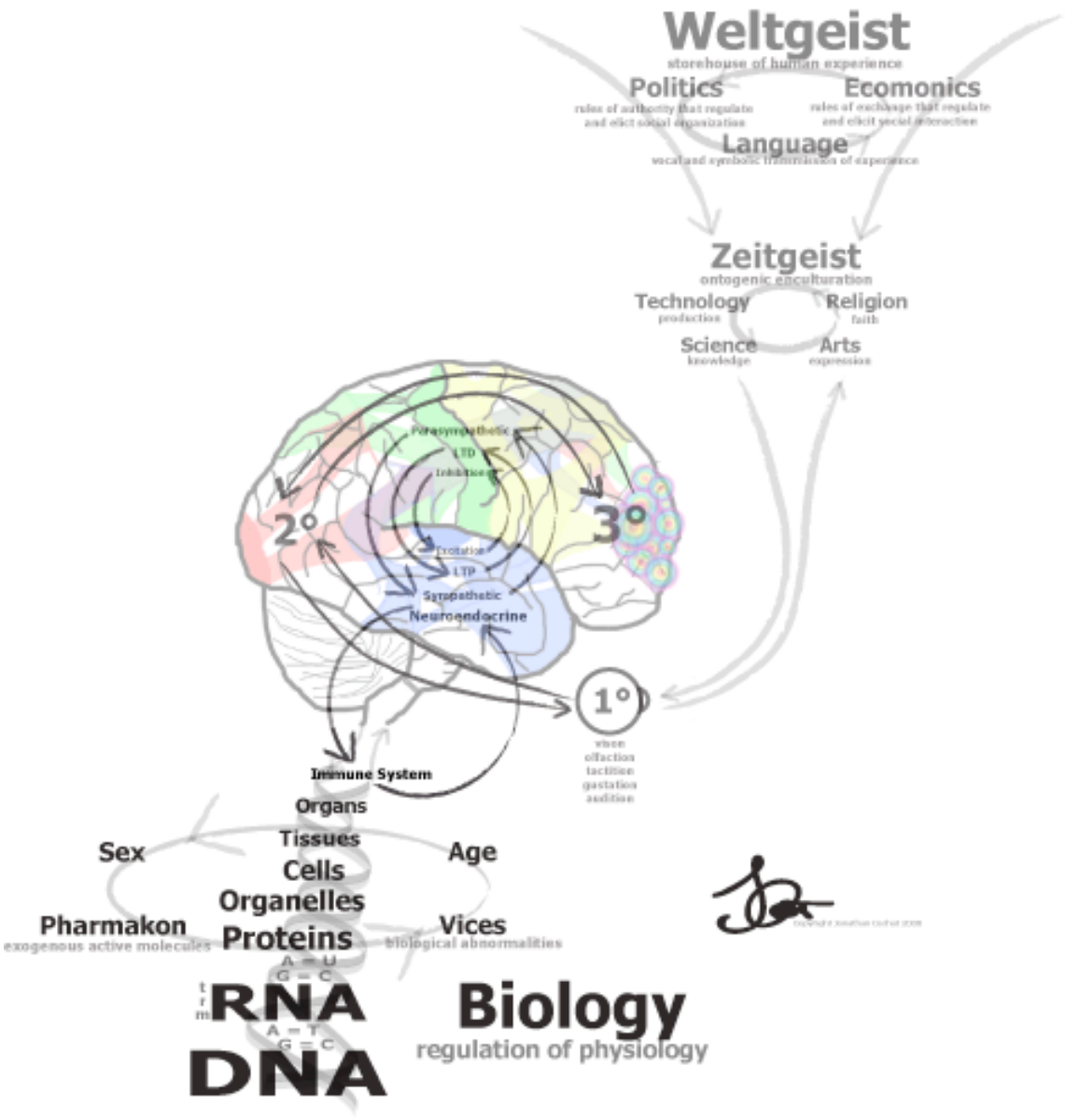
From Genes to Cognition to Social Behavior – a DNA Microarray Research Proposal



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Abstract

A difference in active gene expression in cerebrocortical tissue between humans and non-human primates has been revealed recently with a novel experimental technique – the DNA microarray. Researchers in this field have interpreted their results to suggest that human brain evolution has resulted in an upregulation of gene expression coding proteins functional in synaptic growth, transmission and maintenance, in addition, to facilitating cellular metabolism.

This proposal is to further explore these recent results, while including methodological revisions designed to reduce the potential for uncorrelated interference. Secondly, we hypothesize that a growth in the complexity of social dynamics, especially pronounced in chimpanzees, bonobos and humans, was a major force across the evolutionary changes in primate brain anatomy and physiology. To test this hypothesis, we would like to use a DNA microarray to probe for social and/or cultural correlations in cerebrocortical gene expression between primates of divergent social niches or habitats.

Together, these experiments could solidify an empirical bridge across multiple levels of analysis, linking metaphysical social structures to the regulation of genetic expression, while also elucidating the principles and mechanisms behind the evolution and physiology of the most complex biological machine on earth, the human brain.

Statement of Problem

Specific Aim #1: Replication of recent DNA microarray experiments to confirm upregulation of genes involved with synaptic growth, transmission, maintenance and metabolism and the development of new, non-human primate DNA microarrays in order reduce uncontrolled variability in current experimental designs.

Specific Aim #2: Examine for a link between metaphysical social behaviors and cerebrocortical gene expression.

Introduction

The human brain (*Homo sapiens*), specifically the cerebral cortex, is the most intricate and complex matrix of neuronal communication in existence on this planet. Advances in experimental techniques and research methodologies within the toolbox of the natural sciences have provided significant advances towards understanding its evolution and the selective pressures therein. Specifically, the recent developments in DNA microarray technology have allowed researchers to examine the transient regulation of gene expression of brain tissue both within and between animal species.

DNA microarray plates contain up to 10,000 different, but known gene probes that hybridize or bind with sequences of mRNA (a protein), extracted from a sample tissue. This type of 'shotgun' technique allows for the collection of information rich data sets in a relatively short amount of time. Recent DNA microarray studies have unveiled dimorphisms at the molecular level of analysis in human and chimpanzee brain physiology, furthering an understanding of the neuroscience of cognition and behavior and brain evolution.
Although the human brain around 4x larger than that of our closest evolutionary relatives – the chimpanzees (Pan troglodytes) and bonobos (Pan paniscus) – our brain

surprisingly is comprised of approximately only 50% more neurons (Preuss et al. 2004, 858). The cerebral cortex, or neocortex, is the outermost layer of the brain (its 'bark'), shown in the second row of Figure 1, stained purple. At first glance there appears to be little difference between the chimpanzee and human cerebral cortex other than overall size.

