THE VON ECONOMO NEURONS: FROM CELLS TO BEHAVIOR

Thesis by

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Abstract

The von Economo neurons are one of the few known specializations to hominoid cortical microcircuitry. The recent emergence of this cell type, as well as its localization to subregions of the frontal cortex, suggest its involvement in sophisticated cognitive behaviors. Studies of this cell may thus provide insights into human uniqueness and origin and may additionally be relevant to the treatment and understanding of mental illness.

The first section of this thesis investigates the anatomical details of these cells, including their structure and surface receptor expression. Using a Golgi preparation of a human postmortem brain, I describe the dendritic architecture of this unique population of neurons. We found that, in contrast to layer 5 pyramidal neurons, the von Economo neurons have sparse dendritic trees with symmetric apical and basal components. This confirms that the von Economo cells in both ACC and FI share the architectural characteristics of a single population, and that this population is distinct from other layer 5 neurons. I additionally used immunohistochemistry to probe the receptor expression on these cells, and found that the von Economo neurons strongly express the dopamine D3 and D5 receptors, as well as serotonin-1b and serotonin-2b receptors. Together, these results provide the first detailed anatomical description of a neuron type unique to great apes and humans.

In the second part of this thesis, I explore whether a behavioral stimulus, humor, activates the regions in which this cell occurs. Humor is a hallmark of social discourse and usually depends on the convergence of fast, intuitive assessments with a slow "re-interpretation" of the humor. Because of these characteristics, we thought it likely that humor would activate FI and ACC in addition to other regions in the brain. I used event-related fMRI to differentiate brain activity induced by the hedonic similarities and cognitive differences inherent in cartoons depicting two kinds of humor: visual humor (sight gags) and language-based humor. I found that the brain networks recruited during a humorous experience did indeed include FI and ACC, and that the profile of activation differs according to the type of humor being processed.

Taken together, these projects significantly expand on our knowledge of these unusual cells, and provide a basis that allows us to hypothesize about their function. In the conclusion of this paper, we propose that the role of the von Economo neurons is to facilitate fast decision making in the context of high uncertainty, such as during social interaction.

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Abbreviations

5-HT—5 hydroxy-tryptamine, also called serotonin

ACC—anterior cingulate cortex

BOLD—Blood oxygenation level dependent

DA—dopamine

FI—fronto-insula cortex

fMRI—functional magnetic resonance imaging

VEN—von Economo neuron

"Each has his own tree of ancestors, but at the top of all sits Probably Arboreal."

—Robert Louis Stevenson

"The Astonishing Hypothesis is that 'You,' your joys and your sorrows, your memories and your ambitions, your sense of personal identity and free will, are in fact no more than the behavior of a vast assembly of nerve cells and their associated molecules."

— Sir Francis Crick, The Astonishing Hypothesis

1 Introduction

1.1 Hominoid brain evolution

Humanity resides in the human brain, and, as with any other biological organ, the human brain is shaped by evolution. Because the modern human brain only exists by virtue of the adaptations of our primitive ancestors, it shares features with living nonhuman primates. By taking an evolutionary approach towards the study of the human nervous system, we may begin to see what general features we have in common with our closest relatives, as well as how humans are, neurologically speaking, special.

Of the 181 known species of living primates, only five make up the family known as the hominoids: the humans and great apes (Nusbaum et al., 2006). Behaviorally, the great apes are diverse: orangutans are arboreal while gorillas are terrestrial; bonobos are peaceful while chimpanzees are aggressive; gorillas are polygamous while humans are monogamous (or, at least, less promiscuous than bonobos and chimpanzees). There is no

unifying characteristic that defines great apes in terms of social structure, diet, or sexual behavior. As a family, the great apes appear to be more intelligent than the simians, though this has been notoriously difficult to demonstrate in a laboratory setting. It has been fairly well established, however, that great apes use tools, have some form of self-recognition, and transmit culture; and though these traits are by no means unique in the animal kingdom, they have been demonstrated more consistently in the hominoid family than in any other (Biro et al., 2003; Breuer et al., 2005; de Veer et al., 2003; Nusbaum et al., 2006; Sanz et al., 2004; van Schaik et al., 1999; van Schaik et al., 2003; Whiten et al., 1999). In addition, African apes and humans live in dynamic, highly complex social groups, a characteristic that is extremely difficult to quantify but likely to be an important factor in the evolution of general intelligence. It appears that the brains of great apes and humans have evolved to be flexible and adaptive, capable of identifying optimal responses in the context of a multitude of different circumstances and environments. This cognitive feature seems the most prominent in humans, and is responsible for our colonization of every habitable niche, even at the expense of our fellow primate species (Caldecott and Miles, 2005).

What biological structures underlie the flexible, intelligent behavior of the hominoids? Previous speculations that humans are characterized by large frontal lobes have been replaced by empirical evidence that disproportionately large frontal lobes are, in fact, characteristic of the hominoids (Semendeferi and Damasio, 2000; Semendeferi et al., 1997; Semendeferi et al., 2002). Recent exciting studies designed to identify elevated gene expression or mutation rate in humans have not only identified genes that are likely to have contributed to this frontal lobe expansion (Nusbaum et al., 2006), but also genes

associated with metabolism and synaptic plascticity (Caceres et al., 2003). This line of evidence serves as a useful starting point, so that other experimental methods can be employed to explore the functional and anatomical repercussions of these genetic changes, one case of which is explored elsewhere in this thesis (see section 5.2).

In addition to these known differences in gross brain anatomy and genetics are those internal characteristics of the brain that separate the human minds from the apes, and the hominoids from the simians. Perhaps surprisingly, very few differences have been identified on the cellular or molecular scale. In general the microstructure of brains is surprisingly homogeneous across mammalian species.[1] Cajal's pioneering work in the 1800s resulted in the neuronal doctrine, which states that the neuron is the basic anatomical unit in the brain, and that information flow in the brain is in the form of chemical and electrical messages that pass from neuron to neuron. The circuits formed by populations of neurons throughout the brain are the biological substrates that underlie behaviors. Thus, one might expect systematic differences in this circuitry from species to

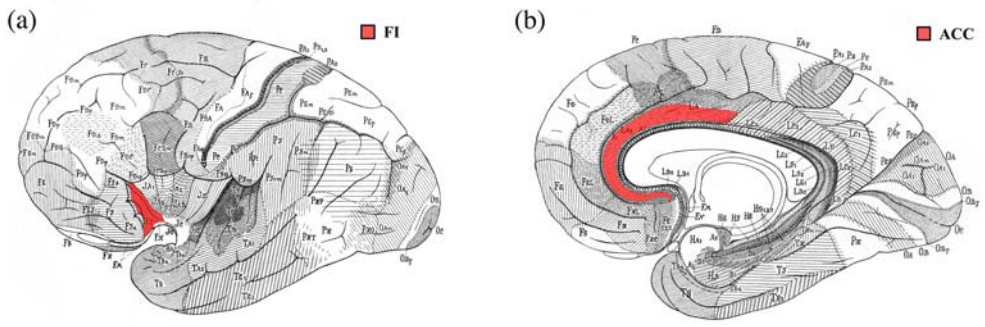

Figure 1. Location of the von Economo neurons in the human brain. (a) Lateral view of the brain with left anterior and fronto-insula (FI) demarcated in red. (b) Medial view of the brain with left anterior and anterior cingulate cortex demarcated in red. Illustrations from von Economo and Koskinas (1929) modified by Atiya Hakeem.

[1] This is a disadvantage if one is looking for an obvious "neural correlate" of human brainpower, but an advantage if one wants to use species other than humans to study how the brain works and how to cure diseases in the brain.

species that correlates with differences in species-specific behavior. In some cases these differences are documented, such as in the primary visual region (Preuss et al., 1999; Sherwood et al., 2003), but these cases are the minority. Indeed, in the frontal lobe—still relatively mysterious but known to be crucial in planning, decision making, behavioral inhibition and social interaction—our knowledge of species-specific differences is sparse.

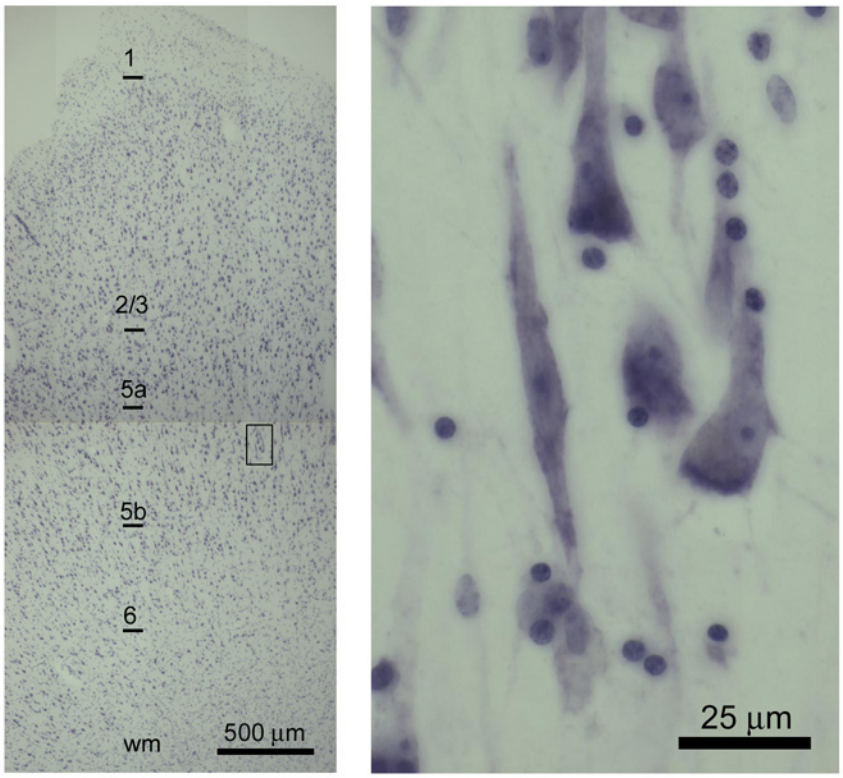

Figure 2. Cresyl-violet stained anterior cingulate in a 53-year-old male human. (a) Low-power photomicrograph montage. (b) z-projection of six 1 um slices collapsed into one depth plane. A single von Economo neuron, center, surrounded by several pyramidal neurons. Photomicrograph taken from boxed area in (a).

1.2 The von Economo neurons

In 1999, Nimchinski and colleagues reported a type of cell that they identified as unique to great apes and humans. At the time, they termed this population the "spindle cells," but to avoid potential confusion with other uses of this name we now refer to them as "von Economo" (VE) cells.

This name is chosen in honor of the neuroanatomist Constantin von Economo, who is the first author of the original 1925 book that contains the classical description of this distinctive class of neurons. Upon inspection of his Golgi preparations of human

cortex, he noted that these large cells were located in layer 5 and restricted to two regions of the human brain: the anterior cingulate cortex (ACC) and in posterior orbitofrontal cortex adjacent to the insula, a region that he termed "fronto-insular" cortex (FI, figure 1). Both of these regions lack a granular layer 4; as in motor cortex, this agranularity may reflect a functional specialization.[2]

In a cresyl violet (Nissl)-stained sample of human or great ape cortex, these cells may be easily distinguished from the neurons around them due to their symmetric, bipolar soma shape and their large size (Figure 2), and it was on the basis of such stains that the phylogenetic uniqueness was determined. The VENs of the anterior cingulate cortex are present in all four living species of great apes and the humans, which implies that they evolved within the last 15 million years (Figure 3). The Von Economo cells in the fronto-insular cortex are present only in the great African apes, and not the orangutan (Allman et al., 2005). This pushes the likely emergence of Von Economo cells in that region to 9 million years ago.

[2] In general, layer 4 is a granular layer that receives input from the thalamus, and layer 5 contains large neurons that project to other cortical and subcortical regions. It follows that sensory cortex has a very large layer 4, whereas motor cortex lacks layer 4 altogether.

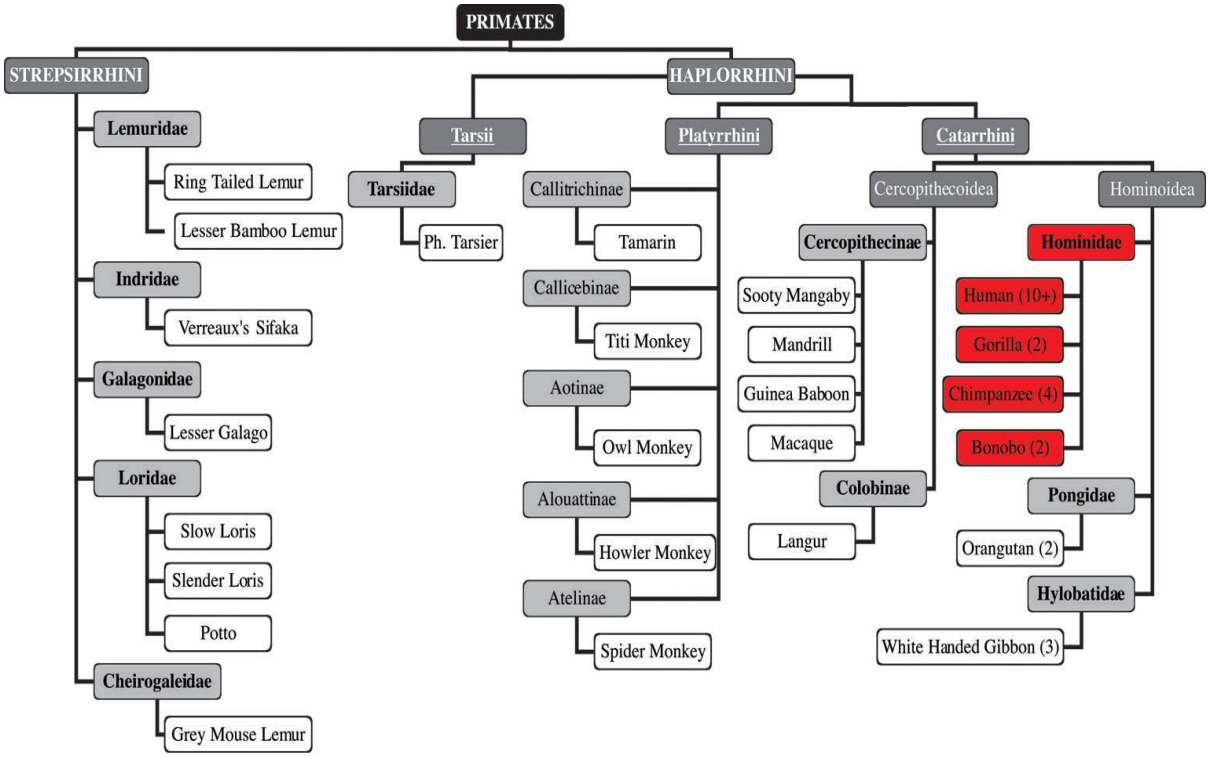

Figure 3 Primate cladogram. Species with VENs in both ACC and FI are in red. The orangutan, the only living non-African great ape, has VENs in ACC but not FI. Number in parenthesis indicates the number of specimens counted stereologically by N. Teatreault and J.Allman. Figure prepared by Atiya Hakeem.

