Published in final edited form as:

Hear Res. 2011 April ; 274(1-2): 5–12. doi:10.1016/j.heares.2010.02.014.

Microvascular Organization of the Cat Inferior Colliculus

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Abstract

Brain neural activity depends critically on the blood supply to a given structure. The blood supply can differ within and between divisions, which may have functional significance. We analyzed the microvascular organization of the cat inferior colliculus (IC) to determine if the capillary distribution is homogenous throughout. The IC consists of the central nucleus (CN), the dorsal cortex (DC), and the lateral cortex (LC), each with different roles in auditory behavior and perception. Plastic-embedded tissue was studied from adult cats in 1 μ m-thick semithin sections stained with toluidine blue; tissue was sampled from the IC in a caudal-rostral series of sections. The architectonic subdivisions were drawn independently based on Golgi impregnations.

We used the nearest neighbor distance (NND) method to quantify capillary density between subdivisions. Overall, the distribution of capillary density was non-homogenous across the IC. We found significant capillary NND differences between the CN and LC (Mann-Whitney test; $p \cdot 0.05$), CN and DC (Mann-Whitney test; $p \cdot 0.05$), and LC and DC (Mann-Whitney test; $p \cdot 0.05$). The CN had the lowest NND values among all three divisions, indicating the highest capillary density. NND values changed gradually as analysis moved from the center of the IC towards the periphery.

The significantly higher microvascular density in the CN may imply that the lemniscal auditory pathway has higher levels of blood flow and metabolic activity than non-lemniscal areas of the IC. The non-homogenous microvascular organization of the IC supports parcellation schemes that delineate three major subdivisions and confirms that the borders between the three regions are not sharp.

Introduction

The inferior colliculus (IC) is a key structure in the central auditory system. The IC is the principal site of convergence for ascending auditory information (Aitkin et al., 1984b), and receives substantial corticofugal input from the auditory cortex and other sensory areas (Aitkin et al., 1981; Aitkin et al., 1978; Winer et al., 1998). Thus, the IC acts as a center of

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*Dr. Jeffery Winer passed away on December 9, 2008 before this study was completed. We gratefully acknowledge his ideas, guidance, and enthusiasm that set the framework for this study. He saw the preliminary results to the microvascular organization of the inferior colliculus, which were presented at Society for Neuroscience 2008. However, he did not see the final version of the manuscript.

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integration between ascending information from brainstem nuclei and descending information from the forebrain (Winer et al., 1998).

The cat IC is divided into three principal subdivisions: the central nucleus (CN), the lateral cortex (LC), and the dorsal cortex (DC) (Ramón y Cajal, 1909; Berman, 1968; Morest and Oliver, 1984; Oliver, 2005). The CN is part of the lemniscal auditory pathway (Winer et al., 2005), tonotopically organized (Rose et al., 1962; Merzenich et al., 1974; Servière et al., 1984; Schreiner et al., 1997; Malmierca et al., 2008), and essential for hearing (Jenkins et al., 1982). The LC receives auditory input from the cochlear nucleus and the superior olive (Shneiderman and Henkel, 1987; Schofield and Cant, 1992; Schofield and Cant, 1996; Oliver et al., 1997; Oliver et al., 1999; Cant and Benson, 2003; Cant and Benson, 2006; Cant and Benson, 2008), and somatosensory input from the dorsal column and trigeminal spinal nuclei (Aitkin et al., 1978; Aitkin et al., 1981; Zhou et al., 2006). LC neurons are responsive to both acoustic and somatosensory stimulation and have been implicated in multisensory integration (Jain and Shore, 2006). Unlike the CN and LC, the DC receives little or no brainstem input (Irvine, 1986). However, the DC receives heavy descending corticofugal input (Diamond et al., 1969; Coleman and Clerici, 1987; Herbert et al., 1991; Winer et al., 1998). DC cells have heterogeneous response properties, and the role of the DC in hearing is unclear (Aitkin et al., 1975; Aitkin et al., 1994).