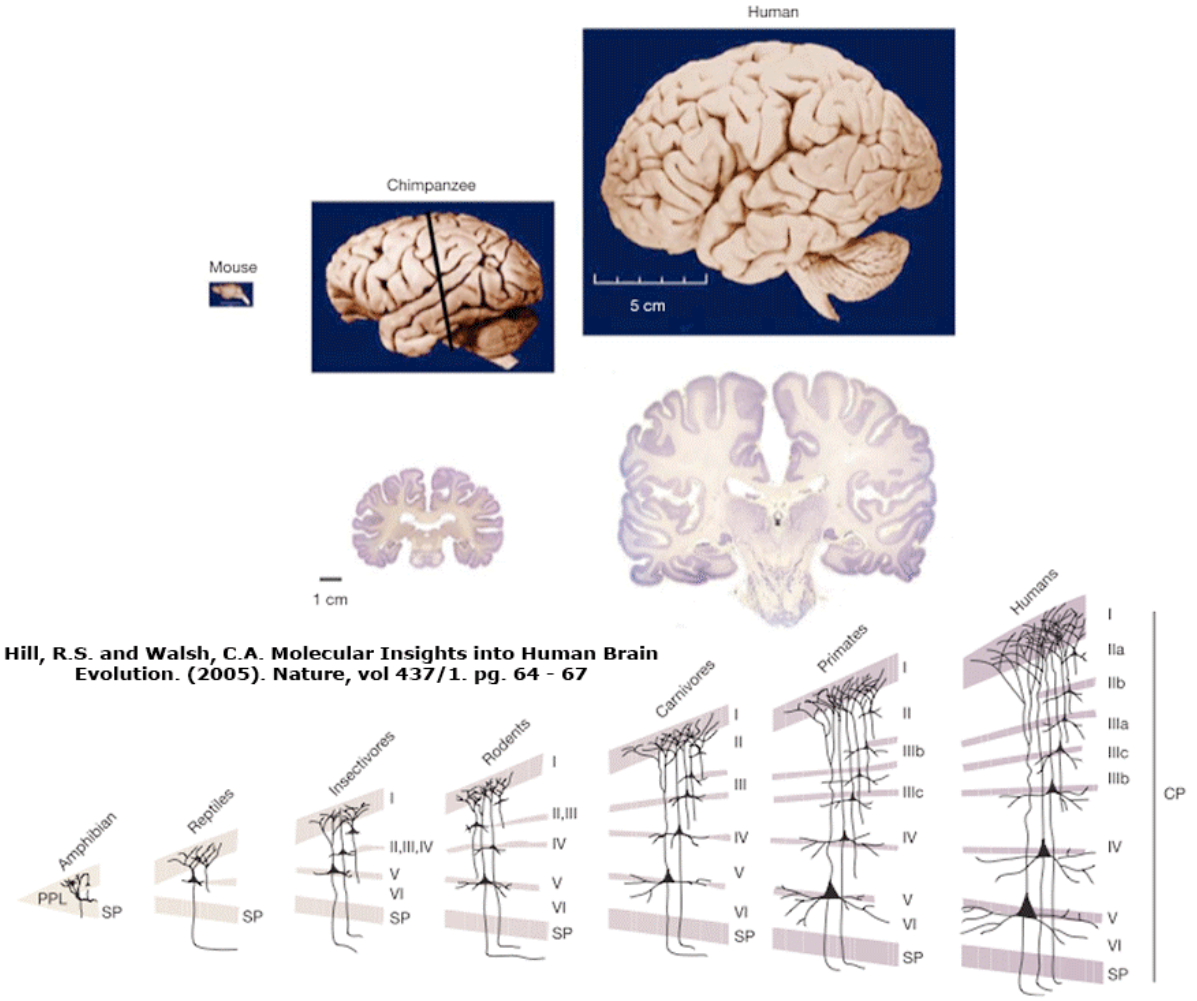


Figure 1 | Differences in cerebral cortical size are associated with differences in the cerebral cortex circuit diagram. The top panel shows side views of the brain of a rodent (mouse), a chimpanzee and a human to show relative sizes. The middle panel shows a cross-section of a human and chimpanzee brain, with the cellular composition of the cortex illustrated in the bottom panel (adapted from ref. 5). The cerebral cortex derives from two developmental cell populations: the primordial plexiform layer (PPL) and the cortical plate (CP). The primordial plexiform layer seems to be homologous to simple cortical structures in Amphibia and Reptilia, and appears first temporally during mammalian brain development. The cortical [illegible] layer into two layers (layer I at the top and the subplate (SP) at the bottom; numbering follows the scheme of ref. 31). Cortical-plate-derived cortical layers are added developmentally from deeper first (VI, V) to more superficial (III, II) last. Cortical-plate-derived cortical layers are progressively elaborated in mammals with larger brains (for example, insectivores have a single layer II/III/IV that is progressively subdivided into II, III, IV, then IIa, IIb, and so on), so that humans have a larger proportion of these late-derived neurons, which project locally or elsewhere within the cortex. Images from the top and middle panels are from the Comparative Brain Atlas (http://www.brainmuseum.org).

However, at the molecular level the differences are perplexing (Figure 1, third row). The cerebral cortex is patterned in layers of unique neurons and it appears that the connections and collisions within this experiential matrix of signal transduction reflect the physiology of human's uniquely elaborated cognitive abilities such as language, morality, social cognition and consciousness.

Together, current neuroscientific research is beginning to strongly suggest that an increase in the complexity and magnitude of synaptic connections between neurons within the cerebrocortical layers was at the foundation of hominoid brain evolution. The

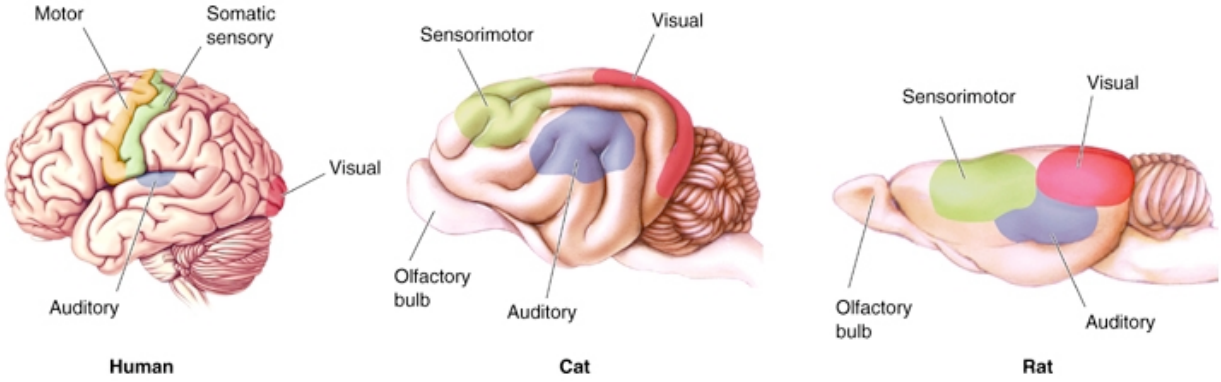
only question which remains is – What external pressures favored such a change in brain physiology?

