Layer III Pyramidal Cells and Cell Column Spacing in HIV-1 Transgenic Rats

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Many studies have shown that subcortical brain areas are affected by Human Immuno-deficiency Virus, type 1 (HIV-1). The first goal of our research was to determine whether HIV-1 anatomically alters cortical brain structures by investigating layer III of the anterior cingulate cortex using a 3-dimensional nearest neighbor analysis. Our second goal was to analyze the same tissue a second time through a 2-dimensional computer analysis cell column program in order to test the dependability of both methods of analysis. We used 6 HIV-1 transgenic (Tg) and 6 age-matched control, female Sprague-Dawley rats as a model for vertically-infected children with HIV-1. The nearest-neighbor distance and mean center-to-center cell spacing distance revealed no significant differences between groups, indicating that layer III of the anterior cingulate cortex is histologically unchanged in rats infected with the virus. These same results were obtained via the 2-dimensional computer analysis, which reiterates the reliability of both stereological analysis programs.

Key Words: cortical physiology; anterior cingulate gyrus; cortical columns; mean center-to-center spacing; nearest-neighbor distance

Introduction

To date, at least 30.6 million people worldwide, including 2.2 million children under the age of 15, are living with Human Immunodeficiency Virus, Type 1 (HIV-1) (UNAIDS/ World Health Organization, 2007). HIV-1 is a progressive disease that becomes Acquired Immunodeficiency Syndrome (AIDS) in its latest and most severe stage. Because there is currently no cure for HIV/AIDS, over three million people die from AIDS-related illnesses each year.

HIV-1 is one of two types of HIV (the other being HIV-2) that is infectious to humans. HIV-1 is a retrovirus and part of the viral family Retroviridae. It functions by attaching to CD4 receptors on CD4 helper lymphocytes, which fight off infection in the body. Once inside the cell, the virus uses the host for viral replication. This process can occur for years without detection. AIDS is said to develop when the viral load, or amount of HIV-1 virus in the blood, progresses such that the CD4 helper lymphocyte count is less than 200 per unit of blood (a normal CD4 count is 600-1500 per unit of blood) and the body can no longer fight simple infections (National Institute of Health, 2007).

Many neuropsychological effects of HIV-1 have been found in symptomatic individuals. Behaviorally, it is known that HIV-1 causes cognitive impairment, such as deficits in prospective memory (Carey et al., 2006) and information processing (Noe, 2002), especially as the disease progresses (Odiase et al., 2006).
HIV-1 seropositive individuals have also shown a decrease in attention, working memory, psychomotor speed, and verbal fluency (Noe, 2002).

HIV-1 has been studied using a variety of models (Ambrose et al., 2007; Reid et al., 2001; Tinkle et al., 1997); however, certain models are especially powerful because the animals are infected with the actual virus. This allows researchers to study the direct effects of HIV without risk of infection while using a non-human model. HIV-1 Tg mice were originally used as a small-animal model for studying HIV-1 in humans (Reid et al., 2001). However, a mouse did not prove to be a good model for HIV-1 because even though some HIV-1 gene expression occurred in most tissues, certain areas of the body, such as skin and muscle, were more expressive than others. Attempts were also made to use a Tat transgene as a model for HIV-1. Tat is a protein that regulates cell death in response to HIV-1 and is thought to be the mechanism by which HIV-1 overcomes the body (Huang et al., 1998). However, the Tat transgene resulted in aversive effects that did not resemble HIV-1 (Reid et al., 2001).

As an alternative, HIV-1 Tg rats have been tested for their susceptibility to the virus (Reid et al., 2001). Confirmation of equal gene expression led to the introduction of HIV-1 Tg rats as the new method for vertically-transmitted HIV-1 research. Deletions of the gag-pol genes from the infectious proviral plasmid (PNL4-3) resulted in a non-infectious HIV-1 gag-pol clone (pEVd1443). The HIV-1 gag-pol clone was incorporated into the Sprague-Dawley rat and a successful animal model was produced.