The subdivisions of the IC were initially defined neuroanatomically with Golgi-impregnations (Morest and Oliver, 1984; Faye Lund and Osen, 1985) and Nissl-stains (Berman, 1968). However, variations in defining borders that stem from different histological methods have led to different depictions of subdivision boundaries (Paloff et al., 1998; Coote and Rees, 2008). Elucidating the boundaries of IC subdivisions has been a topic of current research (Cant and Benson, 2006; Loftus et al., 2008; Coote and Rees, 2008). Cytochrome oxidase (CO), a metabolic marker, has been recently used to define IC subdivisions in the gerbil (Cant and Benson, 2006), cat (Loftus et al., 2008), and rat (Chernock et al., 2004; Loftus et al., 2008). There is high CO activity in the central area compared to the periphery in the IC (Gonzalez-Lima and Jones, 1994; Cant and Benson, 2005; Cant and Benson 2006), but CO activity in the IC is graded and does not show the presence of distinct internal borders (Cant and Benson, 2006). Studies show that CO activity and vascular density are closely but not entirely correlated with each other (Borowsky and Collins, 1989; Fonta and Imbert, 2002; Riddle et al., 1993; Tuor et al., 1994; Weber et al., 2008). Thus, differences in microvascular architecture between IC subdivisions may lend themselves to showing more distinct profiles of IC subdivisions.

Early studies demonstrated that the microvasculature of cortical and subcortical structures in the central nervous system is not homogenous (Craigie, 1920; Craigie, 1921; Dunning and Wolff, 1937). More recent studies have shown that the microvascular architecture reveals stripes and blobs in the primate visual cortex (Zheng et al., 1991), barrels in the rat somatosensory cortex (Cox et al., 1993; Riddle et al., 1993; Woolsey et al., 1996), laminar differences in the cortex (Zheng et al., 1991; Harrison et al., 2002; Grivas et al., 2003; Masamoto, 2003; Tieman et al., 2004; Michaloudi et al., 2005; Weber et al., 2008), and borders of primary and non-primary cortical regions (Zheng et al., 1991; Tieman et al., 2004). Taken together, these findings support the idea that microvascular density profiles may be applied to reveal functionally separate divisions in subcortical structures like the IC. Previous studies showed regional differences in the microvascular architecture in the rat IC (Gross et al., 1986; Gross et al., 1987; Andrew and Paterson, 1989), but these differences were not compared between subdivisions defined by other histological methods. Here, we first examined whether microvascular density in the cat IC differed between subdivisions. Then we investigated whether capillary distribution changed sharply near subdivision boundaries as defined by Golgi impregnations that would indicate the presence of a border.

Material and Methods

2.1. Perfusion and plastic-embedding

All procedures were carried out in accordance with accepted and approved animal care and use guidelines set by the University of California at Berkeley office of laboratory animal care and The National Institutes of Health. Two female cats were anesthetized with sodium pentobarbital (26 mg/kg intravenously), areflexic, and perfused intracranially with fixative containing 4% paraformaldehyde and 0.25% glutaraldehyde, followed by a wash of phosphate buffered saline (PBS). The brain was cut with a Vibratome (TPI, Inc., St. Louis, MO) in the transverse plane into 200 μ m-thick slabs containing the right IC and collected in 0.12 M phosphate buffer (PB), pH 7.4. The slabs were osmicated with 1% OsO₄ in PB for 50 minutes, dehydrated in a series of ethanol washes followed by propylene oxide (Larue and Winer, 1996), and infiltrated overnight in an airtight chamber in 1:1 propylene oxide and epoxy (Araldite, 6005). The next day, the slabs were transferred to a fresh 100% epoxy mixture for 3 hours and mounted on pre-cast epoxy slides. The slide sandwiches were polymerized at 60°C for 16 hours and cooled. The slabs were removed and each slab was mounted on a plastic dowel. One μ m-thick semithin sections were cut on an ultramicrotome with 8 mm-wide glass knives. The sections were stained with 1% toluidine blue solution in PB and coverslipped.

2.2. Data collection for IC microvasculature

Four IC sections spanning the caudal—rostral extent were analyzed in each case. Subdivisions were drawn based on the Golgi parcellation scheme (Morest and Oliver, 1984; Loftus et al., 2008), and the most rostral section of each case was excluded from further analysis because the three subdivisions were not present at this level along the neuraxis. A computer-driven microscope analysis system (Neurolucida; MBF Bioscience, Williston, VT, USA) was used to plot all cross-sectioned blood vessels circular in appearance with a diameter of $\leq 8 \mu$ m at 400 $\times$ final magnification (Fig. 1). Cross-sectioned blood vessels elliptical in appearance were plotted if their minor-axis diameter was $\leq 8 \mu$ m. These criteria are consistent with past studies (Bär et al., 1972; Mato et al., 1979; Conradi et al., 1980; Michaloudi et al., 2005). Hereafter, cross-sectioned capillary profiles meeting the above criteria are called “capillaries.”