In order to understand the evolutionary forces selecting for such a change, it is often beneficial to examine an organism's ontogeny, or their developmental biology across the lifespan.

Neocortical Arealization and Plasticity

Regions of the cerebral cortex, across all of the brains lobes, can be distinguished by functional and structural differences. The cell types and cellular organization within a tissue sample characterize the structural profile of a given region, referred to as its architectonics. More often than not, the architectonics of a region determines its functionality – a primary tenet of anatomy and physiology – 'structure reflects function'.

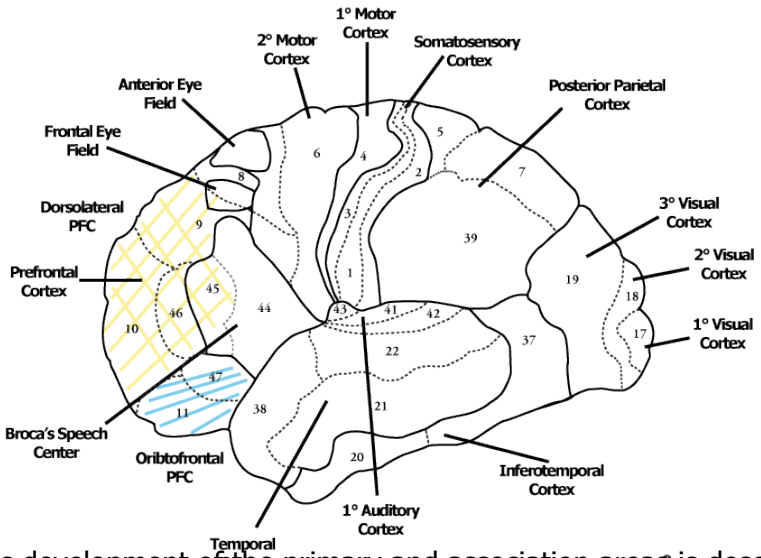
The regions of the cerebral cortex specialized to receive information from the external world through the organism's sensory organs are classified as primary sensory areas (highlighted with color in the illustration below).



Immediately, it is evident that in terms of cortical surface volume, the human brain devotes much less surface area to these primary sensory areas than other mammals.

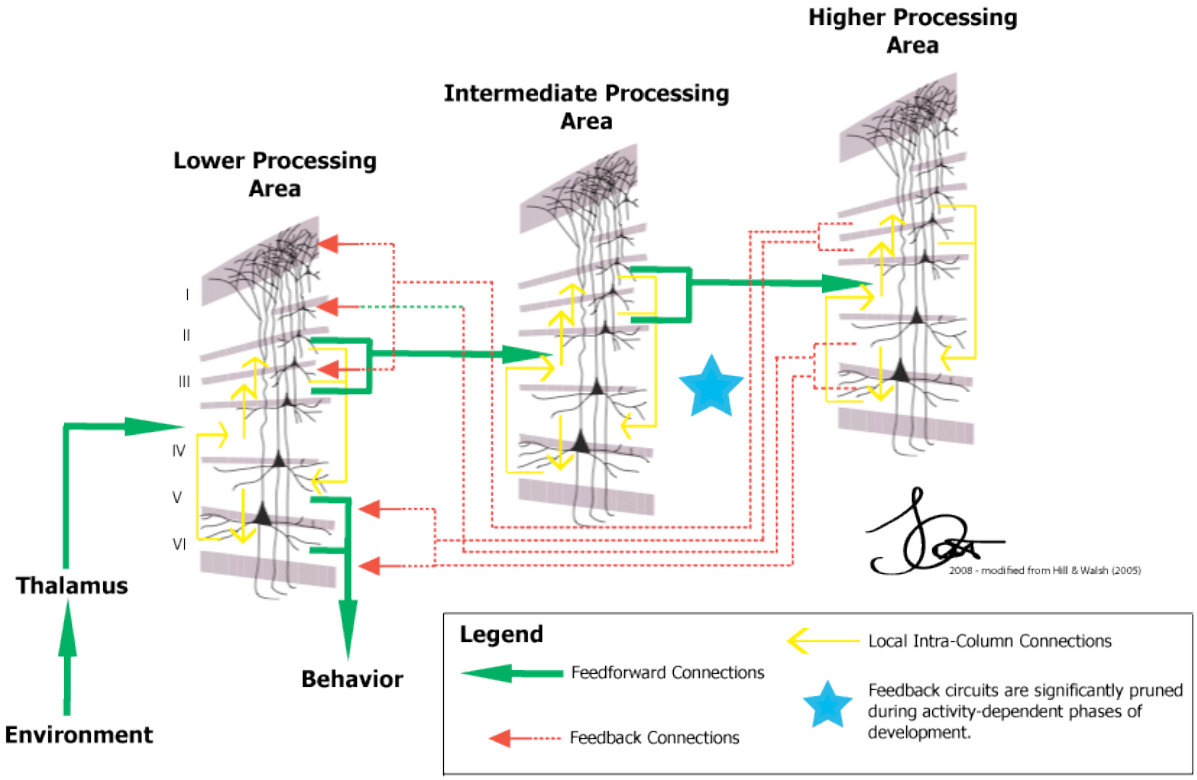
After sensory information is received in the primary areas, higher level processing occurs as the information diffuses out of the primary sensory areas into association areas eventually developing a conscious perception. The critical surface of the human brain, therefore, devotes much more surface area to information processing and integration within these association areas, rather than strike sensation.

Classically discovered by Korbinian Brodmann in 1909, there are number of architectonically distinct regions within the association areas of the primate brain and slight variations are being discovered each and every day (shown below).



The ontogenetic development of the primary and association areas is described as a process of <u>arealization</u>, and is currently best understood in the primary visual cortex of various mammals.