The rats express the HIV-1 provirus with functional deletions of gag and pol without altering expression of Tat or Rev, a viral protein that regulates splicing. These proteins, especially Tat, are good models of HIV-1 because they are the proteins that are activated in the brain when the infection is contracted. Tat protein is considered a viable mechanism for HIV-1 related toxicity (Huang et al., 1998). As a result, HIV-1 Tg rats have proven to show similar symptoms as humans infected with HIV-1, such as neurological changes, respiratory difficulty, and cataracts (Reid et al., 2001).

The vertically infected HIV-1 Tg rat expresses pathology similar to AIDS by five to nine months of age, such as weight loss and neurological abnormalities, which are characterized in rats by circling behavior and hind-limb paralysis (Reid et al., 2001). These rats also suffer from generally enlarged lymph nodes, pale kidneys, and mild pneumonia. The HIV-1 Tg rats used in the following study showed severe cataracts, as well as many behavioral changes. They displayed less exploratory locomotion, total activity, and rearing behaviors, which could indicate a decrease in motivation to explore their environment (unpublished observation).

This is the first study, to our knowledge, which focuses directly on the cortical organization of HIV-1 Tg rats. Earlier studies observed that there were pathological changes in clinically ill rats, such as microscopic hemorrhaging and apoptosis (Reid et al., 2001). Atypical capillaries and endothelial cells, as well as neuronal cell death were also noted.

The highly correlative nature of subcortical neurological deficits in humans and Tg rats infected with HIV-1 led us to believe that there may be broad anatomical brain abnormalities associated with HIV-1. In humans, HIV-1 has been known to result in subcortical brain dysfunction (Dunfee et al., 2006). Functional Magnetic Resonance Imaging (fMRI) scans on the HIV-1 Tg rats displayed a random non-localization of subcortical pathology. This has also been noted in other HIV-1 Tg rats (Reid et al., 2001). Because subcortical deficits have already been established as a problem associated with HIV-1, we decided to investigate the cortex at its most basic level in order to determine whether the cortex is another potential brain area affected by HIV-1.

Since this model affects neurogenesis, we sought to find preliminary evidence that the ontogenetic radial columns were also affected. Corticogeneurogenesis involves the development of highly vertical radial units that are later thought to become the minicolumns of the adult cortex.

It has been argued that the basic unit of the mature neocortex is the minicolumn: an arrangement of neurons (and possibly associated glia) oriented perpendicular to the pial surface.
A typical minicolumn may contain 80-100 neurons (Mountcastle, 1997; Buxhoeveden and Casanova, 2002a). Many minicolumns arranged together via horizontal connections make up a macrocolumn, or cortical column. Macrocolumns typically range between 300-600 \( \mu m \) in diameter depending on cortical region and species. Therefore, we can infer that the number of minicolumns in the cortex or small changes in minicolumn size may alter cortex size both between and within species. It has been hypothesized that the minicolumns within a cortical column share similar morphological properties (Mountcastle, 1997; Buxhoeveden et al., 2006a; Buxhoeveden and Casanova, 2002b).

The current prevailing view on the development of the cerebral cortex asserts that symmetrical and asymmetrical division results in cortical genesis (Kornack and Rakic, 2001). This radial division, also known as the radial unit hypothesis, is widely believed to contribute to the mature morphology of the cell column through radial expansion within ontogenetic cell columns, thus resulting in total cortical surface area (Rakic, 1972, 1978, 1988). Because of this radial migration of progenitor cells, it is possible that minicolumn size is affected by factors that could contribute to ontogenetic column morphology.

