

**COVER SHEET**

**TITLE:** Quantification of interneurons present in postmortem adult Down syndrome brain

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ABSTRACT

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Neuroanatomical abnormalities in the DS brain contribute to specific cognitive deficits in DS individuals. Histopathology has consistently revealed fewer neurons in the DS cerebral cortex. Based on neuron morphology and the developmental timing of the neuron reductions, the missing neurons may be interneurons. We sought to resolve the identity of the neuron subtype that is affected so as to both increase our knowledge of DS neuropathology and our ability to define potential therapies. We identified neurons using immunocytochemistry in post-mortem human brain cerebral cortex, specifically the superior temporal gyrus, and quantified by design-based stereology. Our results reveal a reduction in neuron density in DS STG compared to control, corroborating previous histopathology that reported up to a 50% reduction in the number of neurons in DS cortex. Importantly, comparison of interneuron subtypes (PV, CR) indicate that there is a similar reduction in density of these subtypes in DS STG suggesting that there is a general reduction of interneurons in DS cortex.

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# **Quantification of interneurons present in postmortem adult Down syndrome Brain**

## **Abstract**

Neuroanatomical abnormalities in the DS brain contribute to specific cognitive deficits in DS individuals. Histopathology has consistently revealed fewer neurons in the DS cerebral cortex. Based on neuron morphology and the developmental timing of the neuron reductions, the missing neurons may be interneurons. We sought to resolve the identity of the neuron subtype that is affected so as to both increase our knowledge of DS neuropathology and our ability to define potential therapies. We identified neurons using immunocytochemistry in post-mortem human brain cerebral cortex, specifically the superior temporal gyrus, and quantified by design-based stereology. Our results reveal a reduction in neuron density in DS STG compared to control, corroborating previous histopathology that reported up to a 50% reduction in the number of neurons in DS cortex. Importantly, comparison of interneuron subtypes (PV, CR) indicate that there is a similar reduction in density of these subtypes in DS STG suggesting that there is a general reduction of interneurons in DS cortex.

## **Introduction**

Down syndrome occurs one in every 691 births in the United States making it the most common genetic cause of intellectual disability. However, there is still a significant knowledge gap in understanding the abnormal neuroanatomy that leads to the observed cognitive deficits. This knowledge gap is inhibiting the development of possible therapeutic treatments.

There are two types of neurons in the cortex, interneurons and pyramidal neurons. Pyramidal neurons are the large, long range neurons. Interneurons play a crucial role in neural circuit formation. Cortical interneurons are small, locally projecting inhibitory GABAergic cells that represent about 15% of all neurons in the cortex in rodents and upwards of 20% in primates, including humans [15]. Cortical interneurons are much more numerous and more elaborate in humans to enable the complex functions unique to humans. Further, many neurological and psychiatric diseases have been linked to abnormalities in interneuron function [16, 17] or number [18].

Previous studies have revealed there are fewer neurons in the frontal and temporal regions of the DS cerebral cortex of both developing and mature human DS brain tissue [1-11]. Based on the morphology of neurons and the developmental timing of the neuron reductions observed in DS cortex, the missing neurons may be interneurons.

The temporal lobe (STG) is an important region to analyze in DS for several reasons: 1) thin STG is one of the most consistent gross abnormalities in DS brain [19, 20]; 2) alterations in neuron density and lamination in the STG have been shown during DS cortical development [5]; 3) the STG can be readily identified early in development; 4) the STG is an association cortex and is therefore likely to be involved in the characteristic intellectual

impairment in DS. Therefore, analysis of this area in DS brain will be likely to yield robust and meaningful results.

Mouse models of DS have been used to attempt to better understand cortical development in DS, but these studies contradict data that suggest fewer interneurons are present in human DS cortex. Analysis of DS mouse models show *increased* interneuron generation [12, 13]. Although data from mouse models differ from humans, the data taken together highlights the fact that interneuron development is faulty in DS. The discordance between mouse and human data and the scarcity of human studies in DS limit the access to therapeutic strategies that are effective in humans. It is therefore critical to resolve the discrepancy by analyzing the interneuron populations in vivo.