In mammals, the neocortex consists of six layers each containing a characteristic distribution of neuronal cell types and connections with other cortical and subcortical regions. The cortical layers of the human brain are extensively developed and the neurons within are connected within an almost unimaginably complex matrix of signal transduction (show below).



During embryonic maturation, this laminar patterning is developed from the bottom up – progenitor cells that leave the cell cycle early form the deepest layers and cells born later progressively construct the superficial layers (Sur & Leamey 2001, 251).

The overall scaffolding of the cerebral cortex is regulated by intrinsic determinants (i.e. molecular and genetic) within the proliferative zones of the developing cortex. Once this foundational structure has been developed arealization occurs through extrinsic manipulation of these intrinsic factors. Specifically, the spatiotemporal patterns of sensory input relayed across thalamic afferent neurons are the primary determinate of cortical arealization in almost all examined mammals (Krubitzer & Huffman 2000, 327).

The role of thalamic innervation in cortical arealization is strikingly evident in experiments that reroute the afferent relay connections in the thalamus to a different region of the cerebral cortex. In such experimental designs, retinal axons that normally innervate, or communicate with regions of the primary visual cortex (V1) are physically repositioned to terminate in, what is normally, the primary auditory cortex (A1) on the temporal lobe. If this manipulation occurs early enough in development, the spatiotemporal pattern of visual afferent signaling causes A1 to architectonically develop in such a way that it is quantitatively indistinguishable from typical V1 cytoarchitecture (Roe, Pallas, Kwon & Sur 1992). In other words, the manipulation of sensory input from auditory to visual creates a functional visual cortex where the primary auditory cortex ought to be. In fact, Von Melchner et al., (2000) provides evidence that the rewired organisms actually interpret visual stimuli that activate the rewired projections as visual rather than as auditory. The notion of arealization, supported by experiments such as these, illustrate another fundamental principle of cerebral cortex physiology – plasticity.

Plasticity refers to the ability of cortical networks to adapt or change their response to fluctuations in their input, suggesting that an organism's external environment and subjective experiences have a significant influence the internal regulation of their biology, including their cortical networks. There are numerous experimental designs that illustrate this notion of plasticity. Rats raised in environmentally enriched housing (enhanced sensory, cognitive and motor stimulation) display a variety of cellular, molecular and behavioral differences when compared with rats who were raised in a typical (cage, bedding, lab storage room) or deprived (stressful, dark) environment.

At the genetic level, these cellular changes are mediated by a transient manipulation of genetic expression regulation. Specifically, the input manipulates the expression of proteins involved in synaptic function, signal transduction and cellular plasticity. At the cellular level, these environmentally favorable conditions increase dendritic branching and length, the number of dendritic spines and the size of synapses on some neuronal populations. Furthermore, environmental enrichment has been linked to increases in hippocampal neurogenesis and the integration of these newly born cells into functional circuits (Nithianantharajah & Hannan 2006, 699). The hippocampus is a region of the brain known to play a major role in memory formation and recollection. As such, this environmental enrichment is found to enhance learning and memory, reduce memory decline in aged animals, decrease anxiety and increase exploratory activity in experimental rats (Nithianantharajah & Hannan 2006, 699).

Moving beyond rat models, there are a few clever experimental designs in which plasticity is observed in adult humans. Elbert, Pantev, Wienbruch, Rockstroh and Taub (1995) report that the somatosensory cortex of adults with, as opposed to those without, extended experience playing a stringed instrument (i.e. violin) are significantly different. The areas of the somatosensory cortex representing the fingers on the left hand (used to play) was found to be larger than that of the right fingers, in addition to being larger than the corresponding area in nonmusicians altogether (Nelson & Bloom 1997, 982). Moreover, there is substantial evidence for cortical reorganization following peripheral nervous system injury in adult humans. Somatosensory areas dedicated to the sensation of a amputated limb are subsumed by adjacent cortical neurons enhancing tactile sensation of remaining organs (Ramachandran, 1995).

In summary, the cerebral cortex is arealized into structurally and functionally distinct regions that are ontogenetically developed according to intrinsic and extrinsic influences. However, even after the critical time period of arealization has passed the cerebrocortical networks are plastic, having the ability to adapt in response to changes in the external environment.

Human Brain Evolution and DNA Microarrays

The similarities in brain anatomy and physiology across mammals advocate the theory that neocortical evolution most likely occurred through an elaboration of the biological mechanisms that regulate brain growth and development. Genomic sequence analysis of chimpanzee and human DNA suggests that the inactivation of some genes has occurred (olfactory receptor genes) but that to the addition of novel genes into the genome was not a major force in primate brain evolution (Hill & Walsh 2005, 65).

We can now determine such evolutionary changes by analyzing differences in active gene expression between human and primate brain tissue with a DNA microarray. This experimental technique is extremely useful in that it provides large amounts of expression data as well as the ability to identify entire regulatory networks that drive evolutionary changes in gene expression (Preuss et al. 2004). Caceres et al., (2003) have demonstrated a significant upregulation of gene expression in human cerebrocortical tissue when compared to the cortical tissue of chimpanzees and rhesus macaques (Caceres et al. 2003). Moreover, the genes expressed at higher levels are now implicated to function in glutamatergic excitability (SYN4), plasticity at glutamatergic synapses (CAMK2A), phosphatidylinositol signaling (IMPA1, CDS2), synaptic vesicle release (RAB3GAP, ATP2B1), axonal transport along microtubules (KIF3A, DCTN1), microtubule assembly (MAP1B), and targeting of proteins to postsynaptic densities (USP14) in addition to a number of genes involved with metabolic regulation (Caceres et al. 2003, 13034). Independent studies by Enard et al. (2002), Karaman et al (2003) and Gu & Gu (2003) have confirmed these findings.

Proposed Study #1 – Human Brain Evolution

The upregulation of genes involved with synaptic communication and cellular metabolism reinforce the proposed theory of human brain evolution which emphasizes an increase in the complexity and responsiveness of the cerebrocortical networks to the external world. The primary aim of this research is to replicate previous DNA microarray studies, which the hopes of confirming or negating their results. However, to alleviate

known complications with contemporary DNA microarray experiments this proposal includes slight methodological changes.

The genomic sequences between humans and chimpanzees differ, on average, by 1%-4%. Consequently, the affinity of chimpanzee mRNA to probes made of human DNA is antagonized and can result in false indications on down-regulation simply because the molecular are incapable of binding (Preuss et al. 2004, 852). This non-specific hybridization has been controlled for by statistically normalizing its probability during data analysis; however, our proposal is to include species-specific DNA probes for each microarray.