Relative to other neuronal populations, the VENs develop late in ontogeny as well as phylogeny. They first appear at the 35th week of gestation and only about 15% of the full complement is present at birth (Allman et al., 2005). The adult number is attained by 4 years of age. Whether the VENs emerge by differentiation or migration, there is the possibility that their emergence might be disrupted during postnatal development with dysfunctional consequences.

In all of the great apes and post-natal human brains, the VENs are more numerous in the right FI and ACC than the left (Allman et al., 2005). This hemispheric asymmetry appears to arise after birth, as the VEN are about 6% more numerous in the right hemisphere in the neonate but about 30% more numerous in the adult. This right hemisphere VEN predominance may be related to the right-hemispheric specialization for

the social emotions (Blonder et al., 1993). The fact that this 30% right preference is so tightly regulated and consistent across humans and apes (past the infant period) suggests that this ratio is important for normal functioning and that deviations from it could be dysfunctional.

Little is known about the function of the von Economo cells, despite their unique phylogenetic lineage and their potential importance to human brain pathology. The very features that make these neurons so interesting also make them difficult to study with conventional techniques. Most experimental methods devised to explore single cell function and anatomy are invasive and ultimately require the sacrifice of the animal, which would obviously be inappropriate for the study of the VENs given that they are only present in great apes and humans. However, VENs are a specialization of the circuitry that had been present in a common ancestor to the great apes, and is currently present in other modern anthropoids. Thus, studies of FI and ACC in monkeys can, to some extent, inform our assumptions about the function of the VENs

Monkey studies using anterograde and retrograde tracers indicate the ACC is connected to prefrontal, orbitofrontal, insular and anterior temporal cortices and to the amygdala, hypothalamus, various thalamic nuclei, and the periaqueductal gray (Öngür and Price, 2000; Rempel-Clower and Barbas, 1998; Barbas et al, 1999; Cavada et al, 2000).[3] It is more difficult to localize the region analogous (or homologous) to FI in monkeys, simply because of the absence of definitive cortical landmarks.[4] Agranular

[3] In fact, because these connections are characterized by afferents from multimodal sensory-integration-regions and decision making- regions, and by efferents to motor areas, Francis Crick was moved to speculate that ACC is the site of "free will"! (1994)

[4] Anterior cingulate is always easy to recognize, since by definition – "cingulate" stems from "cingere," meaning "girdle" in Latin – this region wraps around the corpus callosum, the single most recognizable cortical structure in the brain. FI, on the other hand, is one of many protuberances in the lumpy cortical mantle, and the macaque brain is considerably less lumpy than the great ape or human brain. This rules out

regions of anterior insula that extend into orbitofrontal cortex do exist in macaques, however, and these are extensively connected with medial temporal and cingulate limbic structures. (Carmichael and Price, 1994; Carmichael and Price, 1995; Carmichael and Price, 1996). Thus, the regions in monkeys that are presumably homologous to those containing the VENs in hominoids are coupled to each other anatomically.

1.3 Rationale of the approach.

The spatially localized nature of the VENS, in conjunction with the known modularity of the brain, gives us another advantage in guessing their function. The recent explosion of functional magnetic resonance imaging (fMRI) studies has enabled us to identify those paradigms that activate the von Economo regions. This approach is particularly useful in the case of FI, which seems to be more selective than ACC, the latter of which is active in almost every behavior that involves intense concentration or emotion. In fMRI studies, FI and ACC are coactivated by two broad classes of stimuli: Those that involve decision making in the context of high uncertainty, and those that involve social stimuli.

In order to link the VENs directly to behavior, we can use the fMRI literature in conjunction with pharmacological literature. That is, once we know what sort of behaviors the circuits might be involved in, we can look for molecular agents that might mediate those behaviors. Once we have some likely candidates, we probe for these receptors or molecules using specific antibodies, which subsequently allows us to determine whether these molecules act directly on the VENs.

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a simple 1:1 mapping of cortical regions. Furthermore, the outstanding cytoarchitectonic feature of FI is that it contains von Economo cells, which are not present in monkeys. This makes FI impossible to delineate cytoarchitectonically in the monkey brain!

The von Economo cell regions appear to be strongly activated during periods of high uncertainty. In an fMRI study during which subjects were engaged in a simple gambling task, activation in both FI and ACC got increasingly stronger as the uncertainty in the task increased (Critchley et al., 2001). In a similar vein, both regions were activated during a reversal task, in which a subject attempts to maximize reward during a task that changes contingencies when the subject's behavior stabilizes (O'Doherty et al., 2003a). A series of incorrect answers will prompt the subject to switch strategies, at which point both ACC and FI show increased activity. Recordings from individual dopaminergic neurons in the macaque monkey ventral tegmentum reveals a similar pattern of activation. During trials with high uncertainty of reward, dopamine neurons exhibit a gradual increase of firing rate across the duration of the trial (Fiorillo *et al.*, 2003). Dopamine is known to be involved in reward delivery and expectation, as well as contingency learning. This leads us to probe for the various types of dopamine receptors, especially D3, the high-affinity dopamine receptor, which is known to have a limbic distribution and is implicated in mechanisms of drug addiction (Le Foll et al., 2005). Additionally, because of models that suggest that dopamine and serotonin act in opposition to one another during learning, we probed for several types of serotonin receptors (Daw et al., 2002). Because the serotonin receptor class is so large – there are thirteen different types – and because antibodies were only available for a subset of these receptors, we were not able to perform an exhaustive survey of all serotonin receptors. However, in probing five of the receptor subtypes, we did identify several that labeled the VENs, with interesting implications.

Golgi is an old technique and was used by both Cajal and Golgi for the work that earned them the Nobel prize in 1906. Although the technique is notoriously capricious, we found that the appropriate protocol, when combined with a consistent source of brain tissue, yielded beautiful results, which I describe and quantify in Chapter 2. This work allowed me to describe the dendritic architecture of the von Economo neurons, and to compare it with that of their layer 5 pyramidal counterparts. A computational study by Mainen and Sejnowski in 1996 showed that variations in the morphology of the cell can have a large effect on the firing profile of the neuron. Knowing the dendritic morphology of the VENs may thus allow future computational studies (in progress by Sejnowski's group) to project the likely firing pattern of the VENs

Finally, in chapter 3 I describe my own contribution to the fMRI literature, a study on the neurobiology of humor. Because humor is essentially an error response coupled with emotional arousal, and because it is so frequently used in social circumstances, we hypothesized that humor would activate the von Economo regions. We did indeed find evidence supporting this hypothesis, and, in addition, report a novel result that contrasts cognitive differences and affective similarities during the perception of two different types of humor.

2 Anatomy of the von Economo Neurons

2.1 Abstract

The von Economo neurons are one of the few known specializations to hominoid cortical microcircuitry. Here, using a Golgi preparation of a human postmortem brain, we describe the dendritic architecture of this unique population of neurons. We found that, in contrast to layer 5 pyramidal neurons, the von Economo neurons have sparse dendritic tress that have symmetric apical and basal components. We also used immunohistochemistry to probe the receptor expression on these cells, and found that the von Economo neurons strongly express the dopamine D3 and D5 receptors, as well as serotonin-1b and -2b receptors. This receptor profile is consistent with a role in mediating decision making in uncertain contexts. Together, these results provide the first detailed anatomical description of a neuron type unique to great apes and humans.

2.2 Introduction

Von Economo neurons (VENs) are large, bipolar neurons that are located in layer 5 of anterior cingulate cortex and fronto-insula cortex (von Economo and Koskinas, 1929). Elsewhere we have referred to them as the "spindle" neurons, but because of potential confusion with other uses of this term, we now refer to them by the first author of the best classical description of these cells. Unlike most neuron types, the VENs are present in the African apes but are absent in the lesser apes, Old- and New-World monkeys, and prosimians (Nimchinsky *et al.*, 1999). This suggests that they arose in the

hominoid clade within the last 15 million years. The volume of the soma is much larger in humans than in apes, and stereological counts suggest that these cells have proliferated in the human line of descent (Allman et al., 2005; Nimchinsky et al., 1995). The recent emergence of this cell type, as well as its localization to subregions of the prefrontal cortex, suggests its involvement in sophisticated cognitive behaviors. This suggests that studies of this cell may provide insights into human uniqueness and origin. Furthermore, because the force of natural selection has had only a relatively short time to shape their functioning and integration with other cell populations, the VENs may be particularly vulnerable to dysfunction. Our understanding of this cell type may thus be relevant to the treatment and understanding of mental illness.

Despite these important characteristics, little is known about the dendritic morphology of the von Economo neurons or about their neurochemical makeup. Cell morphology is crucial to our understanding of these cells, because neuronal shape is directly related to the computation. For example, dendritic structure can establish intrinsic firing patterns (Mainen and Sejnowski, 1996), perform non-linear operations (Koch et al., 1982), or modulate action potential propogation (Vetter et al., 2001). In the current study, we used a modified Golgi technique that enabled us to quantitatively describe the dendritic architecture of the von Economo cells from a young adult human male. Comparisons of the extended dendritic trees allowed us to determine whether the populations of VENs were consistent across regions, and if and how the dendritic trees of VENs differed from those belonging to layer 5 pyramidal cells.

Another way to gain insight into a cell's function is by cataloguing its receptor expression. Pharmacology studies link certain ligands to behaviors, and therefore the

presence of specific receptors on or in the VENs can indicate a role in those behaviors. In this paper we describe immunohistochemical results that show the presence of the dopamine D3 receptor, and the serotonin-1b and -2b receptors.

2.3 Materials and methods

2.3.1 Golgi

Tissue specimens were obtained via Maryland Brain Bank from a human 23-year-old male (PMI = 18 hours) who suffered sudden cardiac arrest. Toxicology reports indicate that there were no drugs or alcohol present in the body at time of death. The right hemisphere fronto-insula (FI) cortex and anterior cingulate cortex (ACC) were dissected, photographed, placed immediately in a potassium dichromate fixative solution (FD Neurotechnologies, Ellicott City, MD) and mailed overnight to the authors. The specimens were kept in this fixative for 17 days, and then placed in FD Neurotechnologies Solution C for 9 days.

Specimens were sectioned at 200 μm intervals on a freezing microtome, mounted on gelatinized slides, and allowed to dry for 2 to 4 days. They were then Nissl-stained with cresyl violet, processed according to manufacturer's directions (FD Neurotechnologies), and coverslipped in Permount (Fisher Scientific, Fair Lawn, NJ).

Once dry, the specimens were observed using the 4x, 10x, and 40x-oil (N/A = 1.00) objectives of a Recihert Polyvar light microscope equipped with a 10x ocular and a motorized stage. The criteria for classifying a neuron as a VEN was an elongated, large soma in layer five of the FI or ACC, a prominent basal dendrite, and symmetrical

morphology along the horizontal and vertical axes of the cell (Nimchinsky *et al*. 1999). We further constrained the category to include only those neurons that had no additional dendrites or branching for a half-soma's distance along the length of the proximal dendrites. For every von Economo neuron traced, we also traced the nearest complete pyramidal cell that had two or more prominent basal dendrites. Using Neurolucida 6.0 (MicroBrightField Inc, Williston, VT) we created three-dimensional reconstructions of the spines, soma, and dendrites of VENs and pyramidal cells in FI and ACC, and used NeuroExplorer (MicroBrightField) for visualization and to perform Scholl analysis (Scholl, 1953). Statistical comparisons were made with nonparametric tests (Kruskal-Wallis and Wilcoxon rank sum tests) using Matlab 7.0 (Mathworks Inc, Natick, MA).

2.3.2 Immunohistochemistry

Neurologically normal human postmortem tissue was obtained from Maryland Brain Bank, UCLA Brain Bank, and Dr. Bob Jacobs, and stored in 10% formalin until sectioning. Tissue specimens were ruled out if the known medical history of the donor included neuropharmacological compounds (i.e., oxycotin), if tissue lacked immunohistochemical reactivity, or if Nissl-stained tissue was evaluated as abnormal. The six specimens used in the experiment had a postmortem interval ranging from 8 to 22 hours (mean 15, s.d. 4.3), and were from male donors ranging in age from 17 to 80 years (mean 45, s.d. 21.0). Tissue was sectioned perpendicular to the pial surface in 50 μm slices on a vibratome and stored in a 0.1 M phosphate buffer with 0.01% sodium azide (Sigma Chemical, St. Louis, MO). The presence of von Economo neurons in tissue was determined by Nissl stain prior to immunohistochemistry. The tissue was processed free-floating with an antibody (see Table 1) in a stock solution of 1% normal goat or

Receptor type	Manufacturer/Product number	Antigen	Host	Working dilution or concentration
Dopamine D1	Chemicon AB1784	Nine aa peptide corresponding to the 4th extracellular domain in rat (88% homology with human)	Rabbit	5 µg/mL
Dopamine D2	Chemicon AB1558	Amino-terminal peptide sequence (aa 24–34) near or at the ligand binding domain of rat D2R (91% homology with human)	Rabbit	1:2500
Dopamine D3	Chemicon AB1785p	Human peptide. 19 aa peptide sequence from 3rd cytoplasmic region	Rabbit	20 µg/mL
Dopamine D3	Santa Cruz D3DR (H-50)	Human peptide. aa 1–50 amino terminal extracellular region	Rabbit	15–25 µg/mL
Dopamine D4	Chemicon AB1789p	Human peptide. 25 aa sequence within the 4th cytoplasmic domain	Goat	10 µg/mL
Dopamine D5	Chemicon AB1790	Human peptide. 20 aa sequence within the 4th cytoplasmic domain	Goat	10 µg/mL
Serotonin 2b	BD Biosciences 556334	Human peptide.aa 1–58	Mouse	10–15 µg/mL
Serotonin 2b	Santa Cruz SR-2B	Human peptide. aa 387–481	Rabbit	10 µg/mL
Serotonin 1b	Chemicon AB5651	Human peptide. aa 15–28	Rabbit	4 µg/mL
Serotonin 1b	Chemicon AB5410	Rat peptide. Sequence 100% conserved in human, corresponding to aa 277-291	Guinea pig	1:1000

Table 1 Antibodies and concentrations used for immunohistochemistry experiments aa = amino acids

rabbit serum (Sigma) and 0.2% Triton X-100 (Sigma). The primary antibody was detected by a species-specific biotinylated secondary antibody (Chemicon, Temecula, CA, 1:200) and then a commercial horseradish peroxidase complex (Vector Laboratories Elite ABC Kit, Burlingame, CA). Labeling was revealed using 3,3'-diaminobenzidine (DAB, Vector Laboratories) as a chromagen according to manufacturer's instructions. Specimens were mounted on gelatinized slides and dried overnight at room temperature. In some cases, some slides were subject to a intensification process through the application of a 2% osmium tetroxide solution for 1 minute, followed by rinsing in buffer, water, and graded alcohols. Once dehydrated, all specimens were cleared in Histo-clear (National Diagnostics, Atlanta, GA) or xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Negative controls consisted of performing the same experiment in parallel, omitting the primary antibody. Specificity of localization for the Chemicon dopamine D3 receptor antibody (AB1785p) was confirmed by control experiments in which the primary antibody was preadsorbed with a dopamine receptor peptide (Chemicon AG229), which abolished staining (data not shown). Results for the D3, serotonin 1b, and serotonin 2b receptors were obtained for at least five of the six specimens, for both hemispheres, and from both FI and ACC, and were replicated using two different antibodies for each receptor (Table 1), thus ruling out false positive results.