## Methods

### Tissue

We obtained sections of adult postmortem brain tissue from the NICHD Brain and Tissue Bank for Developmental Disorders. We analyzed young adults because we want our focus to be on the adult tissue, but do not want our data to reflect the early onset degeneration that has been shown to occur in DS as early as age 35. We will analyze tissue from five DS individuals and five age and gender matched control subjects (Table1). For the purpose of this paper, we have analyzed two individuals, one control and one with DS. Tissue from the superior temporal gyrus (STG), in the temporal lobe was analyzed. Tissues were sectioned to 50 microns in width using a cryostat.

UMB #	DISORDER	AGE (Yrs, Days)		GA (wks)	SEX	RACE
277	Trisomy 21			21	Male	Caucasian
1553	Control			22		Caucasian
M3287M	Trisomy 21	0	91	37	Male	African American
1296	Control	0	98		Male	African American
832	Trisomy 21	0	339		Male	African American
M1931M	Control	0	332		Male	Caucasian
714	Trisomy 21	1	319		Male	African American
1063	Control	1	123		Male	African American
M3828M	Control	1	306		Male	Caucasian
M2854M	Trisomy 21	15	174		Male	Caucasian
M2843M	Control	15	147		Male	Caucasian
1841	Control	19	289		Male	Caucasian
5277	Trisomy 21	19	352		Male	Caucasian
5654	Control	19	264		Male	Caucasian
M1960	Trisomy 21	19	311		Male	Indian subcontinent
753	Trisomy 21	23	295		Male	Caucasian
1226	Control	23	90		Male	Caucasian
5030	Control	24	333		Male	African American
5341	Trisomy 21	25	304		Male	African American
1544	Control	32	315		Male	Caucasian
4273	Trisomy 21	33	315		Male	Caucasian

Table 1: Age and gender matched individuals used for our study. The individuals used for the results of this paper are indicated in yellow.

## Immunostaining

All neurons were identified by their expression of NeuN. Antigen-antibodies were visualized with avidin-biotin, horseradish peroxidase (HRP) and 3, 3'-Diaminobenzidine (DAB) using standard immunohistochemical techniques. Our data shows that we can immunostain post-mortem human brain sections to identify all neurons and several interneuron subtypes including: Parvalbumin (fig.1), Calretnin, Somatastatin, and Calbindin (see table 2 for antibody specific methods). However, our preliminary data includes analysis for PV, CR, and NeuN only.

MARKER		ANTIBODY INFORMATION	ANTIGEN RETRIEVAL	DILUTION	secondary, ABC	DAB
GABA interneurons	GAD65/67		Vector unmasking 15 minutes 90C			This antibody and method did not work.
all neurons	NeuN	AbCAM ab104225 Rabbit	Vector unmasking 15 minutes 95C	1:500	Visucyte HRP polymer	5 minutes ImPACT
Parvalbumin	PV	Sigma P3088 mouse	universal 5 min 95C or Vector	1:1000	Biotin 2' ABC	5 min
Somatostatin	SST	rat	Vector unmasking 15 minutes 95C	1:100	Biotin 2' ABC	10 minutes
Calretinin	CR	7697 Rabbit	Vector unmasking 15 minutes 95C	1:2000	Biotin 2' ABC	3 minutes
Calbindin	CB	ab1778 Rabbit	Vector unmasking 15 minutes 95C		Biotin 2' ABC	

Table 2: Subtype and antibody specific immunostaining methods. For this paper, we used CR, PV, and NeuN. The GAD65/67 staining was never successful and was therefore omitted from the present study.