2.3. Data and statistical analysis for IC microvasculature

Each IC section were divided into square bins (200 $\times$ 200 μ m). Capillaries within each bin were counted with Canvas $\times$ (ACD systems, Victoria BC, Canada) to produce an array of numbers representing counts in each bin. The array was formatted into a matrix, and Matlab (Mathworks, Natick, MA) was used to generate a pseudocolor plot of IC capillaries.

We used the nearest neighbor distance (NND) method for all statistical analyses. NND is defined as the distance from one capillary to its nearest neighbor. With the capillary plots of each section, we calculated the distance between each capillary and its nearest neighbor using R (R Development Core Team 2008, Vienna, Austria). Capillary density was inferred from NNDs; that is, areas with lower NND values signified higher capillary density. We determined statistical differences of NND values between subdivisions using the Mann-Whitney test (Table 1) with significance set at the $p \leq 0.05$ level. A non-parametric test was employed because the population of capillary NND values did not fit a normal distribution.

To determine whether capillary distribution can be used to locate borders, we determined the NNDs of all capillaries falling within a ROI that intersected a Golgi-based border perpendicularly (Fig 4). A scatter plot of NND values was produced, and a locally estimated

scatter plot smoothing (LOESS) line was generated. A LOESS line represents the weighted averages of NNDs along the distance of the ROI axis.

3. Results

3.1. Low-power views of vascular architecture

The primary trunks of large vessels enter the IC along its dorsolateral surface (Fig. 2A). Large vessels and brain tissue appear white on the capillary plots (Fig. 2B).

The central region in the IC is markedly denser in capillaries compared to the periphery and this nonhomogeneity is present in each of the four IC sections that were analyzed (Fig. 2B). The pseudocolor plots show bins with darker shades ventrally and lighter shades dorsally that transition in a gradual manner (Fig. 2C). More rostrally along the IC caudorostral axis, the rostral pole (RP) has a capillary density similar to that of the CN (Fig. 2B, C). Extracolicular auditory structures nearby (i.e., the dorsal nucleus of the lateral lemniscus) have low to moderate capillary density (Fig. 2A, B). Nonauditory structures (i.e., the superior colliculus) have comparatively lower capillary density compared to auditory structures (Fig. 2A). There is evidence of CN inhomogeneities. Areas in the CN have patches with higher than average capillary density, particularly near the ventro-medial border (compare Fig. 2B, C).

3.2. Comparison of microvasculature between IC subdivisions

The CN consistently has lower NND values compared to those in the LC and DC (Fig. 3). However, differences between the LC and DC show no consistent pattern. In case 1575, the CN has significantly lower NND values than those of the LC (Mann-Whitney Test, $p \leq 0.05$; Table 1B; Fig. 3). Likewise, NND values in the CN are lower than the values in the DC (Mann-Whitney Test, $p \leq 0.05$). The NND differences between LC and DC are significant in two sections (Mann-Whitney Test, $p \leq 0.05$), but not in one other section (Mann-Whitney Test, $p \geq 0.05$). In case 1231, significant differences in NND values are seen between all subdivisions (Mann-Whitney Test, $p \leq 0.05$).

3.3 Determining the presence of IC borders with NND values

We looked for the presence of a sharp border between the subdivisions using NND values (Fig. 4 A-D). Along the region of interest (ROI) that traverses the CN to DC border, average NND values start from $\sim 30 \mu\text{m}$ at the most ventral region of the CN and gradually increase to $\sim 50 \mu\text{m}$ at the most dorsal region of the DC (Fig. 4 A, B). The LOESS lines do not show any sharp changes to indicate a border between the CN and DC, and the frequency of capillaries in these ROIs also gradually decrease (Fig. 4 A, B).

Along the ROI that spans the LC to DC border, average NND values start from $\sim 40 \mu\text{m}$ in the LC and gradually reach $\sim 50 \mu\text{m}$ (Fig. 4C). Again, the LOESS line does not show any sharp changes to indicate a border between the LC and DC, and the gradual decrease in the frequency of capillaries in this ROI also confirms the lack of sharp borders (Fig. 4C).

In the ROI that spans the CN to LC border, the average NND values start from $\sim 30 \mu\text{m}$, and stay constant (slope of about 0) until reaching the LC region, where the NND values increase gradually to $\sim 45 \mu\text{m}$ (Fig. 4D). The LOESS line does not show a sharp change to indicate the presence of a border between the CN and LC, although the change that is observed is more drastic than the change between the CN to DC and LC to DC borders (compare Figs. 4A-D).