For some experiments, an Alexa Fluor conjugated secondary antibody (Molecular Probes, Eugene Oregon) was used instead of the biotinylated antibody, in which case the ABC/DAB steps were omitted and the section was mounted, left to dry, and coverslipped

with Vectashield (Vector labs). Slides were visualized with a Zeiss LSM 510 META NLO equipped with a Coherent Chameleon laser.

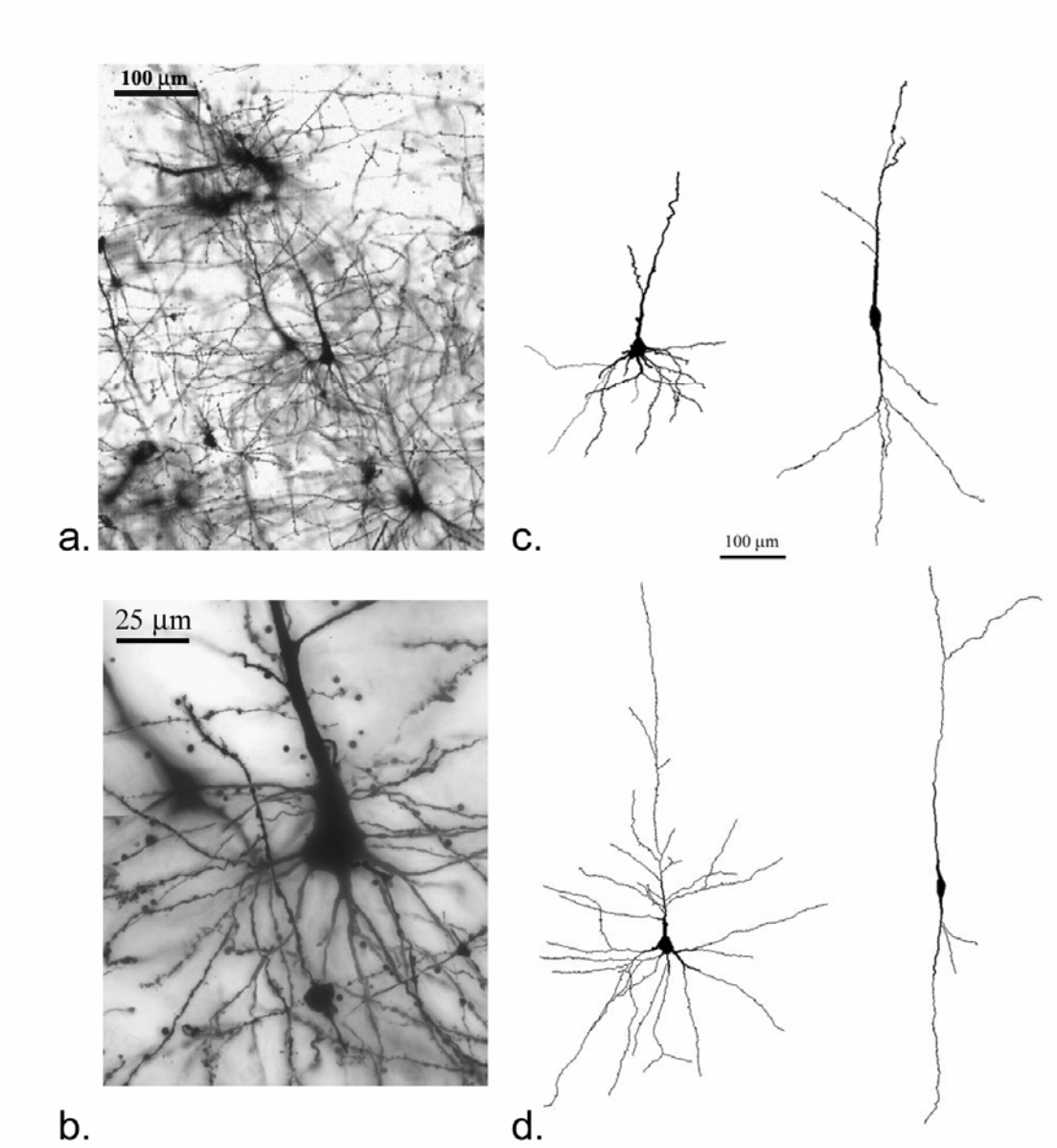

Figure 4. a. Low power photomicrograph of two pyramidal cells in Golgi-stained anterior cingulate cortex, demonstrating the quality of the stain. b High power photomicrograph of pyramidal cell, corresponding to boxed area in (a). Z-projection of 25 slices (taken every 1 µm) projected onto a single plane. c,d Neurolucida tracings of a pyramidal (left) and von Economo (right) neuron from FI (c) and ACC (d). Notice the vertical symmetry and relative sparseness of the VEN dendritic tree. Neurons are oriented so the pial surface is at the top.

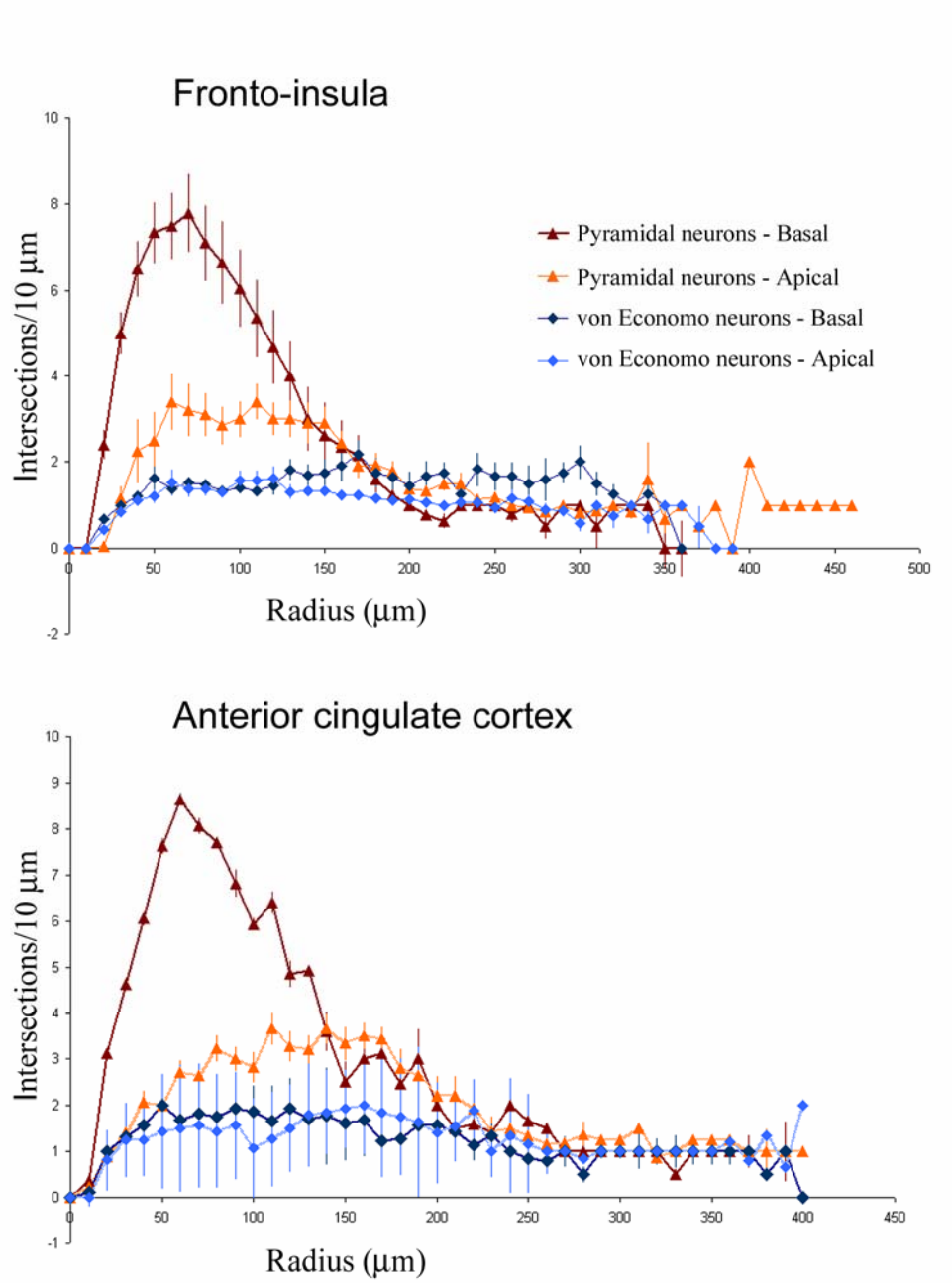

Figure 5 Scholl intersections for FI (top) and ACC (bottom) for pyramidal cells (red triangles, basal tree; orange triangles, apical tree) and von Economo cells (navy diamonds, basal tree; light blue diamonds, apical tree). Note the spike in intersection number that occurs in the pyramidal basal tree that occurs at a radius of 50-100 µm from the soma, and the symmetric intersection number in apical and basal dendritic tress of the VENs in both regions. Error bars represent S.E.M.

2.4 Results

2.4.1 Golgi

Photomicrographs of the specimen sample demonstrate the overall quality of the stain (Figure 4a). Cortical layers were distinguishable on the basis of the Golgi stain. Neurolucida models were created for 17 pyramidal cells and 15 von Economo neurons in ACC, and for 21 pyramidal cells and 20 von Economo neurons in FI. VENs were noted to be symmetric, with their apical and basal dendrites having similar profiles in terms of "branchiness" and length (Figure 4b). In contrast, pyramidal cells had highly branched basal tufts in comparison to their relatively sparse apical trunks (Figure 4c).

We used Scholl analysis to measure dendritic length and the number of branch points ("intersection number") as a function of distance from the soma. Similar to previous findings in macaque temporal lobe (Elston and Rosa, 2000), we found that the peak dendritic complexity of layer V pyramidal cells occurred in the basal tree 50-75 μm from the soma. This spike in dendritic complexity was not present in apical tree of the pyramidal neurons, nor in the apical and basal trees of the VENs (Figure 5).

Between regions (ACC and FI), there were no significant differences in mean total branch length or intersection number for either the pyramidal or von Economo populations ($p>0.25$). Therefore, data from both regions were pooled into a single von Economo group and a single pyramidal group for statistical analyses. When summed overall Scholl radii, neither the total length nor intersection number of the apical and basal dendritic trees of the Von Economo cells differed significantly from one another. In contrast, the basal dendritic trees of the pyramidal neurons contained significantly greater total dendritic length and more Scholl intersections than the apical dendrites of the pyramidal neurons as well as the apical and basal trees of the VENs (Figure 6,

p<0.001). The maximum Scholl radii for the von Economo and pyramidal neurons were not significantly different for either the apical (VEN = 287.14±15.72; pyramidal = 330.52± 17.65) or basal (VEN = 233.43± 14.52, pyramidal = 212.63±10.39) trees (p<0.001, figure 6). Pyramidal cells had a mean total dendritic length 2.5 -fold higher than that of VENs (pyramidal = 2044.3 ±157.1 μm, VENs = 815.8 μm ±66.75).

Spines were distinguishable at 400x magnification. Because the mean total number of spines did not vary by region, data were pooled across ACC and FI. Kruskal-Wallis non parametric ANOVA tests indicated a significant difference in total spine counts between cell and tree types (p<0.001). Post-hoc rank sum tests indicated that the mean sum of spines on the basal pyramidal trees were greater than that of the pyramidal

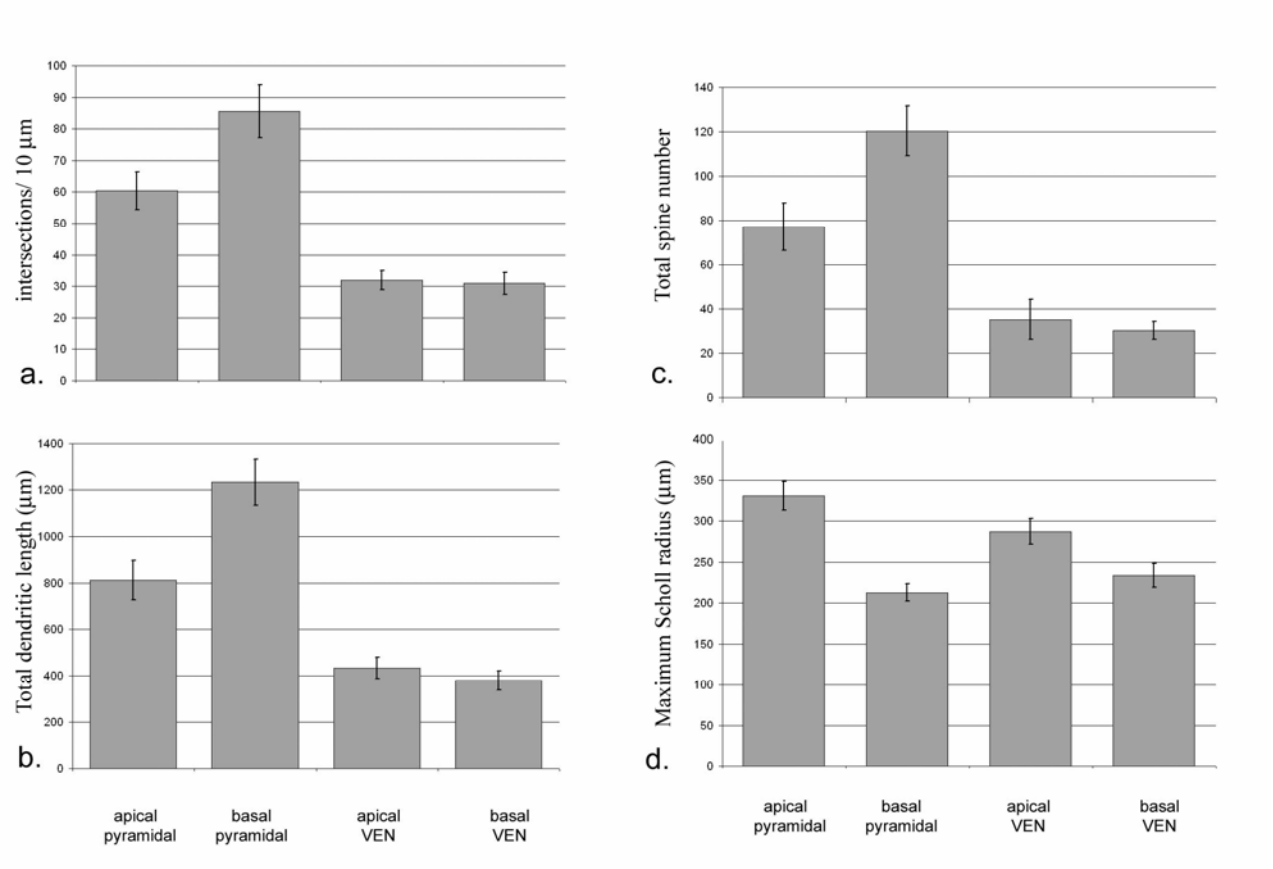

Figure 6 Comparisons of dendritic structure for apical and basal trees of VENs and layer 5 pyramidal cells for (a), total number of Scholl intersections, (b), total dendritic length; (c), spine counts; and (d), maximum scholl radii. Note that, despite significant differences between VENs and pyramidal cells for the first intersections, length, and spine count, there are no significant differences in maximum Scholl radii, suggesting that the observed differences are not due to variations in the degree of Golgi staining. Error bars denote S.E.M.