## Optical Fractionator

The total numbers of parvalbumin (PV), calretnin (CR), and NeuN positive neurons were estimated using the Optical Fractionator (OF) workflow in the MBF Bioscience Stereo Investigator software. A reference point is set before opening the OF workflow. A contour of the 50µm mounted section is traced using the simple click method and the movement of the automated stage. A percentage of the tissue to be counted is initially selected based on the size of the tissue and how

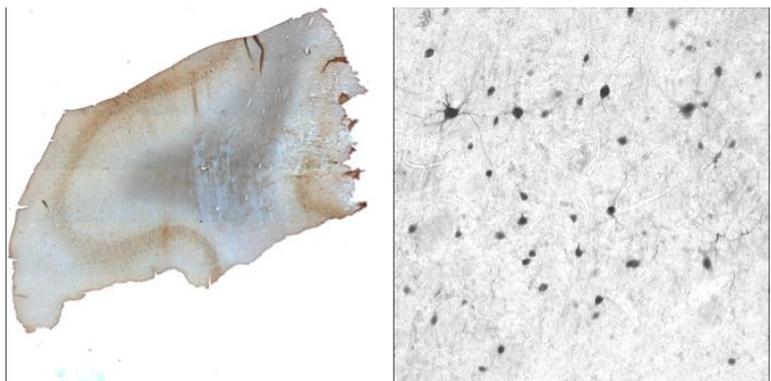


Figure 1: Left, 50 micron section of DS STG stained for PV positive interneurons. Right, staining for PV positive interneurons magnified at 10X.

many positive cells are seen through visually scanning the tissue. Percentages chosen were

PV:5%, CR:3%, NeuN: 2%. This sampling percentage allows us to count every 5-10 sampling sites to obtain an estimate for the whole sample. Section cut thickness is selected in the software to be 50µm and we chose to measure the sample thickness at every sampling site. The counting parameters were chosen so as to get statistically correct results. Our aim was to keep the coefficient of error below 0.1. Once we start counting, the top and bottom of the section sample needs to be set. This measures the thickness of the section. If a positive cell is seen, a marker is placed in the sampling site on top of the cell. This procedure is repeated at every sampling site until all of the sampling sites are visited. At this point, the probe run is ended and the results are exported and saved into Microsoft Excel. The total positive cell count was estimated using the following equation:  $N = \sum Q * \frac{t}{h} * \frac{1}{asf} * \frac{1}{ssf}$ , where  $\sum Q$  is the total number of cells counted,  $t$  the average section thickness and  $h$  the height of the optical dissector, and  $asf$  and  $ssf$  the area sectioning fraction and the section sampling fractions, respectively [10].

### Resample Oversample

The first counts for a sample and stain are considered an Oversample. This means we would be able to obtain statistically similar results by counting a lower percentage of the tissue section. The exact value to be counted in future samples is determined by selecting probe run results and selecting all of the sections counted initially. "Resample" is selected and section interval is entered. When complete,

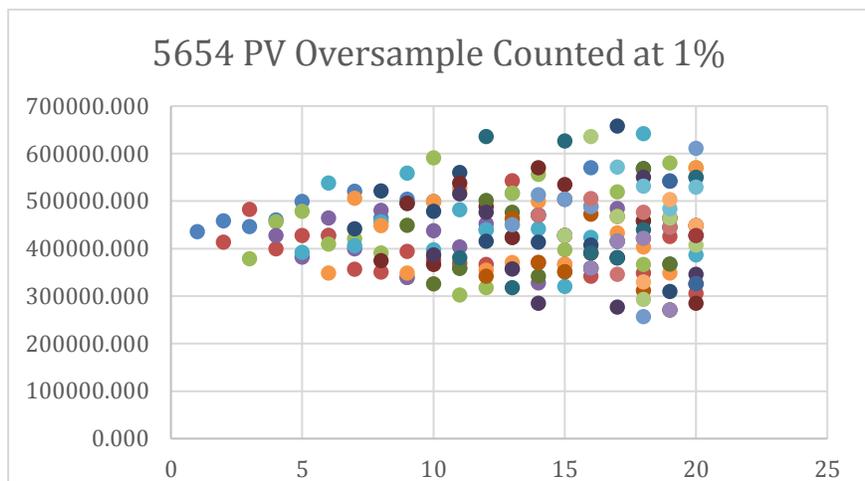


Figure 2: Resample Oversample confirming that 1% is the correct percentage of the tissue to count. As the markers begin to diverge the count's become less accurate. To see the dots beginning to slightly diverge at the second point, indicates that we are not counting too high of a percentage.