4. Discussion

4.1 Methodological considerations for measuring microvasculature

Blood vessels can be identified unambiguously against the toluidine-blue stained neuropil (Gross et al., 1986; Gross et al., 1987; Andrew and Paterson, 1989; Black et al., 1991). This method has advantages over intracardial perfusion of viscous substances (i.e., India ink in gelatin; Yu et al., 1994; Michaloudi et al., 2003; Michaloudi et al., 2005; Grivas et al., 2003) because it eliminates the potential problem of incomplete perfusion. Further, obtaining the absolute counts of capillaries within these 1 μm -thick sections avoids the need for unbiased random sampling using stereological methods (Weibel, 1979).

We only classified cross-sectioned vessels with diameters $\leq 8 \mu\text{m}$. However, larger-sized vessels (i.e., with diameters greater than $8 \mu\text{m}$) were found to constitute less than 4% of the total vessel population in the CN of 24 day-old rat IC (Andrew and Paterson, 1989). In the developing rat, the left and right CN did not have significant differences in capillary numbers, suggesting that the microvascular plan is similar in both IC hemispheres (Andrew and Paterson, 1989). Assuming cat development is similar to the rat vascular development, our results in the right IC likely reflect the microvascular organization of the left IC as well.

A problem faced in quantifying capillary density was the presence of large “holes” in the Neurolucida capillary plots that did not represent brain tissue but large blood vessels. Calculating capillary density strictly as the number of capillaries/brain tissue area led to an underestimation of capillary density in areas with many large blood vessels. Thus, we employed the NND method to attenuate the effects of these gaps in the capillary plots.

4.2 Functional implications for microvascular organization in the IC

Capillary density has been shown to correlate well with metabolic activity (Gross et al., 1987; Borowsky and Collins, 1989; Tuor et al., 1994). Since the CN has the highest capillary density, it is not surprising that the CN also stains most intensely for CO (Loftus et al., 2008). Thus, differences in microvasculature between the IC subdivisions also parallel differences in metabolic activities. The highest microvascular density in the CN implies that the lemniscal or primary auditory pathway presumably is the most metabolically active. Whether this metabolic and vascular organization is conserved in downstream and upstream auditory structures still remains to be known. However, evidence suggests that in the medial geniculate body (MG), a structure one synaptic terminus upstream from the IC, the lemniscal division does not always have the highest microvascular density compared to the non-lemniscal divisions (Song and Winer, 2009).

We showed that the IC microvascular architecture does not show any definite borders of subdivisions. In fact, the microvascular density patterns change gradually between the CN, LC, and DC, which is consistent with the pattern observed in the IC with CO (Cant and Benson, 2006). Qualitatively, microvasculature aligns better with the Golgi-based parcellation scheme (Morest and Oliver, 1984) than the Nissl-based scheme (Berman, 1968) because the border between the CN to DC as seen through microvasculature does not extend as dorsally as the CN to DC border in a Nissl-based parcellation scheme. This is consistent with the finding that capillary density is not correlated with cell density (Baborie and Kuschinsky, 2006).

The RP is a nucleus of the IC that has different ascending projections (Harting and Van Lieshout, 2000) and descending corticofugal connections (Winer et al., 1998) compared to other subdivisions in the IC. In addition, neurons in the RP do not seem to respond to sound stimuli (Oliver, 2005). Although the RP differs connectionally and physiologically from the

three main subdivisions in the IC, based on microvasculature, the RP has similar capillary density than that of the CN (Fig. 2).

4.3 Capillary density and implications for functional magnetic resonance imaging (fMRI)

Functional MRI relies on the blood-oxygen-level-dependent (BOLD) signal to indirectly measure neural activity and requires a close coupling between neural activity and the hemodynamic response. The BOLD signal is derived from local changes in cerebral blood flow (CBF), cerebral blood volume, and oxygenation that change in response to neural activity. An increase in CBF in an active area is the major component in determining a positive BOLD response (Buxton and Frank, 1997), and the main factor in determining the specificity of the signal is the brain's microvascular architecture and density (Logothetis, 2008). Currently, the spatial resolution in animal fMRI studies can be as high as $250 \times 250 \mu\text{m}^2$ with a slice thickness of 2 mm (Goense et al., 2007). While this resolution is useful for studying neural activity in cortical lamina and orientation columns, it is not high enough to study IC subdivision micromodules like the CN fibrodendritic lamina that have each a thickness of 150 to 170 μm (Oliver, 2005). Moreover, spatial resolution in fMRI is unlikely to substantially increase since smaller voxel sizes significantly decrease signal-to-noise and contrast-to-noise ratios (Logothetis, 2008). Thus, fMRI can be useful in studying IC subdivisions as a whole, but its utility may be limited when studying neural activity within subdivisions.