apical, VEN apical, or VEN basal trees ($p<0.005$). The sum of spines on the pyramidal apical tree was greater than that of the VEN apical or basal trees ($p<0.001$). The VEN apical and VEN basal trees had the same mean total numbers of spines ($p=0.98$). We counted the number of dendritic spines per 10 um along the extent of all dendrites on a tree and found that the number of spines on the basal tree of the pyramidal neuron was maximal from 70 to110 μm from the soma, while the maximum number of spines on the apical pyramidal tree occurred at 160 to180 μm distance from the soma. Both the apical and basal trees of the VENs reached the maximum number of spines around 190 to 240 μm from the soma.

age	gender	cause of death	PMI	hemisphere	% of D3 labeled pyramids (number labled/total)	% of D3 labeled spindles (number labled/total)
47	M	Heart attack	15	R	46% (243/522)	78% (47/60)
80	M	Unknown	15	R	35% (98/278)	75% (16/22)
54	M	Coronary artery disease	12	L	56% (60/107)	85% (12/14)
54	M	Coronary artery disease	12	R	56% (85/139)	93% (14/15)

Table 2 Percentage of VENs and pyramidal cells labeled with D3 receptor antibody in four human brain specimens

2.4.2 Immunohistochemistry

2.4.2.1 Dopamine

Dopamine receptors were localized in human brain by immunocytochemistry. The distribution of the D3 receptor in the anterior cingulate cortex and fronto-insula cortex was similar. Labeling was strongest in the deeper layers and was present on the somas and apical dendrites of Von Economo neurons and large pyramidal cells. The apical dendritic shafts of some cells were labeled for 200 μm or greater, long enough to reach the higher layers of cortex (Figure 7a). This profile of labeling was the same for two different antibodies against the D3 receptor, thus ruling out false positives (Table 1). Fluorescent double labeling of the ACC from four hemispheres (three individuals) revealed that a greater proportion of VENs (82.8% ± 8.0) than pyramidal neurons (48.3% ± 10.0) were labeled with the D3 antibody ($p < 0.001$, Table 2, figure 7b). Immunocytochemistry with an antibody recognizing the dopamine D5 receptor revealed a

pattern of labeling similar to the D3 (Figure 7), whereas antibodies to the D1, D2, and D4 receptors did not label von Economo neurons in a recognizable fashion.

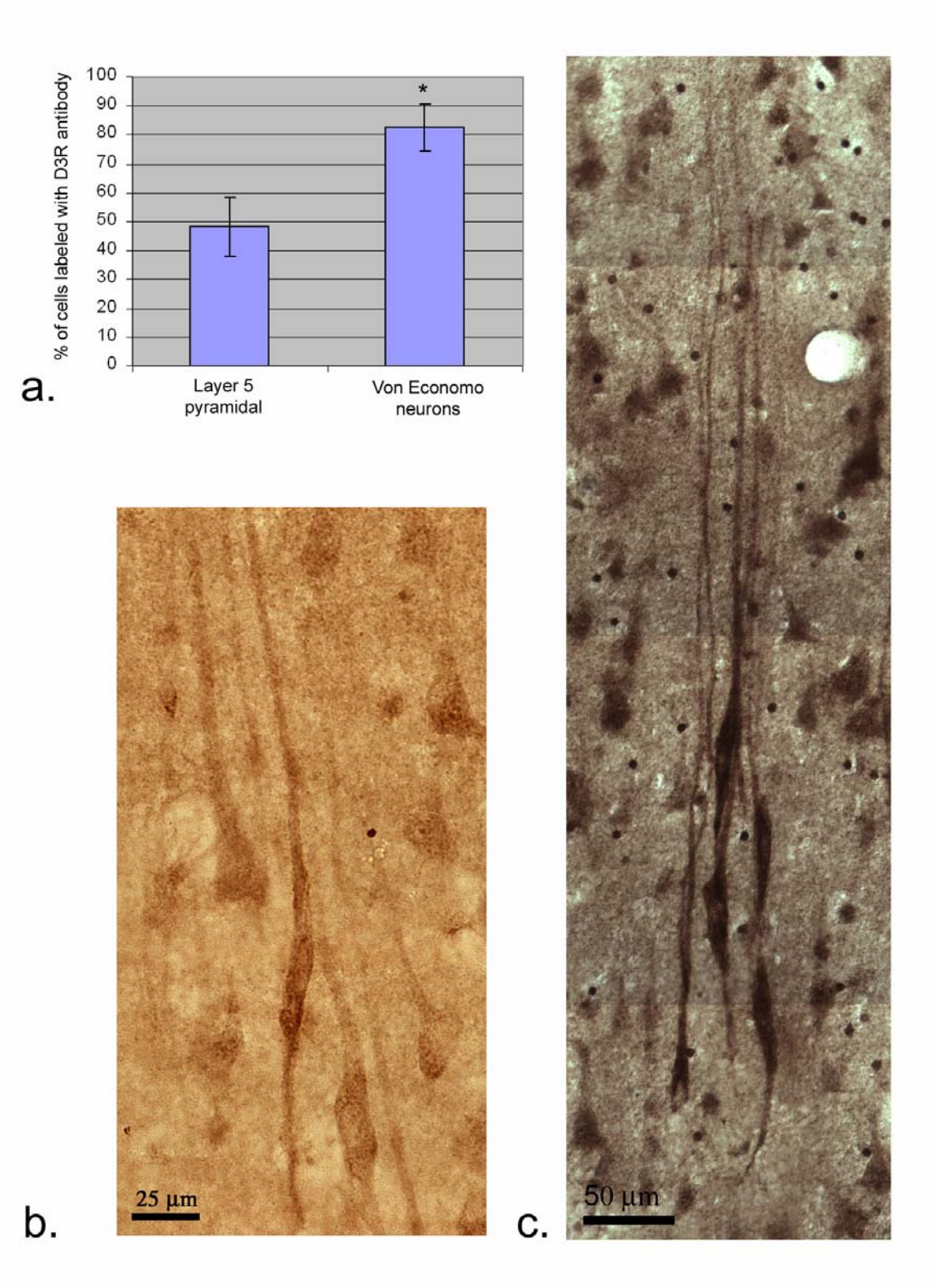

Figure 7 (a.) The percentage of cells labeled with the antibody against the dopamine D3 receptor is significantly lower for layer 5 pyramidal cells compared to von Economo neurons ($p<0.001$). (b) D5 receptor and (c) D3 receptor antibody labeling was evident on the somas and apical dendrites of the von Economo neurons.

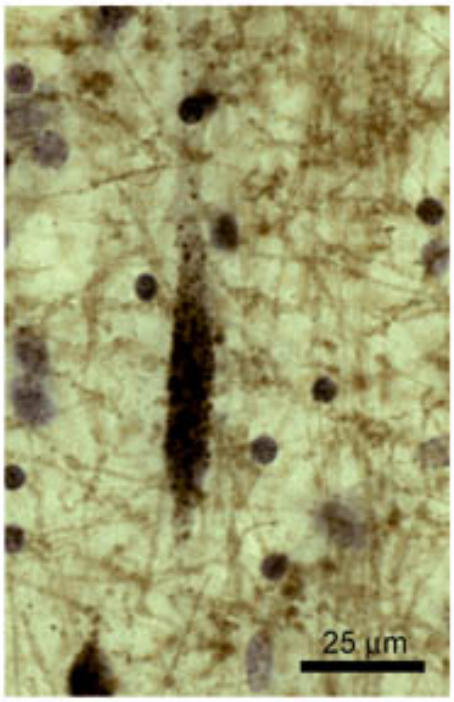

Figure 8 Unlike the D3, D5, and 5-HT 2b receptor antibodies, the 5-HT1b antibody labels the soma but not the apical dendrite of the von Economo neurons

2.4.2.2 5-HT1b

Immunohistochemistry was performed using two different antibodies recognizing different portions of the serotonin 1b receptor (table 1). In the human anterior cingulate cortex, this antibody labeled a network of axons as well as the somas and proximal dendrites of von Economo cells and large pyramidal cells in layer 5 (figure 8). Layers 2 and 3 contained labeled pyramidal somas but few fibers, while layer 6 was nearly confluent with labeled fibers but lacked labeled neurons.

2.4.2.3 5-HT2 receptors

Von Economo cells and other layer 5 pyramidal cells were noted to expess the serotonin 2b receptor, as recognized by two different antibodies. Expression of this receptor was strongly layer 5 specific in human ACC and FI (figure 9a). Expression was strongest on the proximal apical trunk and the soma (figure 9b). von Economo cells were also labeled by an antibody to the 5-HT2a receptor, as were other pyramidal cells in all layers of cortex (data not shown), similar to previous reports. (Jakab and Goldman-Rakic, 1998)

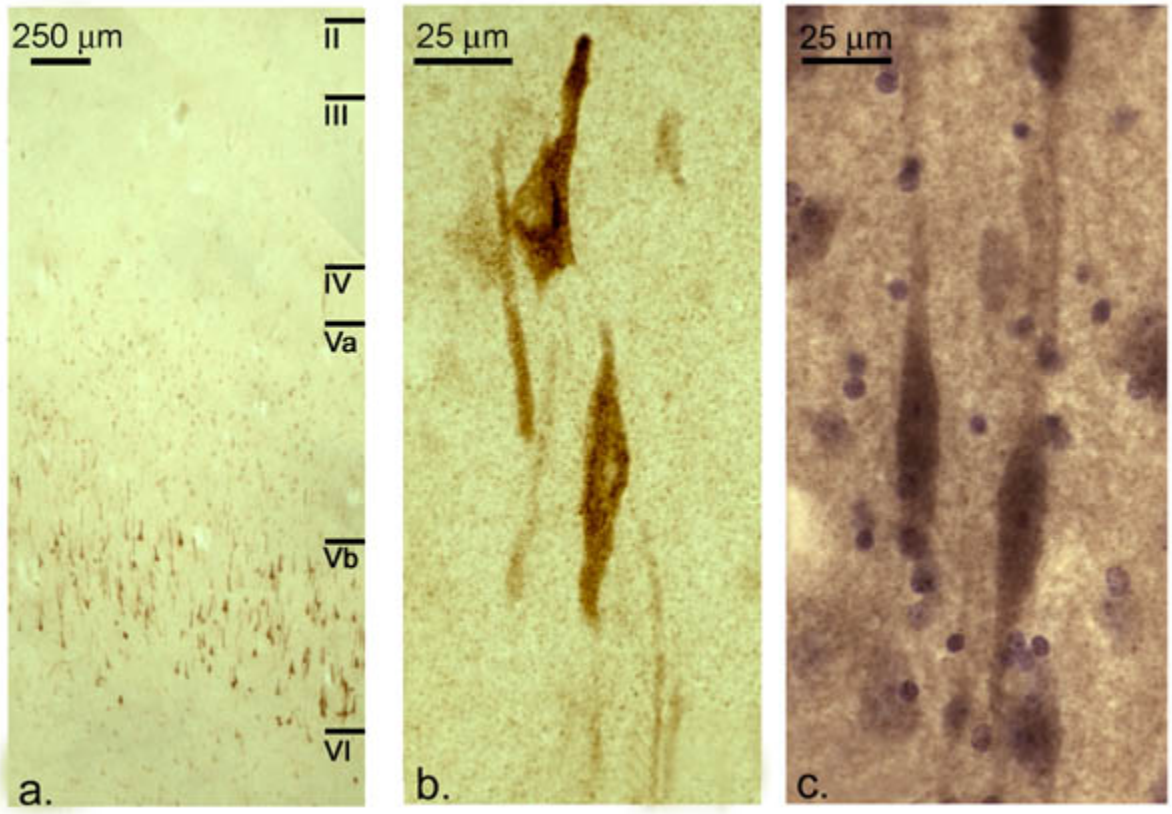

Figure 9 Serotonin 2b antibodies show layer 5 specificity in FI (a) and label the somas and apical trunks of pyramidal and von Economo neurons (b). (a) and (b) are from the same specimen. (c) Two 5-HT2b labeled von Economo neurons from ACC, counterstained with cresyl violet

2.5 Discussion

We used Golgi stains and immunohistochemistry on human brain tissue to characterize the von Economo cells. In doing so, we demonstrated that the von Economo neurons in anterior cingulate and fronto-insula cortex appear to be a single population of cells. Furthermore, we show that the dendritic geometry of the von Economo cells is distinct from that of layer 5 pyramidal cells, and that the VENs express several subtypes of dopamine and serotonin receptors.

2.5.1 Dendritic morphology

The dendritic architecture of neurons reflects the way in which they integrate information (Vetter et al., 2001). Both spines (Nusbaum et al., 2006; Sabatini et al., 2001) and branches (Polsky *et al.*, 2004) can operate as computational compartments, and, compared to their layer 5 pyramidal counterparts, VENs have fewer of both. Studies of rat sensorimotor layer 5 pyramidal cells reveal a relationship between depolarization and output frequency that is linear near the soma and proximal dendrites but non-linear in higher order dendritic branches (Oakley *et al.*, 2001). This suggests that VENs are computationally simple compared to pyramidal neurons.

VENs additionally have only a fraction of the total dendritic length of the average pyramidal cell. The complexity and size of dendritic trees vary with species and brain region. For example, pyramidal dendrites vary according to where they lie in the visual processing stream, with temporal and frontal areas containing neurons of greater complexity than the primary visual area (Elston et al., 2005; Jacobs et al., 2001; Travis et al., 2005). Additionally, Elston found that pyramidal neurons in human prefrontal cortex were more branched and spinous than those in marmoset or macaque monkeys, and that neurons in the cingulate cortex of baboons were similarly more complex than those in vervets and macaques. These results suggest that greater "computational power" comes in the form of more complex and spinous dendrites in the frontal corticies of species that we associate with larger behavioral repertoires. Following this logic, one might hypothesize that a phylogenetically recent neuron type such as the von Economo neuron, found only in great ape and human frontal cortex, would have more extensive dendritic arborizations than the surrounding, presumably more primitive, pyramidal cells.

However, we found that von Economo neurons have fewer spines, fewer intersections, and overall, less dendritic length than their layer 5 pyramidal counterparts, which suggests that the von Economo cells receive, and therefore integrate, fewer inputs than pyramidal neurons. However, it is also possible that the mode of afferent input is different in VENs, which could also account for these differences in structure. Evidence suggests that a substantial amount of communication to the VENs is extrasynaptic (for more discussion, see below). Extrasynaptic transmission renders dendritic and spinious compartmentation superfluous, and could account for the sparse dendritic architecture of the VENs.