Microsoft Excel will open with results. A scatterplot is made graphing the data. Where the point before the data begins to significantly diverge is the value at which the percentage can be decreased by. For example, if the points diverge significantly at the value four, one third of the initial percentage needs to be counted. Once the sample is counted at a lower percentage, "Resample Oversample" is run again to determine if it is possible to further decrease the amount of the section counted. The percentages we chose to use for our counts were PV: 1%, CR: 1%, and NeuN: .5% (see Figure 2).

### Cavalieri Estimator

The Cavalieri Estimator probe is used to estimate the total volume of tissue sampled and counted. A reference point is set to indicate slide position. Sections are set up in the serial selection manager and section interval is selected. The section interval is the interval at

which the tissue is sectioned and stained. Our section intervals were five and seven. Section thickness is also set up in the serial selection manager, giving the program basis for the volume estimate. A marker is selected and named as well as a marking method. We used the paint marker method for our purposes. Markers are placed in the entirety of the region of interest that was counted with the Optical Fractionator Workflow. After all sections are counted, results are found in the probe run list and exported to Microsoft Excel

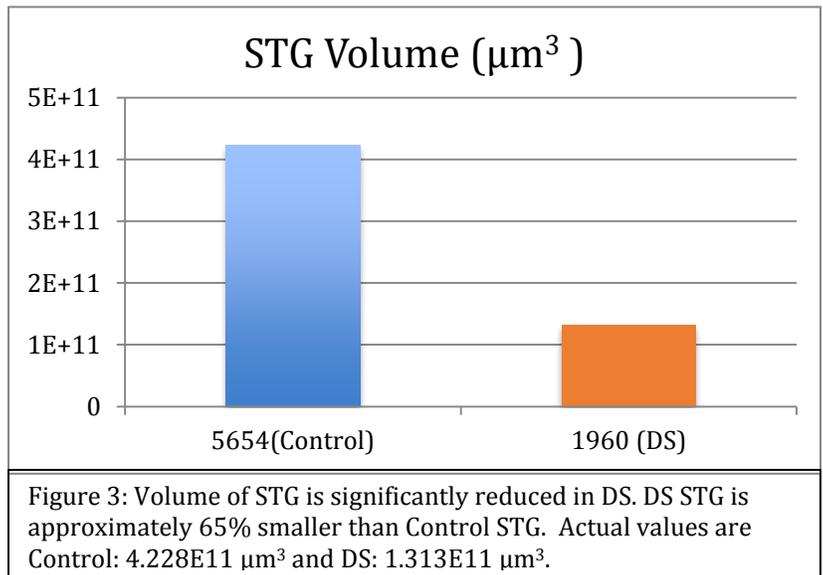
### Density Calculation

To calculate cell density, the total cell population estimate (from the Optical Fractionator Workflow) is divided by the total tissue volume (from the Cavalieri Estimator). To calculate the proportions we compared subtype density to total neuron density.

### Results

#### Tissue Volume

Our results indicate that the DS STG is smaller than the Control STG (Figure 3). Other publications have observed a decrease in thickness of the STG in DS. Our results corroborate this observation [19,20].



#### Total Neuron Density

Golden, J.A. and B.T. Hyman 1994 have found that neuron density in the cerebral cortex is decreased in DS. Our results using stereological methods show that there are fewer neurons in the DS STG and thus, confirm their observations [5]. There are roughly 50% fewer neurons in the DS STG (Figure 4). This result validates our methods as our results are consistent with previous methods have found.

