terminal. Scale bars = 500nm.

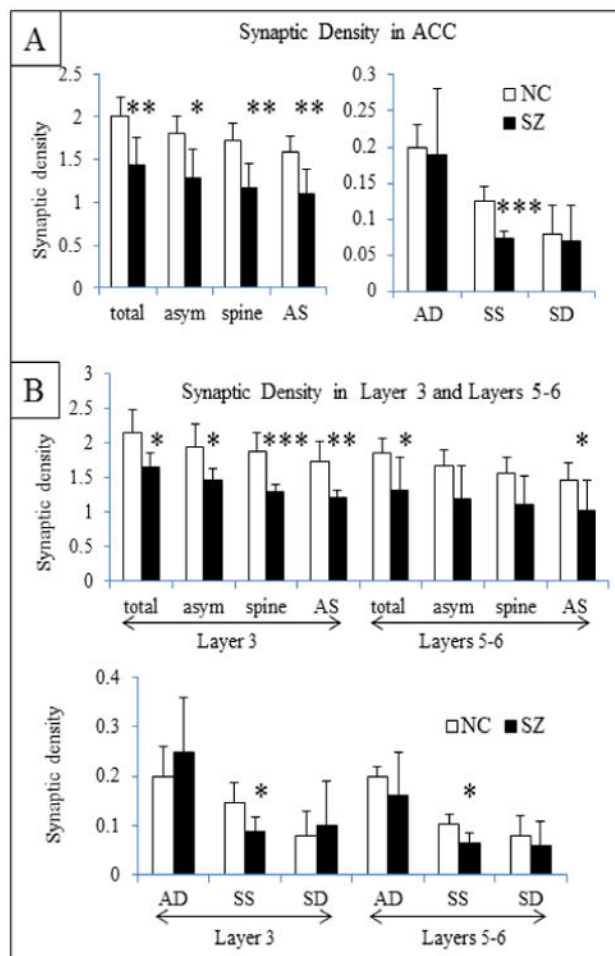


Figure 5. Synaptic density (per $10\mu\text{m}^3$). A) Density of total synapses and specific types of synapses in the ACC. B) Synaptic density in layers 3 and 5-6 of the ACC. Total (all synapses combined), asym (all asymmetric synapses), spine (all axospinous synapses), AS (asymmetric axospinous), AD (asymmetric axodendritic, SS (symmetric axospinous), and SD (symmetric axodendritic) are shown separately for layer 3, and 5/6 combined. T-test results: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

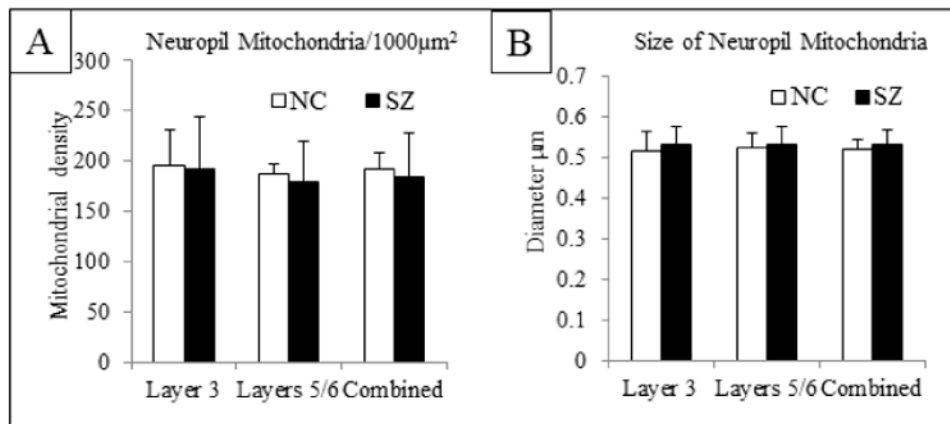


Figure 6. Mitochondria in the neuropil. A) Mitochondrial density per 1000µm² of neuropil indicates no difference in density between NC and SZ in layer 3, layers 5/6 or the combined value. B) The diameter of mitochondria in the neuropil was similar between NC and SZ in layer 3, layers 5/6 and the combined value.