Although we show that the dendritic tree of the average VEN is sparser than that of the average pyramidal cell, previous research shows that the cell bodies of VENs in ACC are, on average, 4.6 times larger than that of layer 5 pyramidal cells in this area (Allman et al, 2002). The VENs' large size suggests that they bear large, rapidly conducting axons, which is a characteristic feature of big neurons in layer 5 elsewhere in the cortex (Allman et al, 2002; Sherwood et al, 2003). The VENs contain an abundance of non-phosphorylated neurofilaments, which is characteristic of neurons bearing large axons (Hof et al, 1996; Nimchinsky et al, 1995, 1996). Lipophilic dye injected into the anterior part of the cingulum bundle backfills VENs in ACC, thus indicating that they project axons into the white matter (Nimchinsky et al, 1995). Thus the function of the VENs may be to provide a rapid relay to other parts of the brain of a simple signal derived from information processed within FI and ACC. However, it is not known where the VENs ultimately project. Studies in monkeys indicate that ACC is connected to prefrontal, orbitofrontal, insular and anterior temporal cortices and to the amygdala,

hypothalamus, various thalamic nuclei, and the periaqueductal gray (Öngür and Price, 2000; Rempel-Clower and Barbas, 1998; Barbas et al, 1999; Cavada et al, 2000).

2.5.2 Immunohistochemistry

While the morphology and connectivity have a large bearing on a neuron's computational properties, neuromodulators such as dopamine and serotonin are crucial as well. In fact, the pattern of input a neuron receives is crucial to the morphology of the dendritic tree. Input patterns guide outgrowth and pruning during development and serve to maintain a stable structure later (Cline, 2001; Wedzony et al., 2005; Wong and Ghosh, 2002). Thus, morphology and receptor expression are synergistic systems that endow a neuron with its particular functional role. Pharmacological and electrophysiological studies provide links between the various receptors and animal behavior. Using immunohistochemistry, we were able to probe some of the receptor types that are strongly expressed in the von Economo cells, which in turn provides evidence with respect to the likely function of this population.

2.5.2.1 Dopamine D3 receptor

Our results indicate that the von Economo neurons, as well as a subpopulation of layer 5 pyramidal neurons, express the D3 dopamine receptor on their somas and apical shafts. We found a similar pattern of labeling for the dopamine D5 receptor. In contrast, antibodies against the dopamine receptors D2, D4, and D1 labeled the von Economo neurons weakly or not at all.

The dopaminergic system projects from the midbrain to the basal ganglia and the frontal cortex, and is the fundamental component of the reward systems that motivate action and signal error and uncertainty. In mammals, there exist two classes of dopamine receptors. The D1 type activates adenylyl cyclase and includes the D1 and D5 receptors, while the D2 type inhibits adenylyl cyclase and includes D2, D3, and D4 and (Missale *et al.*, 1998). Although some studies show that the two classes of dopamine receptors can act synergistically (Hopf *et al.*, 2003), most indicate that they are opposed on the molecular (Aizman *et al.*, 2000; Chase *et al.*, 2004) and behavioral (Chase *et al.*, 2004) levels. For example, D1-class and D2-class agonists can inhibit or facilitate, respectively, pair bonding behavior in voles (Aragona *et al.*, 2006). The von Economo neurons appear to express a receptor from each class (the D5 receptor is D1-like, and the D3 receptor is D2-like), which suggests a dose-dependent effect of dopamine on the cell's output.

The pattern of labeling we observed for both the D3 and D5 receptor is consistent with extrasynaptic transmission of dopamine. Extrasynaptic dopamine action at D3 receptors, originally hypothesized because of the receptor's high affinity (Levesque et al., 1992; Sokoloff et al., 1990) and the disparity between dopaminergic innervation and dopamine receptor expression (Levesque *et al.*, 1992), has been further substantiated by studies confirming that dopamine escapes the synaptic cleft during transmission (Garris *et al.*, 1994; Venton *et al.*, 2003), and that behavior can endogenously elicit changes in extrasynaptic dopamine (Wightman and Robinson, 2002). Our results are similar to the perisomatic immunolabeling of the D5 receptor reported by Paspalas and Goldman-Rakic (2004), who used electron microscopy to demonstrate that the receptors were localized to microdomains specialized for dopamine volume transmission.

The D3 receptor's high (25 nm) affinity for dopamine (Sokoloff *et al.*, 1990) may endow it with a special role in monitoring the expectation of reward under uncertainty (Sokoloff and Schwartz, 2002). When reward is uncertain, the dopamine neurons in the ventral tegmental area exhibit a steady ramp-like increase in activity associated with excited expectancy culminating in the receipt or non-receipt of the reward (Fiorillo et al, 2003). Receptors of varying affinity, such as the D3 and D5 receptors on the von Economo neurons, may serve to encode different aspects (i.e., uncertainty, error, and expected value) of the reward signal. This is also consistent with fMRI activations in ACC and FI during decision making under uncertainty (Critchley et al, 2001), as well as with recent pharmacological, genetic, and behavioral studies that implicate the D3 receptor in motivation when response requirements are high and in self-administration of rewarding drug stimuli in response to environmental cues (Le Foll *et al.*, 2005). These paradigms – high response requirements, and learning based on contextual cues – increase the level of complexity in the task, and, consequently, the uncertainty that reward will be obtained. Interestingly, in humans, performance on an intuitive probabilistic learning task varies depending on a polymorphism in the D3 receptor, a result consistent with the rodent studies (Keri *et al.*, 2005).

2.5.2.2 Serotonin 1b and 2b receptors

As a neurotransmitter in the CNS, serotonin is involved in a plethora of cognitive processes, including mood, anxiety, pain, and aggression. The breadth of these various processes may parallel the breadth of experience that occurs in the periphery, where it mediates smooth muscle growth, blood vessel constriction, and platelet aggregation, among other things (Fozard and Saxena, 1991). Indeed, it has been estimated that 90% of

the body's serotonin is manufactured not in the CNS, but in the chromaffin cells of the gastrointestinal system (Bueno, 2005). Behaviorally, serotonin is known to be involved in aversive responses, which has led to development of models in which serotonin mediates the punishment component to learning in a manner complimentary to the encoding of reward by dopamine (Daw et al, 2002). This model supports the hypothesis that the VENs participate in a circuit involved in adaptive decision making, because they, as well as a subpopulation of the surrounding neurons, strongly express at least two types of serotonin receptor.

The VENs express the 5HT1b serotonin receptor on their somas and proximal dendrites. Although we did not explore whether this type of receptor was coexpressed with dopamine D3 receptors on the same cell, it seems likely that this is the case, given its widespread presence in layer 5 neurons and that activation of this receptor is known to inhibit dopamine release in striatal synapses (Sarhan *et al.*, 1999; Sarhan *et al.*, 2000). This particular receptor subtype appears to mediate behavioral inhibition. Application of a 5HT1b antagonist or knocking out the receptor increases aggressive behavior (Bouwknecht *et al.*, 2001) and sex drive (Rodriguez-Manzo *et al.*, 2002) in rodents, while applying an agonist to the receptor has inhibits both types of behavior (de Almeida et al., 2001; Fernandez-Guasti and Rodriguez-Manzo, 1992).

Less is known about the 5HT2b receptor, the other serotonin receptor that we found to be strongly expressed in VENs. This may be because this receptor is relatively rare in the central nervous system (Baumgarten and Göthert, 1997), although it is strongly expressed in the human stomach and intestines. The gastrointestinal serotonin 2b receptor promotes contractions of the smooth muscles responsible for peristalsis, a

role which appears to be restricted to humans (Borman et al., 2002). Serotonin may serve as an antagonistic signal to dopamine, with serotonin signaling punishment and dopamine signaling reward (Daw et al, 2002). The activation of the serotonin 2b receptor on Von Economo neurons might be related to the capacity of the activity in the stomach and intestines to signal impending danger or punishment (literally "gut feelings") and thus might be an opponent to the dopamine D3 signal of reward expectation. The outcome of these opponent processes could be an evaluation by VENs of the relative likelihood of punishment versus reward and contribute to "gut level" or intuitive decision making in a given behavioral context. ACC and FI are known to have an important role in interoception or the conscious awareness of visceral activity (Craig, 2004). Indeed, evidence shows that an insult to the periphery, such as a challenge to the immune system, can result in a change in serotonin level in the frontal cortex (Gardier *et al.*, 1994). Conversely, the duration of social interaction, which requires rapid, context-dependent behavioral adaptation, is altered via manipulations of the 5HT2b receptor in rats (Duxon *et al.*, 1997). In his theory of "somatic states," Damasio (1995) proposed that this monitoring of sensations arising from the gut is crucial to adaptive decision making. The presence of a serotonergic receptor type on the von Economo cells that is otherwise rare in the brain but strongly expressed in the intestinal tract suggests an interesting extension of the concept that these areas are monitoring activity in the gut. Perhaps the expression of the serotonin 2b receptor on the von Economo cells represents a transposition of this monitoring function from the gut into the brain, which would enable the organism to react much more rapidly to potentially threatening circumstances than if that individual depended solely on sensations arising from the gut. In other words, the strong serotonin

2b expression in the von Economo cells could be a model of the gut reaction in the brain that could enhance rapid decision making.

This immunohistochemical profile dovetails nicely with the fMRI literature, which, due to the highly localized distribution of the VENs, also provides information regarding the types of behaviors that involve the VEN regions. For example, ACC and FI are active when subjects make decisions under a high degree of uncertainty (Critchley et al, 2001). These areas are involved in the subjective experience of pain (Singer et al, 2004a), which is powerfully magnified by uncertainty. These areas are also active when subjects experience guilt, embarrassment and engage in deception (Shin et al, 2000; Berthoz et al, 2002; Spence et al, 2001). ACC and FI are also active in humor (Allman et al., 2005), trust, empathy, and the discrimination of the mental states of others (Singer et al, 2004a; Singer et al, 2004b, Baron-Cohen et al, 1999). All of these social emotions are influenced by the degree of uncertainty involved.

As of yet, we do not know the mechanisms responsible for the differentiation of the complex social emotions that activate FI and ACC, but we do know that the VENs are a recently evolved population that probably serves to relay output of the processing within FI and ACC to other brain structures. Their large size suggests that the VENs may relay a fast intuitive assessment of complex social situations to allow the rapid adjustment of behavior in quickly changing social situations. They can thus be seen as an adaptation supporting the increased complexity of hominoid and especially human social networks. This is reflected in evidence that the capacity for empathy is better developed in chimpanzees than in monkeys (Preston and DeWaal, 2002). We hypothesize that the VENs and associated circuitry enable us to reduce complex social and cultural

dimensions of decision making into a single dimension that facilitates the rapid execution of decisions. Other animals are not encumbered by such elaborate social and cultural contingencies to their decision making and thus do not require such a system for rapid intuitive choice. We are not suggesting that animals lacking VENs lack intuition, but rather that the VENs are a specialization that facilitates rapid intuitive decisions in complex, often social situations.

2.6 Acknowledgments

We thank Sarah Teegarden for her help with the histology and immunohistochemistry, Patrick Codd for help with the Neurolucida tracing, and Dr. Bill Yong for providing us with the specimens for the pilot studies. This work was funded by the Gordon and Betty Moore Foundation, and the Swartz Foundation.

3 Brain Activation During Sight Gags and Language-Dependent Humor

3.1 Abstract

Humor is a hallmark of human discourse. People use it to relieve stress and to facilitate social bonding, as well as for pure enjoyment in the absence of any apparent adaptive value. Although recent studies have revealed that humor acts as an intrinsic reward, which explains why people actively seek to experience and create humor, few have addressed the cognitive aspects of humor. We used event-related fMRI to differentiate brain activity induced by the hedonic similarities and cognitive differences inherent in two kinds of humor: visual humor (sight gags) and language-based humor. Our findings indicate that the brain networks recruited during a humorous experience differ according to the type of humor being processed, with high-level visual areas activated during visual humor and classic language areas activated during language-dependent humor. Our results additionally highlight a common network activated by both types of humor that includes the amygdalar and midbrain regions, which presumably reflect the euphoric component of humor. Furthermore, we found that humor activates anterior cingulate cortex and fronto-insular cortex, two regions in the brain that are known to have phylogenetically recent neuronal circuitry. These results suggest that humor may have co-evolved with another cognitive specialization of the great apes and humans: the ability to navigate through a shifting and complex social space.

3.2 Introduction

The phenomenon of humor is universal among humans (Buss, 1988; Caron, 2002; Miller, 2000) and regarded by some as uniquely human (Bergson et al., 2003; Caron, 2002). Humor may have evolved to function as a coping mechanism. Freud (1960) posited that laughter served to discharge the accumulation of internal tension, an interpretation consistent with empirical observations of humor-induced stress reduction (Berk et al., 1989). In clinical contexts, "laughter therapy" is used to increase pain tolerance (Weisenberg et al., 1995) and immune function (Bennett et al., 2003; McClelland and Cheriff, 1997).

Humor also has a strongly social aspect, and in fact, measurements of extroversion in human subjects have been found to correlate with humor-elicited activity in reward regions as measured by functional magnetic resonance imaging (Mobbs et al., 2005). People are more likely to laugh when part of a crowd than in isolation (Devereux and Ginsburg, 2001; Fridlund, 1991; Smoski and Bachorowski, 2003), and a "sense of humor" in an individual may help raise that individual's social status (Salovey et al., 2000), increase that individual's social support network (Salovey et al., 2000), facilitate pair bonding in romantic relationships (Bippus, 2000; Ziv and Gadish, 1989), and attract compatible mates (Bressler and Balshine, 2005; Bressler et al., in press; Buss, 1988; Cann et al., 1997; Miller, 2000; Murstein, 1985). The role of humor in some of these social interactions has been proposed to differ according to gender (Bressler and Balshine, 2005; Bressler et al., in press; Nusbaum et al., 2006; Smoski and Bachorowski,

2003; Ziv and Gadish, 1989), and, intriguingly, a recent fMRI study suggests differences in brain activity in men and women during the perception of humor (Azim et al., 2005).

Presumably, the draw towards those who make us laugh is derived from the subjective pleasure that is inherent in a humorous experience. Recent imaging papers shed light on this aspect of humor by revealing that humor activates the ventral tegmentum and the ventral striatum (Mobbs et al., 2003), as well as regions associated with emotion, such as the amygdala and insular cortex (Moran et al., 2004). Thus, like the taste of fruit juice (Berns et al., 2001), the sight of an attractive face (Aharon et al., 2001; O'Doherty et al., 2003a), or the scent of vanilla (Gottfried et al., 2002), humor activates components of the system involved in reward processing. However, because humor differs from primary rewards in its cognitive complexity and abstract nature, we may also expect activity in "higher-order" reward regions that mediate association formation and learning. Such regions are thought to be located in frontal cortex, such as the site of ventromedial activation observed by Goel and Dolan (2001), as well as frontal pole, where damage results in a disturbance in the affective response to humorous cartoons and jokes despite retention of the ability to discriminate humorous from non-humorous stimuli (Shammi and Stuss, 1999).