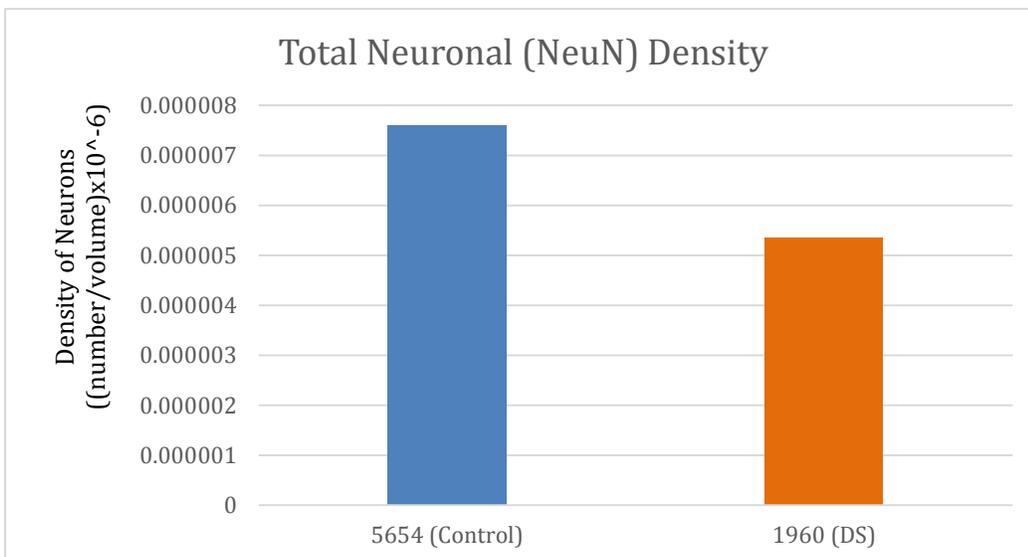
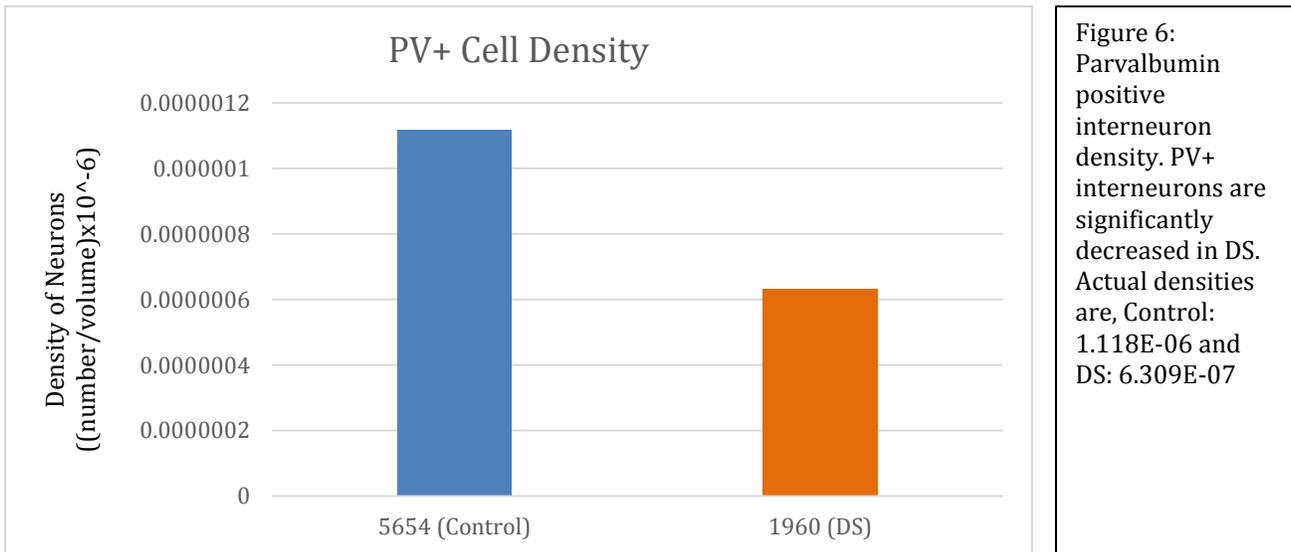