Because of its reiterative and genetic specificity, the cell column has become a descriptive indicator for comparative neuroanatomy and neuropathology (Buxhoeveden et al., 2002b). In the case of the latter, pathological changes in the structure of the cell column is thought to contribute to neuropsychological deficits associated with a variety of neurological conditions. These so-called minicolumnopathies, together with their constituent cell abnormalities, have been observed in neurological disorders such as autism (Buxhoeveden et al., 2006b; Casanova et al., 2002c; Casanova et al., 2003b; Casanova et al., 2002a), Asperger’s syndrome, Rhett’s syndrome (Casanova et al., 2003a), and Alzheimer’s disease (Esiri and Chance, 2006). Evidence for abnormal minicolumn structure has also been demonstrated in psychiatric disorders such as schizophrenia (Buxhoeveden et al., 2000) and dyslexia (Casanova et al., 2002b) and in the anterior cingulate and prelimbic cortices of rats exposed prenatally to cocaine (Buxhoeveden et al., 2006a).

Due to the strong genetic component associated with the radial cell column, we hypothesized that minicolumns and the pyramidal cells, of which minicolumns are comprised, might be affected as a result of the expression of the HIV-transgene in rats. Specifically, we looked for evidence that the size of the columns may have been affected. The first goal of the present study was to investigate the nearest-neighbor distance of pyramidal neurons in lamina III of the anterior cingulate cortex of adult HIV-1 Tg rats.

Lamina III, an associative cortical region from which underlying thalamic input is relayed to other brain areas, is highly favored in studies of cortical organization and physiology (Buxhoeveden and Casanova, 2002a). First, the microvertical organization of the cerebral cortex is most clearly evident in lamina III. Because of the ease with which the columnar orientation of cells and fibers in the cortex may be detected in lamina III, studies which utilize data collection primarily by automated computer analysis and through application of stereological techniques focus primarily on this lamina as cells easily identifiable in histology. Lastly, the anterior cingulate gyrus has presented high responsiveness to pathophysiology in neurodegenerative conditions caused by neurological disorders and substance abuse.

Our second goal was to analyze minicolumn size via a two-dimensional computer analysis cell column program, Image J. These results were then compared to the data obtained from the three-dimensional nearest-neighbor distance analysis. This study marks the first time that these two programs of analysis were used on the same material. We hypothesized that this research would reiterate the reliability of both programs for their use in stereological analysis by resulting in similar findings.

**Materials and Methods**

**Animals**

All animal protocols in this study were approved by the University of South Carolina
Institutional Animal Care and Use Committee (IACUC). Six overiectomized (OVX) female Tg Sprague-Dawley rats were purchased from Harlan Laboratories (Hsd: HIV-1 (SD); Indianapolis, IN). Six age-matched female OVX HIV-1 Sprague-Dawley littermates, which have the transgene but do not express it or display any symptoms characteristic of HIV, were purchased as viral controls. All rats were handled and weighed every second day to reduce handling-induced stress and to check for signs of wasting.

**Tissue Preparation**

Animals in this study were anesthetized and decapitated at 10 months of age. The brains were rapidly dissected and cut through the midline. All brain halves were immediately frozen on powdered dry ice and stored in -30°C Celsius. The left hemispheres were used for histology and the right hemispheres were used for biochemical analysis.

**Tissue Processing - Unfixed Tissue**

Cryosections were cut at 60 um in a Micron cryostat (HM 500M) using disposable low profile stainless steel blades (Accu-Edge, 4689). The cerebellum was removed and the tissue was embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue Tek), and frozen onto a metal chuck. Sections were thaw-mounted on VWR superfrost plus (precleaned, 25 x 75 x 1 mm) slides and kept in -30°C Celsius until staining.

**Nissl Stain for Nearest-Neighbor and Computer Analysis**

Sections were dehydrated in a series of graded ethanol, Nissl stained, and stored in xylene. They were then coverslipped (VWR micro cover glass, 22 x 60 mm, No. 1 1/2) with Cytoseal XYL (Richard-Allan Scientific) xylene-based mounting medium. A nominal section thickness after dehydration and coverslipping resulted in a final mean thickness of 23.13 um. Pictures of the stained tissue were taken for further computer analysis.