The rewarding aspect of humor is only part of the humor phenomenon, however. In order to appreciate a joke, you must first "get" the joke. What exactly is this cognitive mechanism that precedes the mirthful aspect of humor? Some researchers posit that humor requires an element of incongruity or cognitive conflict (Coulson and Williams, 2005; Suls, 1972). Indeed, an ERP study by Coulson and Williams (2005) indicates that,

compared to non-joke stimuli, jokes presented to the left hemisphere elicit larger amplitude N400s, a hallmark of cognitive conflict. Although the slow time resolution of fMRI somewhat hampers the disentanglement of the cognitive from the rewarding aspects of humor, Moran, et al.'s (2004) study used popular television sitcoms as humorous stimuli to gain some insight into this question. They used the onset of a laugh-track as a marker between humor comprehension and appreciation epochs. By observing activation two seconds prior to the onset of laughter, the authors found that brain activity during humor comprehension is distinct from that of humor appreciation, and is characterized by left lateralized activation in the left posterior temporal gyrus and left inferior frontal gyrus.

The affective dimension of humor appears to generalize across modalities; past studies have used both static and dynamic visual imagery (comics and film clips) to elicit humor, as well as auditory delivery of jokes. Some models (Suls, 1972) predict that the re-establishment of coherence – that is, the process of discarding prior assumptions and reinterpreting the joke in a new context -- is crucial to the comprehension of humor. If this is correct, then one should observe increased activation during the re-interpretation that is associated with the modality in which the humor is conceived. Goel and Dolan (2001) broached this question by observing activation associated with different types of auditory humor: semantic jokes and puns. They did indeed find differentiation between the two types of jokes. However, the anatomical sites of semantic and phonological processing are not always easily differentiated, which leaves this result open to interpretation.

In the present study, we used cartoons from "The Far Side" and "The New Yorker" to study brain activation specific to the type of humor portrayed. In cartoons containing language-independent "sight-gag" humor, the humorous element is often a visually improbable predicament, social scene, or action that violates a viewer's initial expectations or assumptions. In cartoons containing language-based humor, the humor may be derived from incongruity between the picture and its descriptive caption, or from a verbal deviation from social norms. Although both types of funny cartoons contain similar levels of complexity, make similar demands on the low-level visual system, and elicit similar feelings of mirth, the cognitive aspect of "getting the joke" differs depending on the sort of incongruity (sight vs. semantic) that needs to be reconciled. This in turn should lead to distinctly different activation patterns associated with the different types of humor. Inversely, both types of humor should produce the same affective result. Thus, as in previous studies, we expect both language-based and sight-gag humor to increase activity in regions associated with reward and emotion, particularly the substantia nigra, nucleus accumbens, amygdala, and insular cortex.

The speculation that humor may be a uniquely human cognitive trait (Bergson et al., 2003; Caron, 2002) prompted our third hypothesis: Humor will activate both anterior cingulate cortex (ACC) and fronto-insula cortex (FI), the two regions in which an evolutionarily recent neuron type, the Von Economo cells (previously termed "spindle neurons"), are present (Allman et al., 2002; Allman et al., 2005). A review of the functional imaging literature reveals that the Von Economo cell regions, particularly FI, are active while reversal learning (O'Doherty et al., 2001), decision making under uncertain conditions (Critchley et al., 2001), and observing bizarre images of

animal/object chimeras (Michelon et al., 2003). Like humor, these paradigms involve incongruity detection and reappraisal, and provided the impetus to formally test the hypothesis that humor activates the Von Economo regions ACC and FI.

3.3 Materials and methods

3.3.1 Subjects

Twenty right-handed healthy volunteers (median age 26 years, range 20-61 years, eight female) gave written consent to participate in this study. Four subjects were discarded from analysis for having three or fewer ratings of "very funny" across all trials. All subjects were fluent English speakers and had normal or corrected-to-normal vision. None had a history of psychiatric illness, and they took no regular medication. The study was approved by the Caltech Internal Review Board.

3.3.2 Stimuli

Stimuli consisted of 100 line drawing cartoons from "The Far Side" by Gary Larson (47 cartoons), or the New Yorker Magazine (various authors, 53 cartoons). 50 of these drawings had been altered slightly so that the humorous element was removed – these were intended to serve as controls for those cartoons found to be humorous. In a preliminary study, we gathered funniness ratings on a scale of 1-10 for each drawing, both with and without captions. From this pilot study, we selected 25 "language-dependent" cartoons, which had mean ratings that were more than one standard deviation

away from their original mean rating in absence of a caption. 25 cartoons that were still within one standard deviation from their mean rating after the caption was removed were categorized as "sight-gag" stimuli, meaning that the humorous element was in the drawing itself, not the caption. Control groups of non-humorous cartoons were selected for each category, language-dependent and sight gag, so that the average number of words in the baseline (unfunny) group was not significantly different from the average number of words in the funny group. Thus, although each subject rated each cartoon separately, there were 50 canonically funny stimuli, as determined by the pilot study, and 50 canonically non-funny control stimuli. Of the 50 canonically funny stimuli, half were language-dependent and half were "sight-gag."

3.3.3 Task

The experiment consisted of an event-related design. Cartoons were presented in random order to subjects, with an interstimulus interval of 300, 600, or 900 ms. We used this short ISI in order to avoid disrupting the "flow" of the humorous stimuli, which we feared might generate a feeling of impatience or anticipation in the subject. Studies suggest that, as long as the ISI is not fixed, using short ISIs can maintain sufficient statistical power in fMRI studies (Elston et al., 1999; Seymour et al., 2004). Subjects were told to observe each cartoon and rate how funny they found it to be, any time after the "rating" cue appeared, four seconds after the stimulus onset. Ratings were done via button box, with one being "very funny," four being "not funny at all," and two and three indicating that it was somewhere in between (note that, due to the limitations of the button box, this rating scale is different from the 1-10 scale used in the pilot study).

3.3.4 Imaging procedure

The functional imaging was conducted by using a 3 Tesla Siemens Trio MRI scanner to acquire gradient echo T2* weighted echo-planar images (EPI) with blood oxygenation level (BOLD) contrast (TR = 2 s, TE=30 ms, flip angle = 90 degrees). Each functional volume consisted of 32 axial slices of 3.2 mm thickness and 3 mm in-plane resolution. Axial slices were acquired 20 degrees above the AC-PC line for each subject to minimize distortion and dropout in the orbitofrontal cortex area. A T-1 weighted structural image was also acquired for each subject using an MP Rage sequence (Siemens).

3.3.5 Imaging analysis

The images were analysed using SPM2 (Wellcome Department of Imaging Neuroscience, London, UK, http://www.fil.ion.ucl.ac.uk/spm/). In order to correct for subject motion, the images were realigned to the first volume. Slice timing correction was applied and images were spatially normalized to a standard MNI template. Spatial smoothing was applied using a Gaussian kernel with a full width at half maximum (FWHM) of 8 mm. Following pre-processing, statistical analysis was carried out using a general linear model, in which each interval (stimulus onset to response time) was convolved with a canonical hemodynamic response function. Analysis of the subjects' behavior indicated that reaction times for an intermediate score (three on the scale of one to four) were significantly longer ($p<0.05$), possibly because of the cognitive effort

required to assign a score in this intermediate range. For this reason, only those cartoons which were rated with a one (least funny) or a four (most funny) by the subject were contrasted when exploring the main effect of humor, although all four scores were included as regressors. We additionally undertook a parametric analysis, in which linear increases in BOLD activation were correlated with the subjective rating of each image.

To look at modality-specific activation, we compared activation during the language-dependent funny cartoons and the visually funny cartoons (25 each), as determined in the pilot study, versus two matched unfunny cartoon control conditions (25 each). Control cartoons were selected for each group so that the average number of words in the cartoon did not differ significantly between funny and nonfunny control conditions. Head movements as determined by the motion correction preprocessing step were used as regressors of no interest. We performed a two way ANOVA, which allowed us to parse the main effects of cartoon humor (funny vs. not funny), the main effects of cartoon type (visual vs. verbal), and the interaction between the two factors. To identify directionality of the response [i.e., (language modulated humor) > (visually modulated humor) and vice versa], we subsequently performed t-tests. We additionally calculated the difference in betas [(βlanguage humor – βlanguage controls) – (βvisual humor – βvisual controls)], and vice versa, for each subject at the peak voxel for each of these contrasts in order to generate the population means. To determine the betas at these voxels, the peak voxel from each of the two second level t-tests was used as the center of a sphere with a radius of 10 mm. For each individual, we then found the peak voxel within this sphere and recorded the betas for all four regressors to determine population means.

Regions of activity were determined using a human brain atlas (Duvernoy, 1991). The SPM-based toolbox MarsBaR (Brett et al., 2002) was used to perform ROI analyses. We used canonical, MNI-atlas based regions of interest (ROIs) for corrections of the caudate, putamen, globus pallidus. Small volume correction for nucleus accumbens was accomplished by centering a sphere of 6.4 mm radius (based on reports that the mean volume of the structure is 1.1 cc in a group of normal human controls (Deshmukh et al., 2005)) at the coordinates (6, 2, -4) and (-6, 2, -4) as reported by Mobbs, et al. (2003). A ROI for ACC was delineated in order to approximate Brodmann's area 24. We drew a line connecting the genu and splenium on an average image created from the 16 normalized anatomical images. The extension of a perpendicular at the midpoint of this line across the cingulate cortex marked the posterior boundary of our anterior cingulate ROI. In the case of FI small volume correction, unnormalized anatomical scans for each individual were imported into MRIcro. The experimenter with extensive experience in locating region FI in human brain histology preparations (JMA) demarcated region FI on each anatomical scan. Normalizing and then averaging these images provided a region of interest used for small volume correction in MarsBaR.

3.4 Results

3.4.1 Behavior

Four subjects were discarded from analysis for having three or fewer ratings of "very funny" across all trials. Across the remaining 16 subjects, 19% of cartoons were

scored as “very funny” and 40% scored as “not funny at all.” Of those cartoons rated “very funny,” about half were Far Side (mean 46.3%, s.d 10.9). There was no significant difference in ratings between Far Side and New Yorker cartoons (Far Side mean rating = 1.82, 0.28 s.d., New Yorker mean rating = 1.80, s.d. 0.21), nor was there a significant difference in the number of language and the number of visual cartoons selected as funny ($p = 0.90$; Figure 10a). Mean ratings for the canonically humorous cartoons (as determined in the pilot study) were significantly higher than the mean ratings for control cartoons ($p<0.01$, Figure 10b). Mean ratings for language-dependent and visual cartoons were not significantly different. Reaction times (mean 7.04 s, 2.95 s.d.) for cartoons rated “very funny” and “not funny at all” were not significantly different, though reaction times for an intermediate score of 3 on a 1-4 scale were significantly higher.

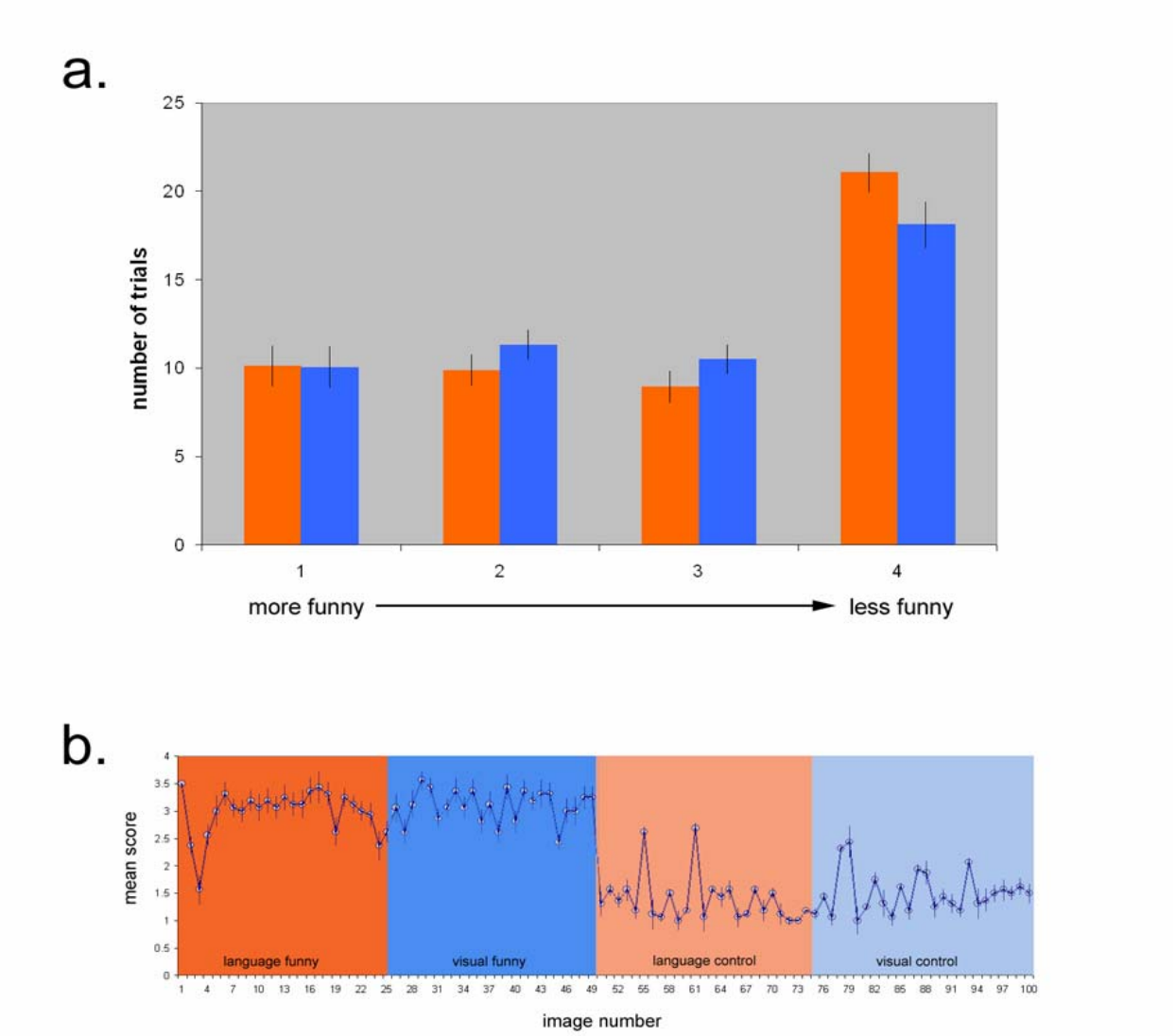

Figure 6 (a) Mean distribution of trial types across rating (1-4, with 4 being the most funny) and category (language based, red; visual, blue) for all 16 subjects. (b) Mean score (1-4, with 4 being the most funny) for each cartoon, computed across the 16 fMRI subjects. Cartoons 1-25 (red block) were canonically funny language cartoons, as determined in the pilot study, and cartoons 26-50 (blue block) were canonically funny visual cartoons. Cartoons 51-75 (pink block) were control language cartoons, while cartoons 76-100 (light blue block) were control visual cartoons. Note the relatively low mean scores of the control cartoons relative to funny cartoons.