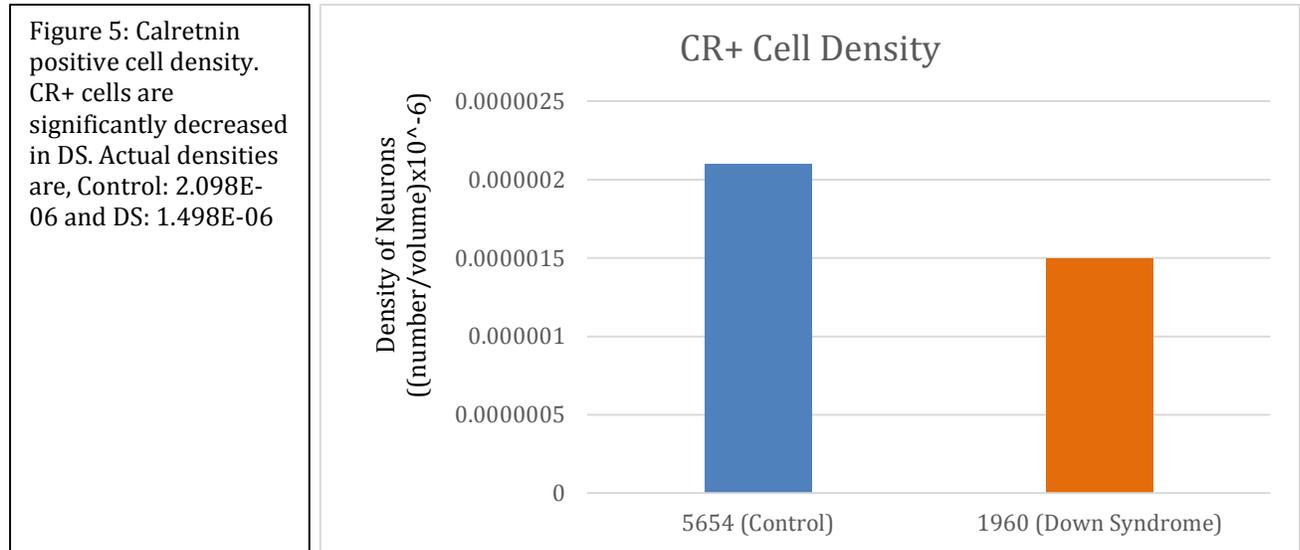