An assumption in BOLD imaging studies is that changes in the BOLD signal are linearly related to neural activity. However, a more recent study showed with combined Laser Doppler Flowmetry and electrophysiological recordings that CBF and neural activity have a nonlinear relationship: large differences in CBF may not necessarily equate to large differences in neural activity (Jones et al., 2004). These findings support studies that found CBF disproportionately “floods” active areas of cortex (Malonek and Grinvald, 1996; Nemoto et al., 1999; Shtoyerman et al., 2000), as if “watering the entire garden for the sake of one thirsty flower” (Malonek and Grinvald, 1996). Interestingly, layer IV in the cortex exhibits the greatest increases in CBF (Gerrits et al., 2000), and contains the highest density of capillaries (Harrison et al., 2002; Weber et al., 2008). These findings suggest that capillary density may play a role in the nonlinear relationship between CBF (by inference the BOLD signal) and neural activity.

We find here that the CN has higher capillary density than the LC and DC. In a situation where each IC subdivision displays similar levels of neural activity, the CN may have a lower threshold for inducing the same BOLD response compared to the LC or DC by virtue of its higher structural capacity to induce more blood flow. Understanding the exact relationship between neural activity and the hemodynamic response is essential for functional brain imaging studies. Further studies need to be done to unveil the role of microvasculature in this intricate interplay.

Acknowledgments

We thank Jason Chung for his help with plotting blood vessels, David Larue and Katie Smith for technical assistance, and Karl Rohe for statistical assistance. We give special thanks to Dr. Nell Cant and Dr. Doug Oliver whose detailed and thoughtful comments refined the presentation of this study. Supported by USPHS grant R01DC02319-29.

Abbreviations

BIC brachium of the inferior colliculus

BICN	brachium of the inferior colliculus, nucleus
BOLD	blood-oxygen-level-dependent
CBF	cerebral blood flow
cic	commissure of the inferior colliculus
CN	central nucleus
CO	cytochrome oxidase
DC	dorsal cortex
dnll	dorsal nucleus of the lateral lemniscus
IC	inferior colliculus
LC	lateral cortex
LOESS	locally estimated scatterplot smoothing
MG	medial geniculate body
NND	nearest neighbor distance
pag	periaqueductal gray
RP	rostral pole
ROI	region of interest
SC	superior colliculus
VLN	ventrolateral nucleus of the lateral cortex

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Fig. 1. Photomicrograph of a 1 μm -thick semithin section through the IC stained with Toluidine-blue at 400 $\times$ magnification. Capillary profiles are marked with an asterisk and those with red blood cells fixed inside the lumen are circled. Several cell bodies are indicated by arrows. Scale bar represents 10 μm .

Fig. 2.

(A) Photomicrographs of case 1575. Four 1 µm-thick IC sections stained with toluidine-blue are shown from caudal to rostral. The number next to each section refers to the percent distance rostral from the caudal tip of the IC. Several primary trunks of large vessels entering the IC are marked with an asterisk. (B) Neurolucida plots of all capillary profiles of sections in (A). Several large blood vessels are marked with an asterisk. (C) Pseudocolor plots of all capillary profiles in (A). Each bin represents the number of capillaries in a 200×200 µm² area. Scale bar represents 1 mm.

Fig. 3.

(A) Bar plots of means and standard errors of CN, LC, and DC NNDs for case 1575. The x-axis refers to the 0.25, 0.50 and 0.65 levels through the IC in case 1575 (Fig. 2). The most rostral section was excluded because the three subdivisions were not present at that level. n.s. = not significant. (B) Bar plots of means and standard errors of CN, LC, and DC NNDs for case 1231. The x-axis refers to the 0.27, 0.40 and 0.63 levels through the IC in case 1231. Asterisks denote significant differences.

Fig. 4.