3.4.2 Functional imaging

As predicted, comparison of the humor versus control states revealed activation in both of the Von Economo cell regions: bilateral fronto-insula (right, $p<0.03$; left, $p<0.01$;

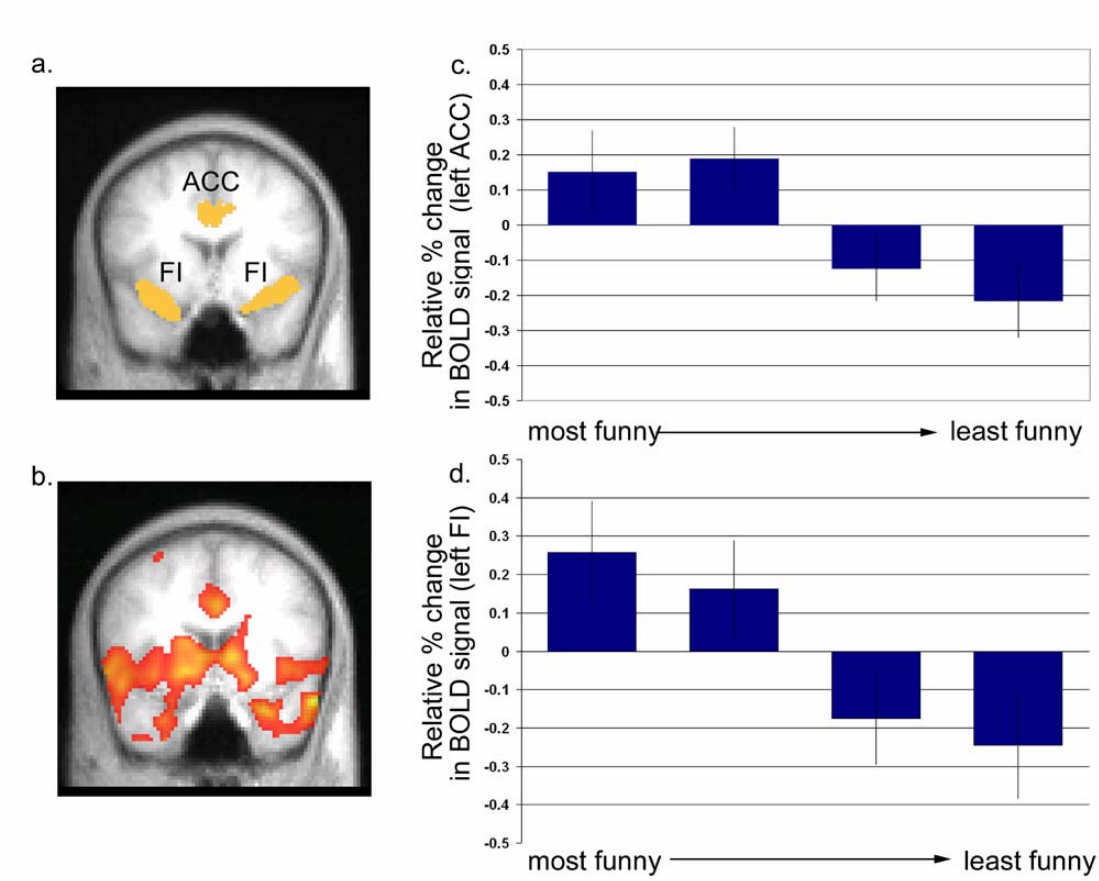

Figure 7 (a) Coronal view of anterior cingulate (ACC) and fronto-insula (FI) cortex ROIs (yellow) overlaid on an average of the subjects' anatomical images. (b) Coronal slice showing regions with significant ($p<0.001$, uncorrected) increases in activity with increasing ratings of funniness. (c) Relative percent change in ACC across all subjects. Error bars represent S.E.M. (d) Relative percent change in FI across all subjects. Error bars represent S.E.M.

corrected for corrected for multiple comparisons across a small volume of interest) and left anterior cingulate cortex ($p<0.03$ corrected for multiple comparisons across a small volume of interest) (Figures 11 and 12). Additional activation was similar to that reported earlier, namely an extended network involving the limbic system and reward areas: bilateral putamen, bilateral nucleus accumbens, and left insula all survived small volume correction ($p<0.05$).

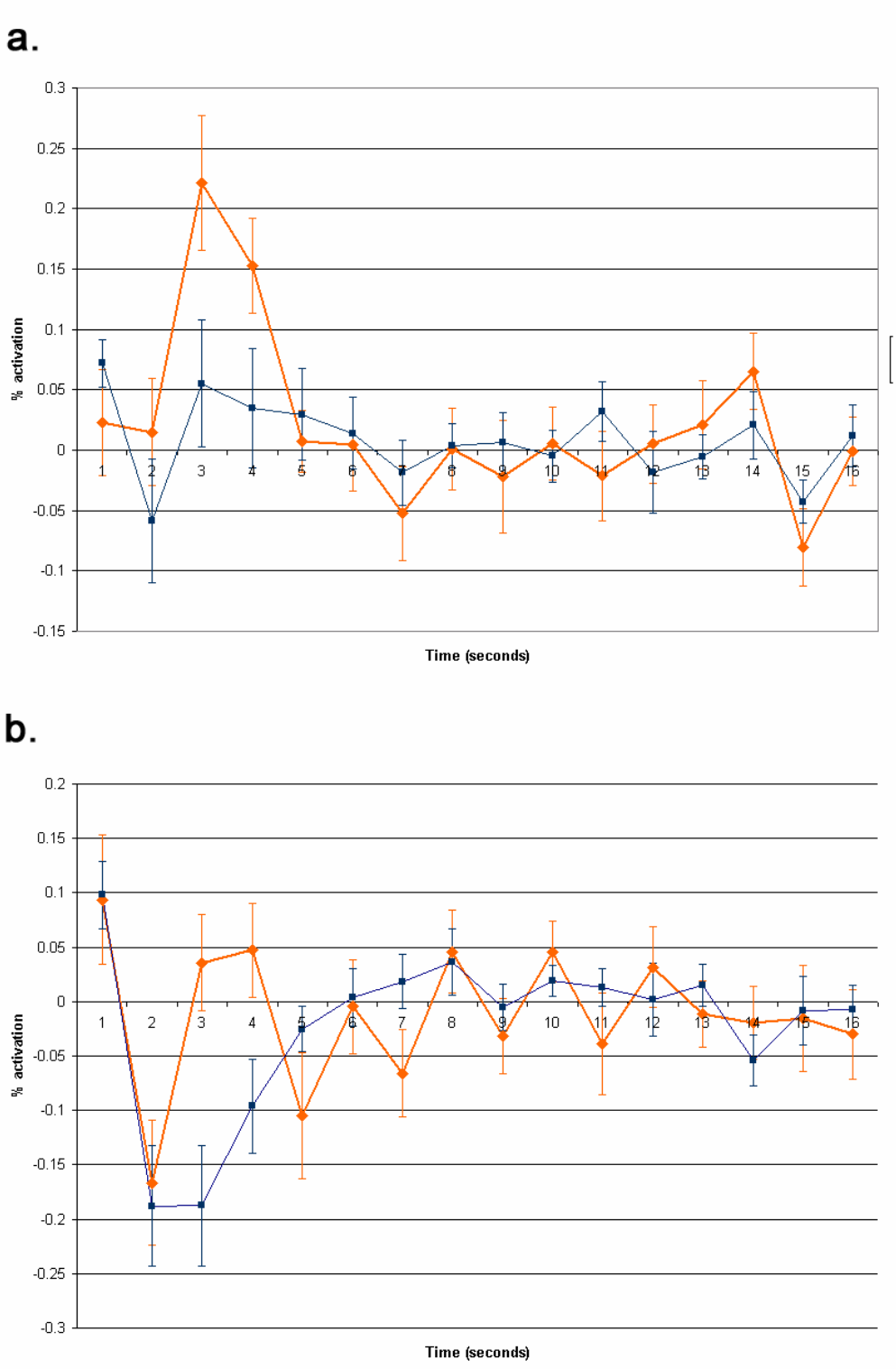

Figure 12 Timecourse of activation for (a) frontoinsula and (b) anterior cingulate cortex. Responses to those images rated as "most funny" are shown in red, and those rated "least funny" are shown in blue. Bars denote S.E.M.

The parametric analysis, which we undertook to explore which areas of activity covaried with the funniness ratings, yielded results similar to those of the funny vs. unfunny contrast described above. Regions of covariance included bilateral superior temporal sulcus, substantia nigra, and caudate; left putamen; left superior frontal gyrus, including dorsolateral prefrontal cortex; and left hippocampus and entorhinal cortex ($p<0.0005$; Table 3). Bilateral anterior cingulate cortex, fronto-insula, and insula proper all suvived small volume correction for the parametric model ($p<0.03$), as did caudate, putamen, nucleus accumbens, and amygdala (figure 13). Using a two-way t-test, we found sex differences in the parametric response similar to those found by Azim and others (2005), with women having greater activity in the middle frontal gyrus, inferior temporal lobe, posterior cingulate, and fusiform gyrus, among other places ($p<0.005$ uncorrected; figure 14). There were no regions with significantly greater activity in men compared to women.

Brain region	L/R	coordinates (x y z) of peak voxel	Z-score
superior temporal sulcus	L, R	-48 -60 20	5.56
middle temporal gyrus	R	56 12 -22	5.45
substantia nigra	R, L	6 -6 -12	5.36
superior parietal gyrus	L	-2 -56 46	5.09
hippocampus	L	-60 -14 -22	4.78
entorhinal area	L	-30 -4 -30	4.7
superior temporal gyrus	L, R	-58 14 -8	4.68
superior frontal gyrus, perigenual anterior cingulate gyrus*	L	-6 56 36	4.64
head of caudate	L, R	-6 -2 12	4.62
putamen	L	-18 6 -4	4.51
dorsal anterior cingulate gyrus*	R	2 10 32	4.45
temporal pole, anterior insula**	L	-42 28 -24	3.91

Table 3 Brain regions that display increasing activation with increasing scores of "funniness." ($p<0.001$). *includes anterior cingulate gyrus. **includes fronto-insula.

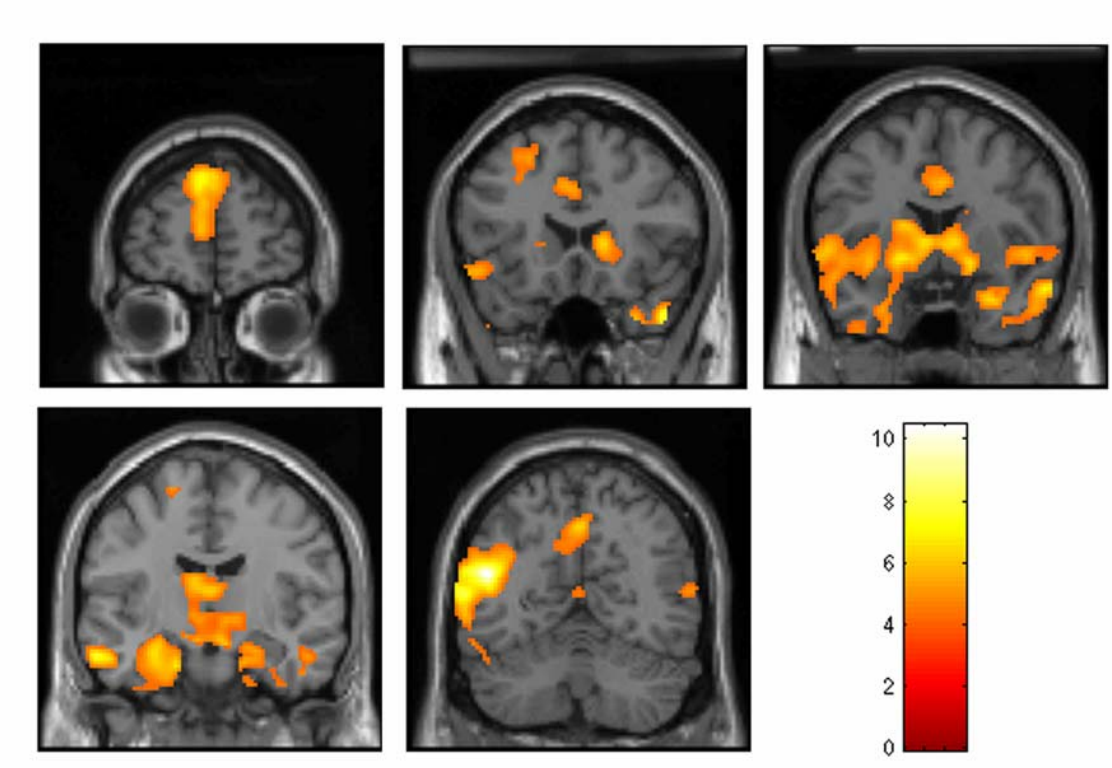

Figure 8 Coronal views of group contrast map for activity that correlates linearly with cartoon rating (increased activity with higher rating of funniness).

A two-way ANOVA revealed the differences in activity due to the main effects of humor, the main effects of humor type, and the interaction between these two factors (Figure 15). Interaction effects between the language-dependent and sight-gag humor categories revealed the functional dissociation between the two different types of humor (Figures 16 and 17, Table 4). Activity that was elicited by language-based humor compared to visual humor included the middle temporal gyrus, the inferior frontal gyrus, and the inferior temporal gyrus, regions functionally defined as Wenicke's area, Broca's area, and the basal temporal language area, respectively (Table 4a) (Benson, 1993; Friederici, 2002; Just et al., 1996). Application of a liberal probability threshold ($p<0.05$, uncorrected for multiple comparisons), suggested a more extended region of activity in the middle temporal gyrus that extended up the length of the temporal lobe (Figure 18).