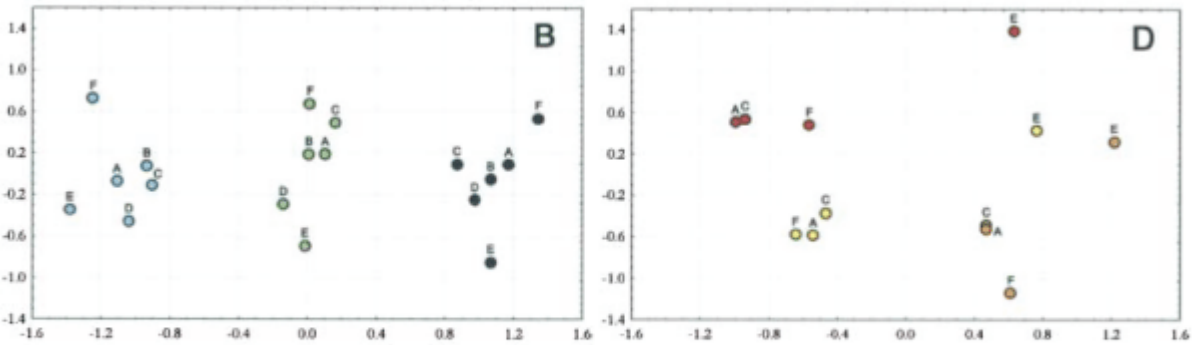
A second caveat while interpreting the results of DNA microarrays is that a species-unique characteristic could have evolved after the two species diverged (Preuss et al. 2004, 852). For example, a gene that is expressed at a significantly greater level in humans than chimpanzees could have been upregulated within the human phylogeny or even downregulated within the chimpanzee phylogeny after speciation occurred.

Lastly, the difficulty of obtaining tissue samples from Great Apes has resulted in most studies including Old World monkeys (i.e. macaques or rhesus monkeys). This research would like to specifically compare and contrast active gene expression in the cerebrocortical tissue of humans (*Homo sapiens*), chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*). Although the branches of phylogenetic relationships are actively debated, it is believed that *Homo sapiens* and *Pan troglodytes* shared a common ancestor around 5 - 7 million years ago, after which *Pan troglodytes* and *Pan paniscus* diverged around 1.5 - 3.0 million years ago (Blount 1990, 703). Genetically, bonobos are just as related to humans as chimpanzees, if not more so. Behaviorally, however, humans, chimpanzees and bonobos are very similar and, effectively, only differ in the terms of politics (social dynamics) and mating behaviors.

Proposed Study #2 – The Social Brain

A DNA microarray analysis by Khaitovich et al. (2004) demonstrated that gene expression levels in the cerebral cortex, caudate nucleus and cerebellum differ to a greater degree within respective species (human-human, chimpanzee-chimpanzee), rather than between species (humans-chimpanzees) (Khaitovich et al. 2004, 1468). Furthermore, the differences between individual humans was pronounced to such a degree that they exceeded human-chimp differences, while also grouping in a manner that allowed researchers to predict which human individual the tissue sample came from. In other words, the data obtained from chimpanzees clustered around which area of the brain the tissue originated, while the human data organized according to the individual from which the sample was obtained. The data plot from this study is given below;

Multidimensional scaling plots of gene expression differences identified within Humans and Chimpanzees
(Khaitovich et al., 2004, pg. 1464)



Plot B - Expression differences within the human cerebral cortex.
Plot D - Expression differences within the chimpanzee cerebral cortex.

Colors refer to individuals. (A) Broca's area; (B) homolog of Broca's area in the right hemisphere; (C) prefrontal cortex; (D) premotor cortex; (E) primary visual cortex; (F) anterior cingulate cortex.

Khaitovich et al., (2004) suggest this pronounced individualization in humans is a product of the human cerebral cortex being "more influenced by environmental and physiological conditions than the other brain regions and that the humans differ more than the chimpanzees in living conditions" (Khaitovich et al., 2004, 1468). In chimpanzees, bonobos and, to the greatest extent, in humans these external living conditions are dominated by social dynamics and interactions commonly referred to in humans as culture or society.

It appears that the primary adaptive value of ontogenetic arealization and developmental plasticity is the ability to utilize variable sensory information patterns that help the individual navigate their external world successfully. Within primate evolution and especially for humans, a primary source of this information is found within the complex arrangements of social organization and exchange (Geary and Huffman 2002, 681). Therefore, it seems plausible that a direct correlation between social complexity and cortical complex exists, and more so, could have been a driving force in human brain evolution.

Correlations with the cerebrocortical physiology and social behavior have been implicated across academic disciplines. To evolutionary biology, relative brain size, in both birds and mammals, is significantly associated with the attributes of each species' social dynamics. In primates, specifically, a larger brain size is precisely linked to increasing complex social dynamics measured by solitary, pairbonded, harem and multimale mating systems (Shultz & Dunbar 2007, 2429). Within social psychology, cross-cultural comparisons have revealed variable cognitive dispositions, or habits of thought, associated with a region's sociohistorical inheritance and the situated individual's ontogenetic maturation. For example, East Asians observably tend to be more holistic, directing attention and assigning causality to an entire field with little reliance on categories or formal logic, whereas Westerners tend to be more analytic, focusing attention to objects with their experiential field and using categorical knowledge, formal logic and rule to understand its behavior (Nisbett et al. 2001, 291).

By matching social variability (i.e. age, sex, socioeconomic status, hormones) between representative population samples from different localities, this proposal would like to explore any correspondence between the regulation of cortical gene expression and metaphysical social structures.

Conclusively, the utilization of DNA microarrays to probe phylogenetic and cross-cultural diversity in cerebrocortical gene expression purposely allows our research proposal to address the most fundamental aspects of human brain evolution and physiology. The empirical reduction of conscious processes to the physiology of cerebrocortical circuitry has been understandably difficult due to the inconceivable complexity of the synaptic networks contained within. However, a DNA microarray analysis of this nature could provide a window into the physiology of subjective human cognition. Furthering this understanding is not only intellectually valuable, but also has the potential to direct future biomedical research aimed at the treatment and mitigation of neurodegenerative diseases (Alzheimer's, Parkinson's etc)

Research Design and Methods

Specific Aim #1: Replication of recent DNA microarray experiments to confirm upregulation of genes involved with synaptic growth, transmission, maintenance and metabolism and the development of new, non-human primate DNA microarrays in order reduce uncontrolled variability in current experimental designs.