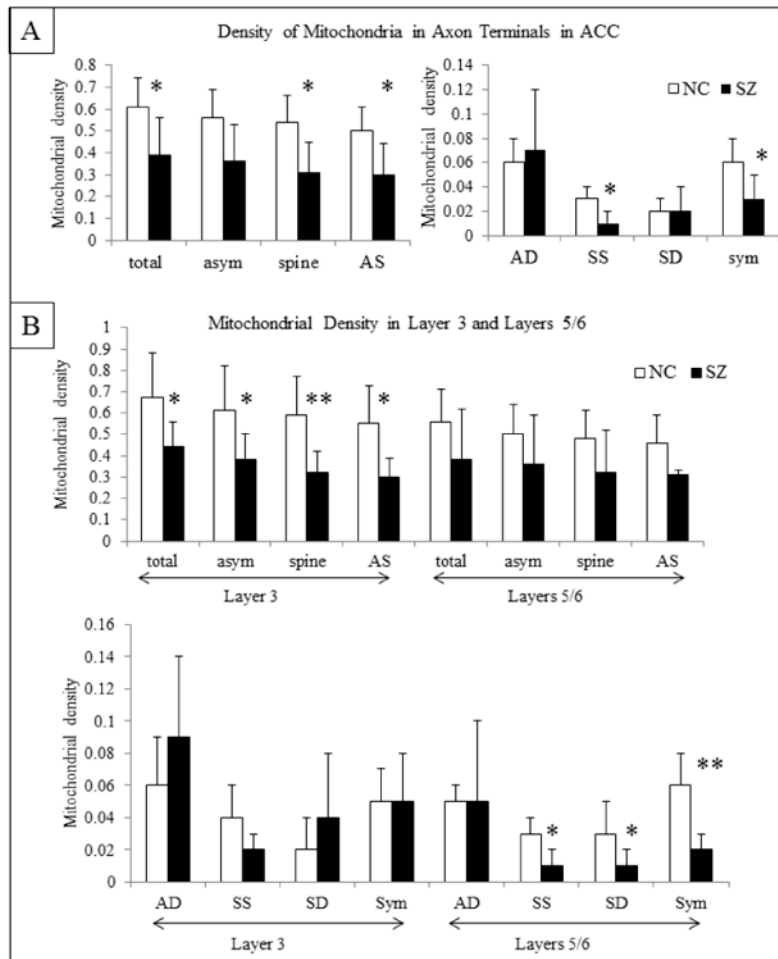


Figure 7. Mitochondria in axon terminals (per $10\mu\text{m}^3$) in the combined ACC (A) and in Layers 3 and 5/6 (B). Abbreviations: the same as Fig. 5. Sym, symmetric synapses. T-test results: *, $p < 0.05$; **, $p < 0.01$.

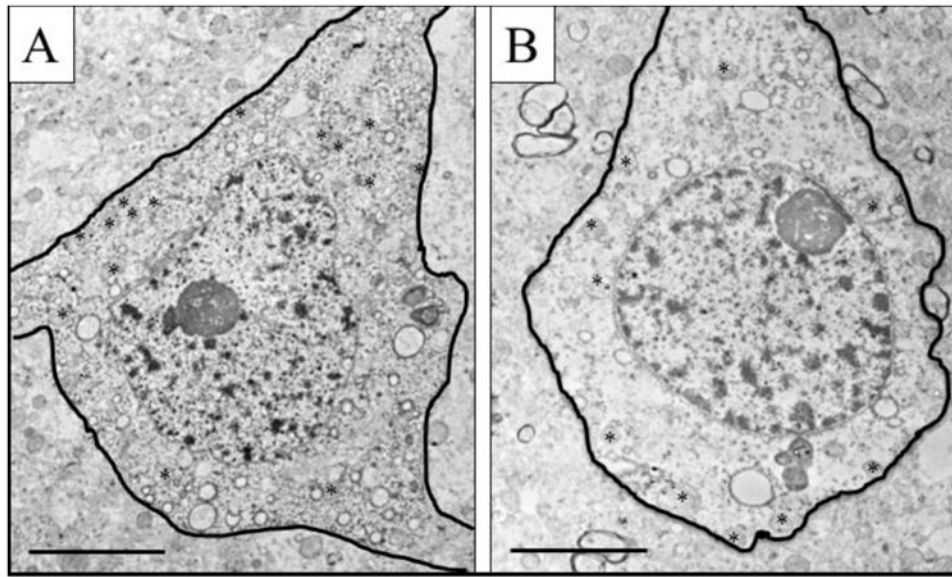


Figure 8. Mitochondria in neuronal cell bodies. Representative electron micrographs of pyramidal cells in NC and SZ. The soma is outlined in black; asterisks indicate mitochondria. Scale bar = 5 μ m.