Figure 4: Total neuronal density in DS versus Control. Total neurons are significantly decreased in DS. Actual values are Control: 7.601E-6 and DS: 5.346E-6

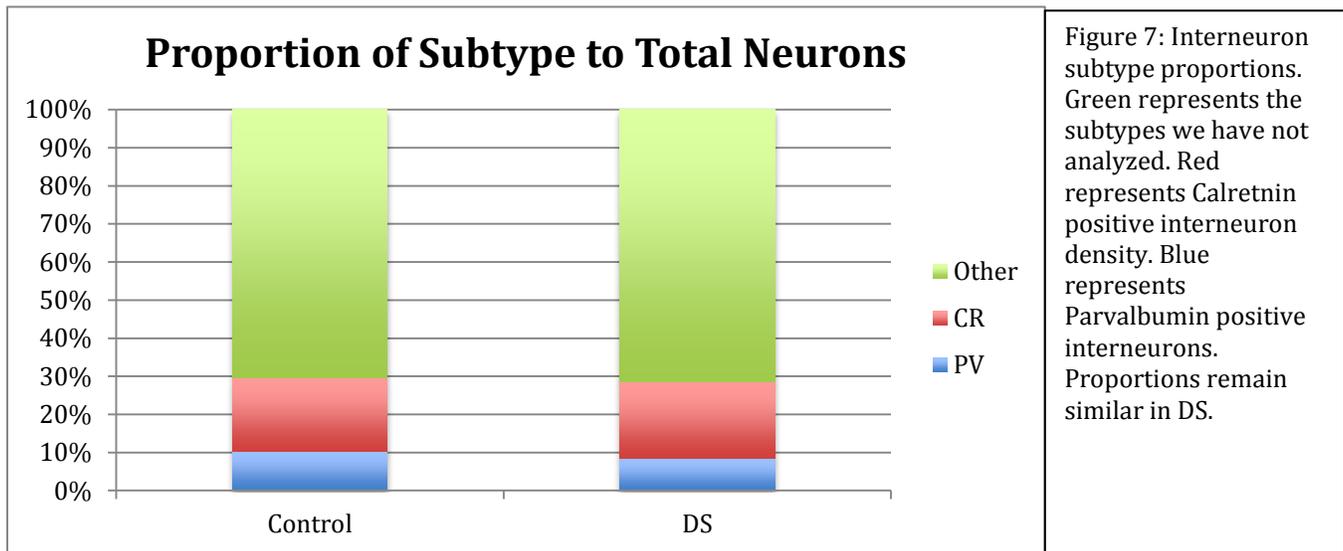
### Subtype Specific Density

We analyzed both Calretinin and parvalbumin interneuron subtypes in both control and DS tissue. Our results indicate a similar pattern in the decrease in density. Both subtypes indicate that the DS samples have significantly decreased density, about 50% (Figures 5 and 6).



### Proportion of subtype over total neurons

To look at subtype specific discrepancies in DS and control, we compared the subtype over total interneuron proportions. Our results show consistent decreases in interneuron subtype proportions (figure 7). The proportions of CR and PV to total neurons are similar in control and DS. Our stereological data shows a consistent decrease in DS while maintaining the same proportions as the control samples. The consistent proportions among DS and control suggest that the density of all of the interneuron subtypes is decreased in DS.



## Discussion

Our aim was to confirm the hypothesis that there are fewer neurons and interneurons in the Down syndrome STG. Previous studies have shown that abnormalities in this region are among the most consistent in DS [5,19,20]. Previous methods used to quantify interneurons in the DS brain are out dated. The experimental results of previous methods were in need of validation. Our data showing a decrease in STG volume in DS corroborates what previous data has shown [19, 20]. Our data indicates that the DS STG is about 60% smaller than the control STG. This could cause some of the phenotypic variants characteristic of DS.

The changes in total neurons as well as parvalbumin positive and calretinin positive interneurons show consistent decreases in DS. Both subtypes have approximately 50% less density in DS when compared to their age matched control. Our data suggests that the decrease in DS STG neuron density is more than 50% when compared to control. These results confirm our hypothesis that there are fewer neurons and subtype positive interneurons in the DS STG, suggesting an overall decrease in interneurons. The consistent decrease across both subtypes and total neurons suggests that faulty interneuron development in DS affects all subtypes at various stages in development. These results confirm that faulty interneuron development typical in DS leads to reduced generation of interneurons.

The proportions of subtype specific interneurons to total interneurons remain constant in DS and control. However, all of the neurons and interneurons are decreased in DS. This result suggests that all interneuron subtypes are affected by abnormal development equally. Not just one subtype appears to be affected.

It is important to confirm these results with the analysis of more brain tissue as well as more interneuron subtypes. Our experiment faced many challenges that we are working to overcome. We only have access to a limited amount of brain tissue. The tissue we have obtained is extremely delicate and difficult to work with. All of the tissue stains slightly

different so each has to be tested before staining a whole sample. However, we are working with what we have and will have enough data to confirm our results.

Our study provides human specific data for interneurons in the DS brain. Using human brain tissue is the only way to confirm neuron deficits and faulty development in DS. It is an important step towards the research of human specific DS therapies. These results inform modeling of human trisomy 21 pluripotent stem cells to identify whether interneuron progenitors have defects in specification, proliferation or migration that result in the presence of fewer interneurons in the DS cortex. Through the use of human trisomy 21 pluripotent stem cells, neurodevelopment and degeneration in DS can be studied as well as a vast number of other issues characteristic to Down syndrome.

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