Determination of IC borders using the 0.50 level-section in case 1575. **(A)** Capillaries in the rectangular ROI were analyzed. The CN to DC border is indicated by a bold line. The NNDs of capillaries within the ROI were calculated and a scatter plot with a LOESS line is shown. The y-axis refers to the NNDs in micrometers of all the capillaries within the ROI and the x-axis refers to the distance in millimeters along the ROI axis. The histogram shows the frequency of capillaries within the ROI in the y-axis and the distance in millimeters along the ROI axis in the x-axis. **(B)** Capillaries in the rectangular ROI were analyzed. The CN to DC border is bolded. Convention is the same as in (A). **(C)** Capillaries in the rectangular ROI were analyzed. The LC to DC border is bolded. Convention is the same as in (A). **(D)** Capillaries in the rectangular ROI were analyzed. The CN to LC border is bolded. Convention is the same as in (A).

Table 1

(A) Mean, median, and range of CN, LC, and DC NNDs in μm for case 1575 and case 1231. The numbers on the left column refer to specific sections. The most rostral section in each case was excluded because the three subdivisions were not present at that level. (B) Results for non-parametric Mann-Whitney Test comparing differences in NNDs between subdivisions for case 1575 and 1231. Significant differences (shaded gray) was set at the $p \leq 0.05$ level.

A	Case 1575		Mean $\pm$ SD	Median	Range	
					Min	Max
0.25		CN	31.3 $\pm$ 10.7	30.8	Min: 6.1,	Max: 134.3
		LC	36.8 $\pm$ 14.1	35.7	Min: 6.9,	Max: 94.9
		DC	35.5 $\pm$ 12.2	34.5	Min: 5.1,	Max: 124.4
0.50		CN	30.0 $\pm$ 9.4	29.4	Min: 5.4,	Max: 83.6
		LC	36.2 $\pm$ 14.2	34.5	Min: 6.3,	Max: 93.3
		DC	36.9 $\pm$ 13.9	35.4	Min: 6.5,	Max: 124.1
0.65		CN	30.1 $\pm$ 9.5	29.6	Min: 8.9,	Max: 96.5
		LC	38.8 $\pm$ 14.0	37.3	Min: 10.9,	Max: 98.5
		DC	37.5 $\pm$ 14.9	35.8	Min: 4.3,	Max: 155.9
	Case 1231		Mean $\pm$ SD	Median	Range	
					Min	Max
0.27		CN	28.5 $\pm$ 8.7	28.1	Min: 7.2,	Max: 83.4
		LC	36.1 $\pm$ 13.6	34.7	Min: 6.6,	Max: 81.9
		DC	34.9 $\pm$ 13.5	33.1	Min: 5.6,	Max: 129.7
0.40		CN	28.7 $\pm$ 8.7	28.4	Min: 7.2,	Max: 109.5
		LC	37.9 $\pm$ 14.5	36.5	Min: 10.8,	Max: 135.3
		DC	34.3 $\pm$ 12.5	32.7	Min: 7.9,	Max: 116.4
0.63		CN	26.6 $\pm$ 7.9	26.1	Min: 6.3,	Max: 68.4
		LC	29.1 $\pm$ 11.8	27.5	Min: 7.1,	Max: 136.6
		DC	31.9 $\pm$ 11.2	30.7	Min: 2.7,	Max: 102.3
B	Case 1575		0.50		0.65	
	CN/LC	Significant ($p = 3.7 \times 10^{-8}$)	Significant ($p < 2.2 \times 10^{-16}$)		Significant ($p < 2.2 \times 10^{-16}$)	
	CN/DC	Significant ($p < 2.2 \times 10^{-16}$)	Significant ($p < 2.2 \times 10^{-16}$)		Significant ($p < 2.2 \times 10^{-16}$)	
	LC/DC	Significant ($p = 0.050$)	Not Significant ($p = 0.064$)		Significant ($p = 0.00060$)	

A	Case 1575		Mean $\pm$ SO	Median	Range
	Case 1231	0.27	0.40		0.63
	CN/LC	Significant ($p < 2.2 \times 10^{-16}$)	Significant ($p < 2.2 \times 10^{-16}$)		Significant ($p = 9.69 \times 10^{-11}$)
	CN/DC	Significant ($p < 2.2 \times 10^{-16}$)	Significant ($p < 2.2 \times 10^{-16}$)		Significant ($p < 2.2 \times 10^{-16}$)
	LC/DC	Significant ($p = 0.0091$)	Significant ($p = 4.19 \times 10^{-12}$)		Significant ($p < 2.2 \times 10^{-16}$)