The objective of this experiment is to develop an DNA microarray with non-human primate oligonucleotide probes in order to improve assay results; Specifically to develop microarray probes for Chimpanzee and Bonobo genes. Once this is completed, the objective is to then replicate previous microarray experiments between humans and non-human primates, in order to validate or negate previous results and also gauge the benefits in the newly developed arrays.

The development of the Chimpanzee and Bonobos microarrays will be completed using the sequenced genomes of each species, respectively. The genomes will be fragmented into the individual probe sequences and hybridized to the chip surfaces. After the probes have been designed, the actual construction of the array chip will be contracted to an outside vendor (i.e. Affymetrix3). Internal control probes will also be included on each chip to verify that hybridization is successful and that equal amounts of cDNA are loaded with each sample.

Replication of past microarray studies will allow us to determine whether or not the newly developed arrays provide more accurate expression data for non-human primates (Chimpanzees and Bonobos). This process begins with the isolation of cerebrocortical tissue from multiple areas of the Human, Chimpanzee and Bonobo brain. The areas of interest include the frontal pole (FP), medial frontal gyrus (MFG), inferior parietal lobule (IPL), anterior inferotemporal cortex (ALT), superior temporal gyrus (STG), and temporal pole (TP). Each of these regions have been well characterized by previous microarray studies (Caceres et al., (2003) and Preuess et al., 2004).

Both male and female test subjects will be used to examine for differences in cortical gene expression as a result of biological sex, or fluctuating hormone levels.

It terms of age, it would be optimal of human samples ranged from 18 to 25 years old and the primates around 10 to 12 years old. Although this age range is somewhat

arbitrary, the important parameter is that the cortex of the subject has reached maturation.

Total RNA will be isolated from the tissue samples and purified based on protocols given in Whiesnant (2008). Double stranded cDNA will be synthesized based on Invitrogen (2008) and then hybridized onto the array using the protocol available in Affymetrix (2006). The expression levels will then be assessed and statistically analyzed.

By comparing our data with that from previous microarray studies, we will be able to gauge the improvements we expect to see with our newly developed arrays. However, a few additional steps are needed to further validate that the non-human primate arrays will give more accurate expression data than the previous studies gave. Although we are very confident that our non-human primate arrays will be able to eliminate the errors caused by sequence differences, Western Blot analyses will allow us to validate this expectation.

A few, well-characterized genes associated with neuron development and cell metabolism within the cortex (D1x1 and ACADSB) are expected to be actively regulated in humans, Chimpanzees and Bonobos at basal levels (Caceres et al., (2003) & Preuess et al., (2004). A western blot will allow us to determine the relative level of these proteins in each tissue sample. It is expected that the expression levels observed in the western blot would be very similar to that observed in the microarray.

The process of a western blot begins by extracting proteins from Human, Chimpanzee and Bonobos cortical tissues and running them on an electrophoresis gel. Once the migration is complete, the proteins are transferred onto a membrane and probed with the appropriate antibodies. Antibodies, such as GAP-DH, will be included on the blots to control for possible error in sample loading. These results will allow us to further validate the expected improvements with the non-human primate arrays. For example, if the level of protein expression for Chimpanzee and Bonobo samples better correlates with the expression levels from either the past study array data or our human array data, then we will be able to conclude that our non-human primate arrays do not improve assay results.

Specific Aim #2: Examine for a link between metaphysical social behaviors and cerebrocortical gene expression.

Several studies have addressed the idea that environment plays a key role in cortical gene expression and cortical development in humans, and that environmental variability gives rise to differentially expressed genes in the cortex. We wish to further investigate this area of research by specifically searching for a link between gene expression and social structures. Our intentions are to eventually form a link between the regulation of gene expression and aspect of the external social environment within the evolutionary development of the human cerebral cortex. Therefore, the objective of this experiment is to use DNA microarrays to determine if gene expression in the cerebral cortex is similar between individuals under similar social structures and divergent between individuals who are members of different social environments. Moreover, we would like to be able to claim that it is precisely this variation in social structures that is responsible for an observed contrast in cerebrocortical physiology.

Once test subjects have been found and controlled for social variability, tissue samples will be obtained and processed in the same manner described above. This DNA microarray will allow us to determine if genes are differentially expressed between varying populations and to quantify these differences. Similar to the above experiment, if further validation of expression level differences is needed, Western Blots or real-time RT-PCR will be used. These methods would include internal controls such as GAP-DH or a universal 18s primer, and either total protein or total RNA would be extracted from tissues samples to run these experiments. Western Blots and PCR would allow us to verify that the differences we see on the arrays are also seen in a follow up experiments.

Expected Results

Human versus Primate Expression Levels We expect to observe a difference in gene expression levels between Humans, Chimpanzees and Bonobos based on data obtained from the novel microarrays. In Humans, we expect an upregulation of genes functioning in the regulation of cerebrocortical development, neuronal growth, lipid and RNA metabolism, cell maintenance/growth and mRNA processing, as compared to the non-human primates (Caceres et al., 2006). Specifically, we hope to confirm the upregulation of genes already reported in previous microarray experiments, shown below;

Table 2 | **Genes with the most consistent expression changes in the human brain in microarray studies**

Gene	Fold change*	Validation‡	Protein function‖	Human disorder (OMIM reference number)¶
Upregulated genes				
SMAD1	5.1–6.4	RT-PCR	Regulation of transcription/signal transduction	
GTF2I	2.5–4.2	RT-PCR	Regulation of transcription/signal transduction	Williams–Beuren syndrome (194050)
CROC4	2.8–2.9	cDNA arrays	Regulation of transcription?	
C21orf33	5.3		Regulation of transcription?	
ZFP36L2	4.1		Regulation of transcription/cell proliferation	
PMS2L5	3.0		DNA repair/mismatch repair	
SF3A3	10.5		RNA processing/RNA splicing	
RGL1	3.1		Signal transduction	
PDE4DIP	9.3		Signal transduction?	
ENTPD6	2.1	cDNA arrays	Nucleotide metabolism/hydrolase activity	
CA2	11.5	RT-PCR, *in situ* hybridization	One-carbon compound metabolism/carbonate dehydratase activity	Osteopetrosis with renal tubular acidosis/ marble brain disease (259730)
NAGPA	5.2	cDNA arrays	Carbohydrate metabolism/protein modification	
GM2A	10.2		Lipid metabolism/sphingolipid catabolism	Tay–Sachs disease, AB variant (272750)
SPTLC1	18.0		Lipid metabolism/sphingolipid metabolism	Hereditary sensory neuropathy type I (162400)
PRDX6	2.9		Lipid metabolism/response to oxidative stress	
OSBPL8	9.6		Lipid transport/steroid metabolism	
GOSR1	12.4	RT-PCR§	Intracellular protein transport	
HSPA2	5.2–9.4	RT-PCR	Chaperone activity/heat-shock protein activity	
COL6A1	12.5	cDNA arrays RT-PCR	Cell adhesion/extracellular matrix	Bethlem myopathy (158810)
THBS4	5.3	RT-PCR	Cell adhesion and motility/extracellular matrix	Premature coronary heart disease (600715)
WIRE	9.6		Actin cytoskeleton organization?	
Downregulated genes				
TWIST1	–6.7	RT-PCR, *in situ* hybridization	Regulation of transcription/skeletal development	Saethre–Chotzen syndrome (101400)
DDX17	–10.8	RT-PCR	RNA processing/RNA helicase activity	
ACADSB	–3.0		Lipid metabolism/energy pathways	2-Methylbutyrylglycinuria (600301)