**Section Analyses**

Pyramidal cell spacing and minicolumn organization were analyzed through nearest-neighbor distance using StereoInvestigator, as well as through a modified version of Image J, a program downloaded from The National Institute of Health. For both programs, refer to Figure 1 for clarification on measures taken.

**Figure 1.** A sample of Nissl stained tissue. Outlines of soma are shown in blue. The green line between the soma indicates the measure taken for “neuronal space.” Red dots are markers of nuclei within each cell and are joined by a red line, which is exemplary of the mean center-to-center cell spacing and nearest neighbor distance taken during analysis.

**Nearest-Neighbor Analysis**

Every sixth section of the anterior cingulate cortex was analyzed using nearest-neighbor analysis method developed by Schmitz et al. (2002) and Microbrightfield StereoInvestigator software. We used an E-800 light microscope at 600X with oil immersion lens. The block advance was 60 um with a mounted section thickness of 25 um and 3 um guard zones. Three areas of interest (66.43 x 91.96 um) were used for analysis in the StereoInvestigator program, with eight sampling sites each (50 x 50 um). The experimenter was responsible for setting the parameters (as indicated above), while the program provided randomized sampling sites from which the data was to be extracted. The parent pyramidal cell for each sampling site was only counted when more than half of the soma was within the area, under the condition that no part of the cell was touching the lower left area of the sampling site, as this may lead to
unintentional double-counting of somata. Once the parent pyramidal cell was marked, surrounding cells were only counted when the edges of the entire soma were visible.

A single experimenter was responsible for running the nearest-neighbor analysis via the StereoInvestigator program. The experimenter was blind to the type of tissue being analyzed (control vs. HIV-1 Tg). As shown by the standard error bars of the means in Figures 2 and 3, the variability due to manual analysis was minimal.

Computer Analysis of Cell Columns

Our method is a modification of previous computer analysis methods for pyramidal cell assays (Buxhoeveden et al., 2006a). Photographs of the Nissl-stained tissue were taken at 100x total magnification and digitized for further study. Computer analysis was conducted using the Image J program. The digitized images were transferred to a processor and underwent thresholding, watershed, and conversion to a binary image, all of which were automated processes of the program.

The operator was responsible for choosing a threshold level (20 pixels) for cell size that would eliminate glia and interneurons from the measurement, and to outline the region of interest (ROI) within the image. The operator also checked both the digitized and binary images for uneven lighting, artifact, and other factors that could affect reliable measurements. The calculation of spacing distance was based on counts of pixel-dense elements in the y dimension collapsed onto the x or horizontal plane measured over multiple levels of the image. Horizontal lines of one-pixel depths were run throughout the entire ROI and the final results were summed and displayed. The program measures horizontal spacing of cells as it descends in the vertical plane rather than assuming the presence of vertical units.

Although there are a total of six layers in the cortex, we focused on layer III where cell columns are generally one pyramidal cell wide at any point in cortical depth (Seldon, 1981a; Seldon, 1981b). Cell columns in the adult cortex are basically defined by pyramidal cell arrangement and make up the majority of the cortical neurons. As neuropil space increases during development, the columns and thus the pyramidal cells which comprise them separate further (Mountcastle, 1997). Therefore, measuring the mean spacing distance between pyramidal cells is an appropriate measure of the mean spacing distance between cell columns.

The major parameter measurements used were mean neuropil space and mean cell spacing distance. The neuropil space is measured from the edge to edge of cell soma, representing the space between columns, an area which is densely packed with axons, dendrites, glial cells, and fiber systems. The cell spacing distance is measured from cell nuclei to neighboring cell nuclei. This measurement includes the neuropil space, as well as the size of the cell soma.

Statistical Analysis

Statistical analysis for nearest-neighbor distance and cell columns was performed using an unpaired, two-tailed t-test with 95% confidence interval using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California USA. All error bars represent the standard error of the mean (SEM). An alpha level of .05 was used for all statistical tests.