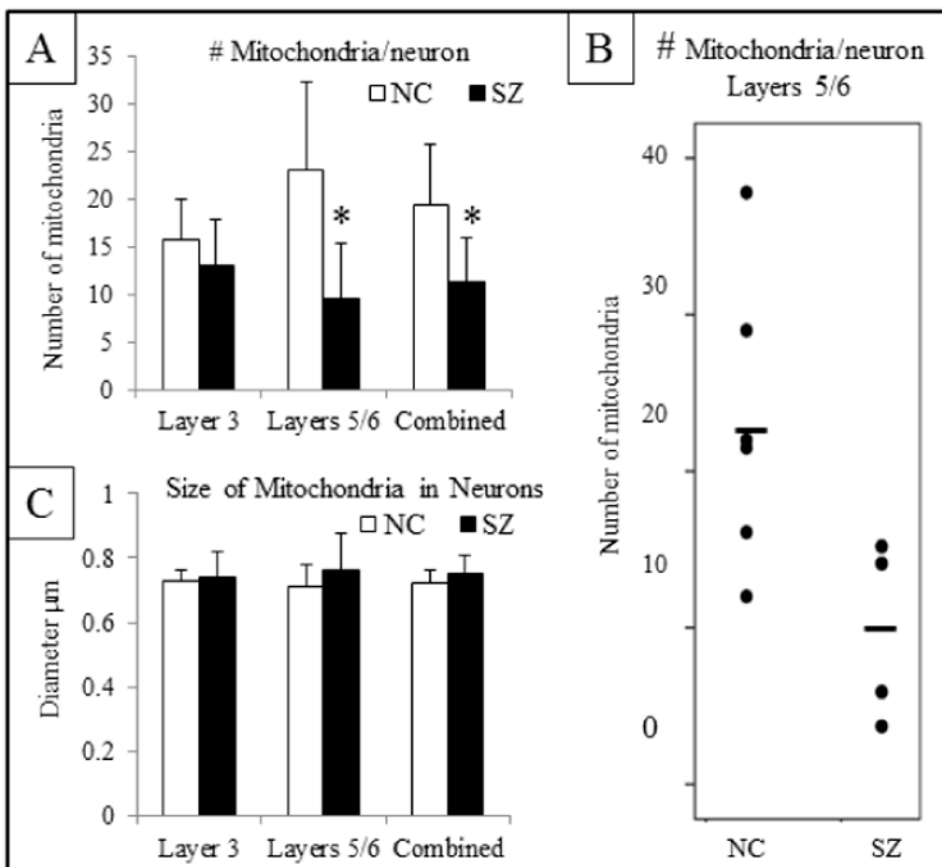


Figure 9. Mitochondrial quantification in neuronal somata for Layer 3, Layers 5/6 and then the combined value. A) Number of mitochondria per soma in a single section. There are significantly fewer mitochondria per neuron in the combined ACC and in layers 5/6 in SZ vs. NCs. B) Scatter plot of the number of mitochondria per neuron in Layers 5/6. C) Diameter of mitochondria indicates no difference in size of mitochondria in SZ.

Table 1

Demographic information for the cases.

NCs	source	ARS	PMI	Nicotine	alcoholism	P values
1	MBC	32CF	7.0	yes	no	age: 0.311
2	MBC	45AAF	6.75	na	yes	race: 0.598
3	MBC	40AAM	7.0	yes	no	sex: 1.000
4	ABC	87CM	7.5	no	no	PMI: 0.425
5	ABC	32CF	7.0	yes	no	nicotine: 0.459
6	ABC	61CM	4.0	yes	no	alcoholism:0.747
mean		49.5	6.54			
SD		21.3	1.27			

SZ	source	ARS	PMI	Nicotine	EtOH	medication
1	MBC	60AAM	5.0	na	no	Olanzapine
2	MBC	60AAF	6.0	na	no	typical
3	ABC	56CM	7.5	yes	yes	Olanzapine Quetiapine
4	ABC	70CF	5.0	yes	no	Depakote; Abilify
mean		61.5	5.88			
SD		6.0	1.18			

NCs, normal controls; ARS, age, race, sex; PMI, postmortem interval in hours; C, Caucasian; AA, African American; M, male; F, female; P values indicate the results of independent t-tests or Pearson Chi-Square tests between groups.

The proportion of axon terminals (AT) containing at least one mitochondrion was 30.5% in NCs and 26% in SZs, for all layers combined. The percentage of symmetric axospinous synapses in all layers combined that contained at least one mitochondrion in SZ was 67% of that of NCs ($p<0.03$). This change was confined to layers 5/6 where <10% of these terminals contained at least one mitochondrion. Abbreviations are the same as those in Figure 5.

Table II

	% of AT w/ 1 mitochondrion	total	asym	spine	AS	AD	SS	SD	sym
combo	NC	30.5	30.8	30.9	31.4	26.4	29.0	34.0	27.1
	SZ	26.4	27.0	25.4	25.9	32.6	17.5*	15.2 t	18.3*
L3	NC	30.2	30.8	30.6	31.1	28.6	23.4	19.5	23.2
	SZ	26.3	26.0	24.2	24.3	35.3	21.4	26.4	25.9
L5/6	NC	30.1	30.1	30.7	31.0	24.0	29.4	40.8	31.0
	SZ	26.2	27.9	26.5	27.3	30.8	17.3	4.0*	9.4**

* $p<0.05$;

** $p<0.014$.