*Average fold change in gene expression levels between human and chimpanzee cortex in the Enard *et al.*[33] and Cáceres *et al.*[32] oligonucleotide array data. Positive and negative values correspond to genes with higher and lower expression in humans, respectively. When two different probe sets of the same gene show significant expression differences, both values are shown. ‡Independent confirmation by Cáceres *et al.*[32] of expression differences in human cortex using real-time RT-PCR, cDNA microarray analysis and *in situ* hybridization. §M.C., unpublished results. ‖Function of the protein encoded by the gene according to the information available in the SOURCE (source.stanford.edu), LocusLink (www.ncbi.nlm.nih.gov/LocusLink), OMIM (www.ncbi.nlm.nih.gov/OMIM) and AmiGO (www.godatabase.org) databases. Inferred functions are indicated by question marks. ¶Human disorders that are associated with mutations affecting the gene listed can be found in the OMIM (www.ncbi.nlm.nih.gov/OMIM) database. The OMIM reference numbers are in brackets. RT-PCR, A type of PCR in which RNA is converted into DNA, which is then amplified.

Preuss, T.M., Caceres, M., Oldham, M. & Geschwind, D. Human Brain Evolution; Insights from Microarrays. (2004). Nature. 5. pg 857

Additionally, in Human samples we expect an upregulation of genes associated with synaptogenesis and neuronal growth, but not listed in the table above. These include *Emx2*, *Pax6* (neocortical genes regulating development), *Dlx1* and *Dlx2* (forebrain-specific regulatory genes). Caceres et al., (2003) found these genes to be upregulated in humans as compared to non-human primates and link this upregulation with the overall larger size, neuronal activity and cognitive function of the human brain.

It is also expected that our data will report a different fold change in gene expression between each species as compared to previous results. This discrepancy arises from our use of the non-human primate arrays, specifically developed for the chimpanzee and bonobo samples, but hopefully will reflect a more accurate measure of difference.

The significance of more accurate non-human primate arrays available for experimental use is widespread. These microarrays will allow future studies to better characterize and understand the genetic foundations of cortical physiology within and between species, including humans. This has intellectual value in simply understanding the human brain, but also will permit different perspectives into biomedical research in the future. As Social Neuroscience research is already revealing, external influence on the body from social factors to architecture can both positively and negatively effect an individuals health. Also, our newly developed arrays will better equip researchers with the tools needed to shed light on the evolutionary forces that advanced the human brain to its present abilities. We intend to draw correlations between the observed upregulation of these key genes in humans (as compared to Chimpanzees and Bonobos) and the selective factors driving primate brain evolution some 8-7 million years ago. This aspect of our study will be further elaborated on in the future, though it is important to note now the importance of developing better methodological techniques to complete such goals.

There are potential roadblocks in the development of DNA microarrays containing the genome of Chimpanzees and Bonobos. One of the unavoidable consequences of developing an entirely new microarray is that the first few attempts will likely not be completely successful. We expect that a period of trial and error will be necessary in order to produce a working, consistent microarray. Moreover, if the optimized microarrays do not yield significant improvements in data sets, we might have to conclude that some other, outside factor must be involved. Previous studies have shown that differences between human and non-human primate expression levels do exist, and if we do not see these we will have to modify our experimental design to include additional controls (such as Chimpanzees and Bonobos from the same genetic line).

Expression Level Verification after Array Development The expression level data from our arrays will be further validated with Western Blot analyses. We expect that the expression levels of key genes will correlate with the amount of protein synthesized by each gene. In general, we expect to see greater amounts of proteins associated with these key genes in Humans as compared to Chimpanzees and Bonobos.

In particular, we propose to analyze proteins coded by D1x1 and ACADSB with Western Blots, which are well-characterized genes associated with neuron development and cell metabolism within the cortex. We expect the relative amounts of protein to correlate

with expression levels of their associated genes on the arrays, and expect their concentrations to be higher in Humans than in Chimpanzees and Bonobos.

We also propose to use these Western Blots to support the expected improvement from our novel microarrays. Basically, the Western Blots should help us determine if our arrays more accurately show the levels of gene expression for Chimpanzees and Bonobos as compared to past experiments that profiled these animals using arrays with human oligonucleotide probes. If Western Blot data implies that our arrays do not improve expression results (meaning they do not eliminate error from genome sequence differences), we will again have to return the parameters of our study and better define our controls.

Differences in Expression Levels between Genders Previous studies measuring active gene expression in the Human brain have found sexual dimorphisms and there has been little research done towards determining if such dimorphisms exist in none human primates. Array profiles compared between males and females of the same species have revealed differentially expressed genes on the Y chromosome (DBY, SMCY, UTY, RPS4Y, and USP9Y) and X (XIST) chromosomes in humans (Vawter et al., 2004). Although the biological significance of these genes is not well understood, we still foresee the need to control for them and expect our experimental design to further characterize these differences between males and females. We are unsure of what differences, if any, will arise between male and female Chimpanzees and Bonobos.

Cross-Cultural Expression Levels Finally, we propose to examine expression level differences between individuals from different social environments. This aspect of the study intends to strengthen the argument that cerebrocortical gene expression is influenced by variations in the regional social environments or, for humans, the culture. Recent genomic reviews generally agree that genes vary from 5% to 15% across populations and an individual's membership to a particular population can be predicted based on this genomic information (Witherspoon et al., 2007). We propose that our microarray data for humans will give similar results, but allow us to take the conclusion one step further. Our experiment will be meticulously designed with controls that could possibility reveal statistical correlations between cortical gene expression and specific social factors such as political arrangements, economic institutions, language, social bonding, social connectedness and perhaps even cognitive strategies (i.e. holistic or analytical) which are actualized in different cultures in different ways.