Results

Nearest-Neighbor Distance

The nearest-neighbor distance analysis revealed no significant difference between the HIV-1 Tg rats ($M = 16.37, SEM = \pm 0.6763$) and their corresponding control group ($M = 16.56, SEM = \pm 0.3873$), $t(12) = .2561, p > .05$ (see Figure 2). Results remained valid when controlled for tissue thickness, as the HIV-1 Tg and control rats did not significantly differ (see Figure 3) when analysis was performed to calculate their average tissue thickness. Analysis of resulting tissue depth is necessary since material is often compressed during mounting of the sample.
Layer III Pyramidal Cells and Cell Column Spacing in HIV-1 Transgenic Rats
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Comparison of Stereological Analyses

The Nearest-Neighbor Analysis and the computer analysis via the Image J program independently fail to conclude a significant difference in average distance to a nearest-neighbor cell and mean cell spacing distance, respectively. By resulting in similar findings, the data provide supportive evidence of the reliability of both the three-dimensional nearest-neighbor analysis and the two-dimensional computer analysis programs (see Figure 5).

Computer Analysis of Cell Columns

Results from the Image J program show that the mean center-to-center cell spacing distance in layer III of the anterior cingulate cortex of HIV-1 Tg rats ($M = 20.84, SEM = ± 0.5163$) was similar to their age-matched controls ($M = 20.89, SEM = ± 0.3916$), $t(10) = .07716, p > .05$ (see Figure 4). These findings also held true for neuropil space, $t(10) = .08979, p > .05$, as HIV-1 Tg rats ($M = 14.02, SEM = ± 0.6611$) and their age-matched controls ($M = 13.95, SEM = ± 0.3709$) showed similar results.
Discussion

Because HIV-1 in adults is known to affect subcortical processes in the brain (Martin et al., 2001), it was originally hypothesized that children infected with HIV-1 from the zygotic stage of development may have greater cortical abnormalities as well. Our study strongly suggests that the cortex, at least in layer III of the anterior cingulate cortex, is histologically unaffected by HIV-1. These findings correlate with previous research which focuses on subcortical rather than cortical brain pathology as a major area of dysfunction in HIV-1 patients (Anes and Ellis, 2007; Martin et al., 2001; Goodkin et al., 2001).

The anterior cingulate cortex is involved in a variety of autonomic and cognitive functions, such as blood pressure regulation and control of emotion and decision-making tasks. Because we did not find histological abnormalities in the HIV-1 Tg rats investigated in this study, it may be inferred that children vertically-infected with HIV-1 are also spared from deficits in these areas.

Although we did not find significant results indicating anatomical changes in layer III of the anterior cingulate, it is possible that prospective studies will find abnormalities in other areas, such as in layers V and VI, which are responsible for motor skills (Calvin, 1998). Data from such a study may support recent behavioral findings discussed earlier in the paper.

It is important to note the small sample size used in this study. We do feel that our data is powerful in that the nearest-neighbor distance, as well as the mean center-to-center cell spacing distance, is nearly equivalent between the HIV-1 Tg and control rats. However, further research using a larger sample size is suggested, as it would be beneficial in confirming our results.

This study also demonstrated the reliability of both the column program and the three dimensional study, as they produced similar results in the same tissue. This encouraging outcome independently substantiates the results and confirms the relative accuracy of the two-dimensional analysis, a method that is considerably easier to use and is less time consuming. Future research will attempt to expand the aims of the present study by examining pyramidal cell nearest-neighbor distance and cell columns in lamina II, V, and VI of the anterior cingulate and prelimbic regions. We also intend to analyze apical dendrite bundles and myelin bundles since these are anatomical correlates of the pyramidal cell columns (Buxhoeveden and Casanova, 2002a). It is also possible that minicolumns are changed in other ways that may include alterations between inhibition and excitation, cell size and type, synaptic organization, and intrinsic and extrinsic circuits.

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