It is evident that the social dynamics of human populations varies widely across different regions of the world, and we would like to know if these variations influence the degree to which some genes are differentially expressed. There are a few genes expected to show pronounced regional variation (*duffy, ABCC11* and *SLC24A5*). These genes are called ancestry-informative markers (AIMs) and are used to reliably situate individuals within broad, geographically based groupings or self-identified races (Witherspoon et al., 2007).

It terms of particular genes that are actively regulated during higher-level cognitive operations of cortical physiology, we are completely unsure. We are unsure if such correlations exist, but have chosen to use DNA microarrays in particular for this experiment, in that the large data sets produced can be statistically evaluated for such 'social expression patterns'. Aside from lesion studies and disease profiles, there is very

little empirical data that would permits a move from the cellular and molecular dynamics of cerebrocortical physiology to the dynamics of personality and social behavior. It is this absence of knowledge, however, that motivates our experimental design. There is always the possibility of failure, but we feel that will properly designed controls and microarrays there is great potential in future research to uncover the biological foundations of consciousness, social behavior and the characterization of diseases that are detrimental to both.

We intend to use this part of our study to strengthen the argument that ontogenetic development of the primate cerebral cortex is extremely responsive external stimuli, particular social stimuli. Furthermore, that primate brain evolution can be characterized by precise but substantial changes in the social dynamics (i.e. mating and parenting behavior) of early hominoid populations.

If such correlations could be supported, the implications of this study about be numerous. Foremost, it would suggest that a human's cerebral cortex, the foundation of their personality, intelligence and subjective self, and its development is not only biologically altered by variations in human cultures, but is genetically altered as well. Additionally, we hope to proceed with this research in the future to illustrate that ontogenetic, or generational, variations in cerebrocortical anatomy and physiology which arise from chances in social dynamics is significant enough to drive genetic divergence between individuals and, as such, evolutionary changes in the primate brain. We are currently unaware of any ongoing research that has the potential to deliver such exciting results.

In conclusion, we believe that this type of DNA microarray research will be critical for better characterizing and understanding the relationship between the regulation of gene expression in the primate cerebral cortex, cerebrocortical structure and function, as it relates to higher level cognition, and how each of these levels of analysis are influenced by an individual's external environment, particularly the social dynamics during ontogenetic development. While also clarifying how levels of cerebrocortical gene expression in *Homo sapiens* compares to that of our closest living relatives and relates to primate brain evolution. If such implications can be made, we hope to further develop this methodology to better understand how variations in metaphysical social structures relates to psychological and neurodegenerative disease profiles, with the goal of one day suggesting that in order to truly diagnose and treat these uniquely human disease we ought to be looking outside of the brain rather than deep within it.

Bibliography

"Affymetrix HG-U133 Set Gene Chip Protocol (2006)." Affymetrix. 6 Oct. 2008 <http://www.affymetrix.com>.
"CDNA Synthesis Protocol." Invitrogen. 6 Oct. 2008 <http://www.invitrogen.com>.

Blount et al. Issues in Bonobo (Pan Paniscus) Sexual Behavior. American Anthropologist (1990) vol. 92 (3) pp. 702-714

Caceres, M., Suwyn, C., Maddox, M., Thomas, J., W., Pruess, T., M. Increased Cortical Expression of Two Synaptogenic Thrombospondins in Human Brain Evolution. (2006). Cerebral Cortex. 17. pg. 2312-2321.

Caceres, Mario, Joel Lachuer, Matthew Zapala, John Redmond, Lili Kudo, Daniel

Geschwind, David Lockhart, Todd Preuss, and Carrolee Barlow. "Elevated gene expression levels distinguish human from non-human primate brains." PNAS 100 (2003): 13030-3035.

Elbert et al. Increased Cortical Representation of the Fingers of the Left Hand in String Players. Science (1995)

Enard et al. Intra- and interspecific variation in primate gene expression patterns. Science (2002) vol. 296 (5566) pp. 340-3

Geary et al. Brain and Cognitive Evolution: Forms of Modularity and Functions of Mind. Psychological Bulletin (2002) vol. 128 (5) pp. 667-698

Hill, R.S. and Walsh, C.A. Molecular Insights into Human Brain Evolution. (2005). Nature, vol 437/1. pg. 64 - 67

Khaitovich et al. Regional Patterns of Gene Expression in Human and Chimpanzee Brains. Genome Research (2004) vol. 14 (8) pp. 1462-1473

Krubitzer et al. Arealization of the Neocortex in Mammals: Genetic and Epigenetic Contributions to the Phenotype. Brain, Behavior and Evolution (2000) vol. 55 pp. 322-335

Nelson et al. Child Development and Neuroscience. Child Development (1997) vol. 68 (5) pp. 970-987

Nisbett et al. Culture and systems of thought: Holistic versus analytic cognition. Psychological Review (2001) vol. 108 pp. 291-310

Nithianantharajah et al. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci (2006) vol. 7 (9) pp. 697-709

Preuss, T. M., Caceres, M., Oldham, M., C., Geschwind. Human Brain Evolution: Insights From Microarrays. (2004). Nature. 5. pg. .850-860.

Ramachandran et al. Touching the phantom limb. Nature (1995)
Roe, A.W., S.L. Pallas, Y. Kwon and M. Sur (1992) Visual projections routed to the auditory pathway in ferrets: Receptive fields of visual neurons in primary auditory cortex. J. Neurosci. 12: 3651-3664.

Shultz et al. The evolution of the social brain: anthropoid primates contrast with other vertebrates. Proceedings of the Royal Society B: Biological Sciences (2007) vol. 274 (1624) pp. 2429-2436

Sur et al. Development and Plasticity of Cortical Areas and Networks. Nat Rev Neurosci (2001) vol. 2 pp. 251-262

Vawter, M., P., et. al. Gender-Specific Gene Expression in Post Morterm Human Brain: Localization to Sex Chromosomes (2004). Neuropsychopharmacology. 29. pg 373-384.

Von Melchner, L., Pallas, S.L. & Sur, M. Visual Behavior mediated by retinal projections directed to the auditory pathway. Nature 404, 871-875 (2000).

Whisenant, Thomas. "RNA Extraction Protocol." 6 Oct. 2008 <http://www.scripps.edu/researchservices/dna_array/pdf%20files/rna%20extraction%20protocol.pdf>.

Witherspoon, D., J., et. al. Genetic Similarities Within and Between Human Populations. (2007). Genetics, 176. pg. 351-359.