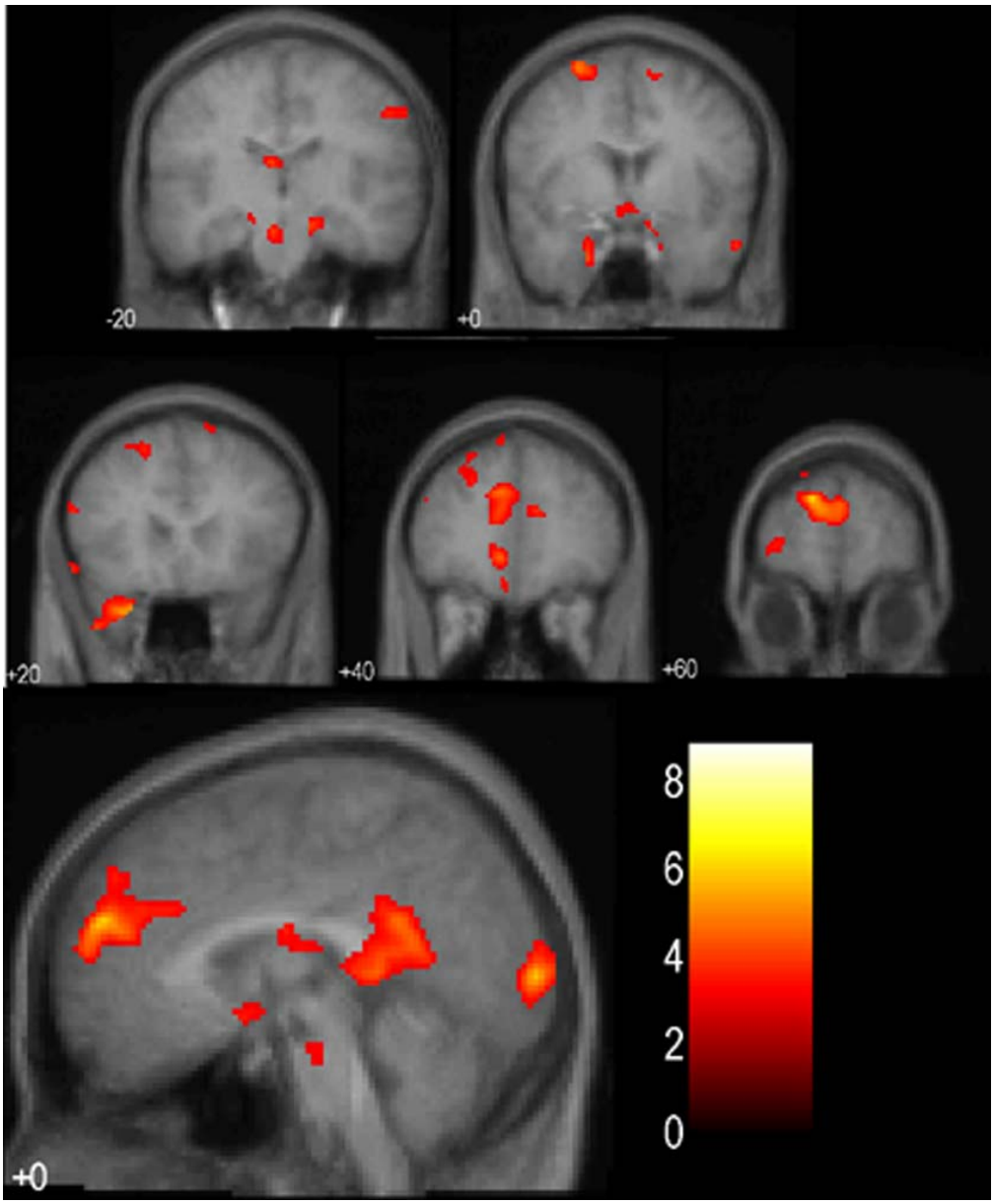

Figure 9 Statistical parametric analysis in which women had greater activity than men, overlaid on the average of the female structural scans ($p<0.005$, uncorrected). Similar to results reported by Azim and others (2005), regions included bilateral middle frontal gyrus and primary visual cortex, left medial orbitofrontal cortex (gyrus rectus and medial orbital gyrus), superior frontal gyrus, and inferior temporal cortex, and right posterior cingulate (ordered from most to least significant; not an exhaustive list). Right, but not left, nucleus accumbens was more active in women than men after ROI analysis as described in methods ($p<0.05$, corrected over small volume of interest). This differs from previously described results, which found the nucleus accumbens to be the site of greatest activation difference between sexes (Azim et al., 2005)

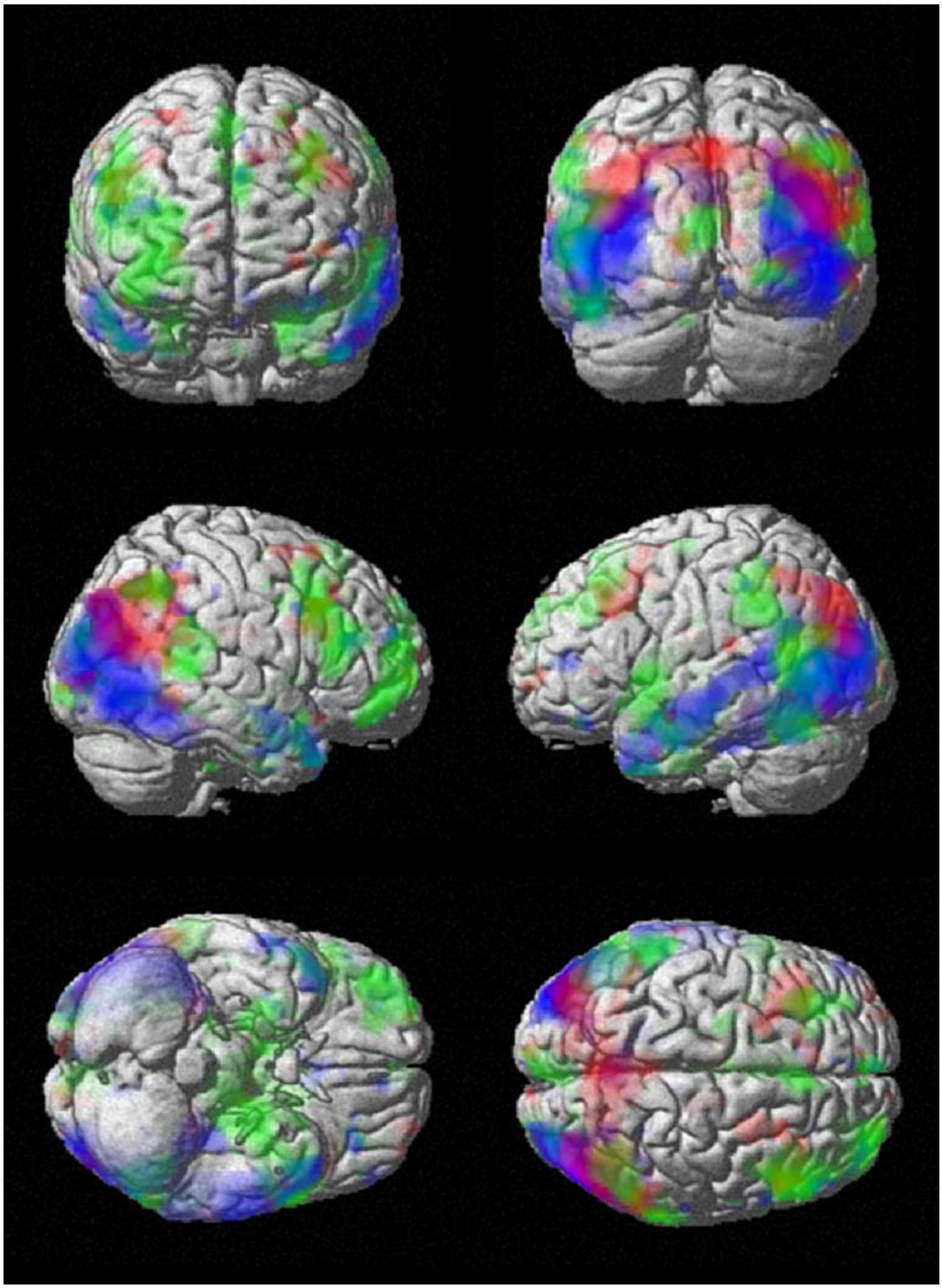

Figure 10 Surface projections of color-coded statistical parametric maps (SPMs) the results of a two-way ANOVA ($p<0.005$, uncorrected) overlaid onto canonical single-subject anatomic rendering. Green indicates the main effect of humor (humorous cartoon vs. control), blue indicates the main effect of cartoon type (language vs. visual), and red indications regions for which there is an interaction between these two effects. Violet indicates the regions that show variations in activity according to cartoon type (language vs. visual) as well as to the interaction. Trials were parsed into categories (funny or not funny, visual or language; 25 trials of each type) in a canonical fashion for all subjects.

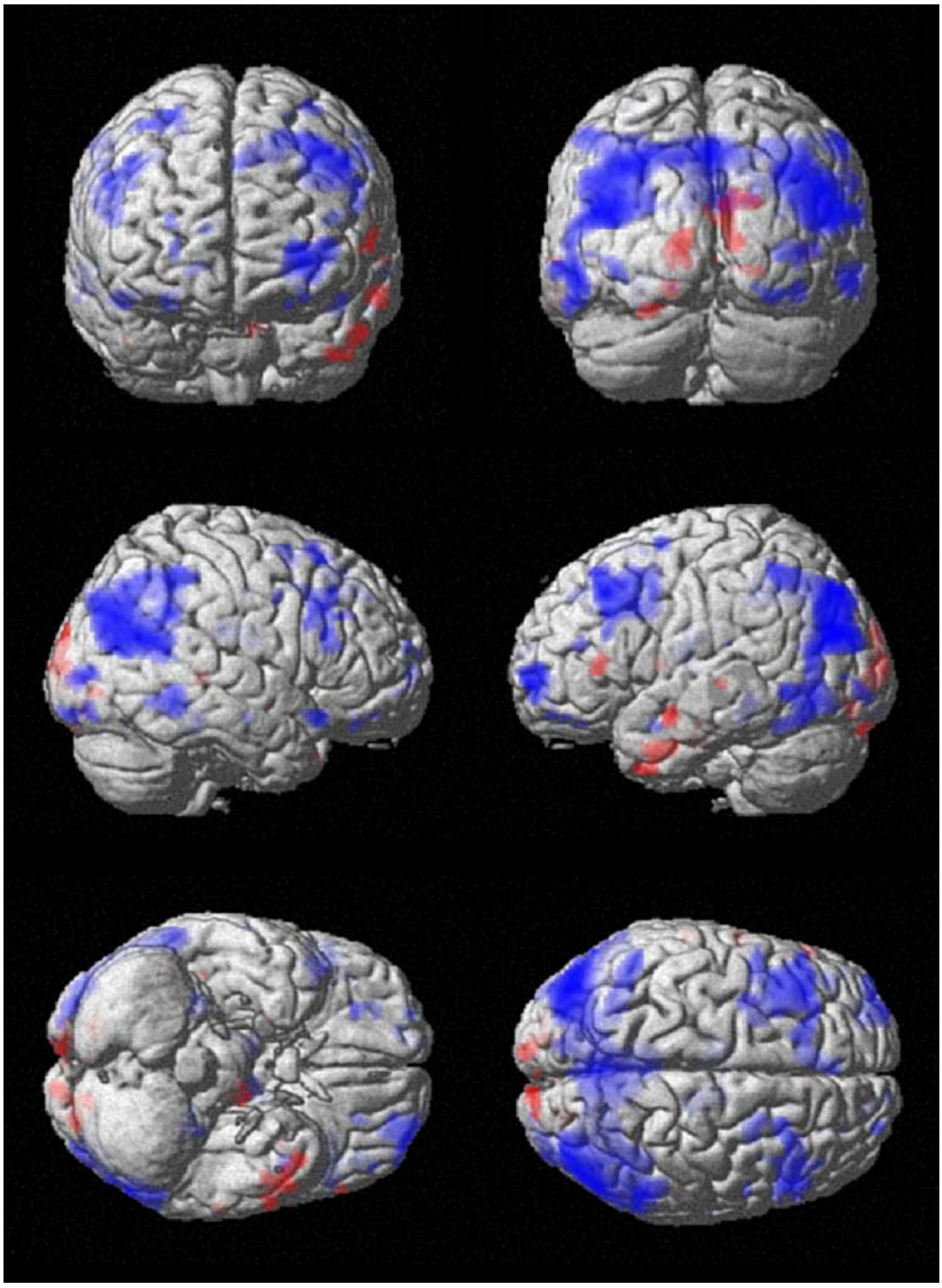

Figure 11 Surface projections of color coded statistical parametric maps (SPMs) showing the results of second-level t-tests ($p<0.005$, uncorrected) overlaid onto canonical single subject anatomic rendering. Blue indicates those regions where [(visual humor – visual control) > (language-based humor > language-based control)]; red indicates the opposite.

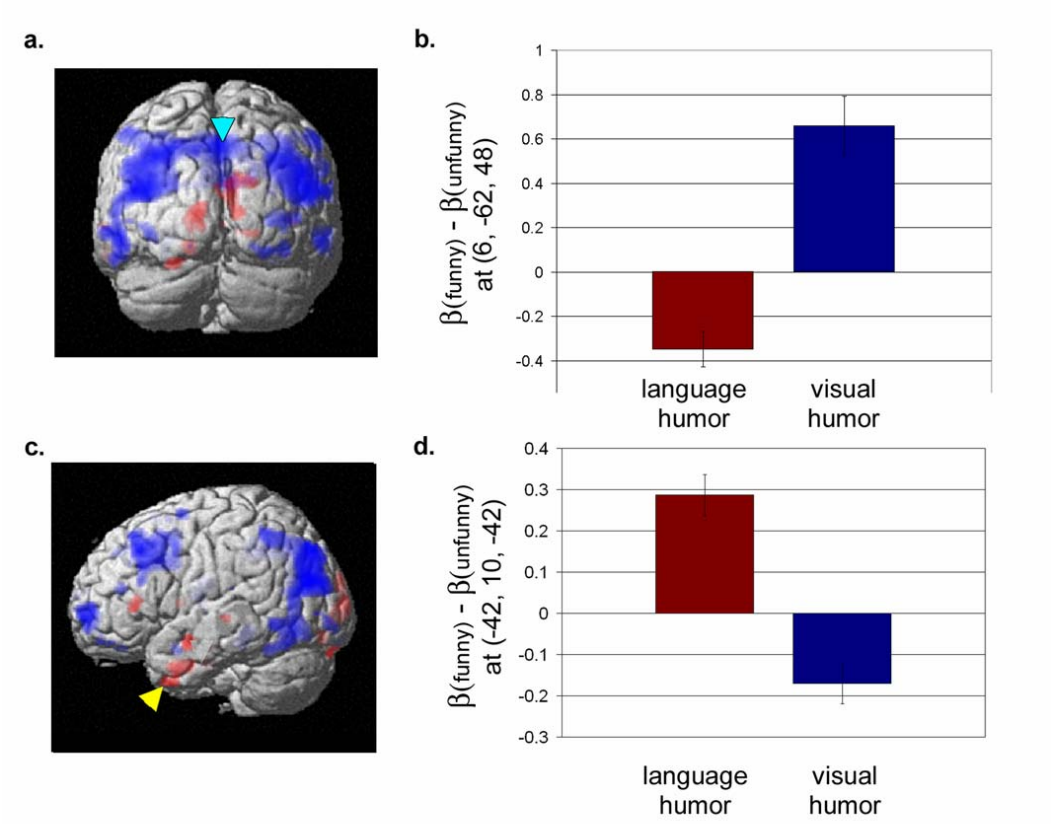

Figure 17 (a) Replication of surface projection from Figure 17, with peak voxel modulated by visual humor > language humor indicated by the cyan arrowhead. (b) Mean differences in betas across all subjects for the voxel indicated in a. Red bar, differences in betas for funny trials minus the betas for control trials for language-based cartoons; blue bar, differences in betas for funny trials minus betas for control trials for sight-gag cartoons. (c) Replication of surface projection from Figure 4, with peak voxel modulated by language humor > visual humor indicated by the yellow arrowhead. (d) Mean differences in betas across all subjects for the voxel indicated in c. Red bar, differences in betas for funny trials minus the betas for control trials for language-based cartoons; blue bar, differences in betas for funny trials minus betas for control trials for sight-gag cartoons. Note differences in y-axis scale between (b) and (d). Error bars represent S.E.M. in both graphs.

In contrast, the reverse comparison [(visually funny cartoons – visual controls) > (language based funny cartoons – language controls)], activated broad swaths of bilateral higher-order visual cortex, including the horizontal posterior segment of the superior temporal sulcus, the middle occipital gyrus, and the precuneus (Table 4b, Figures 16, and 17).

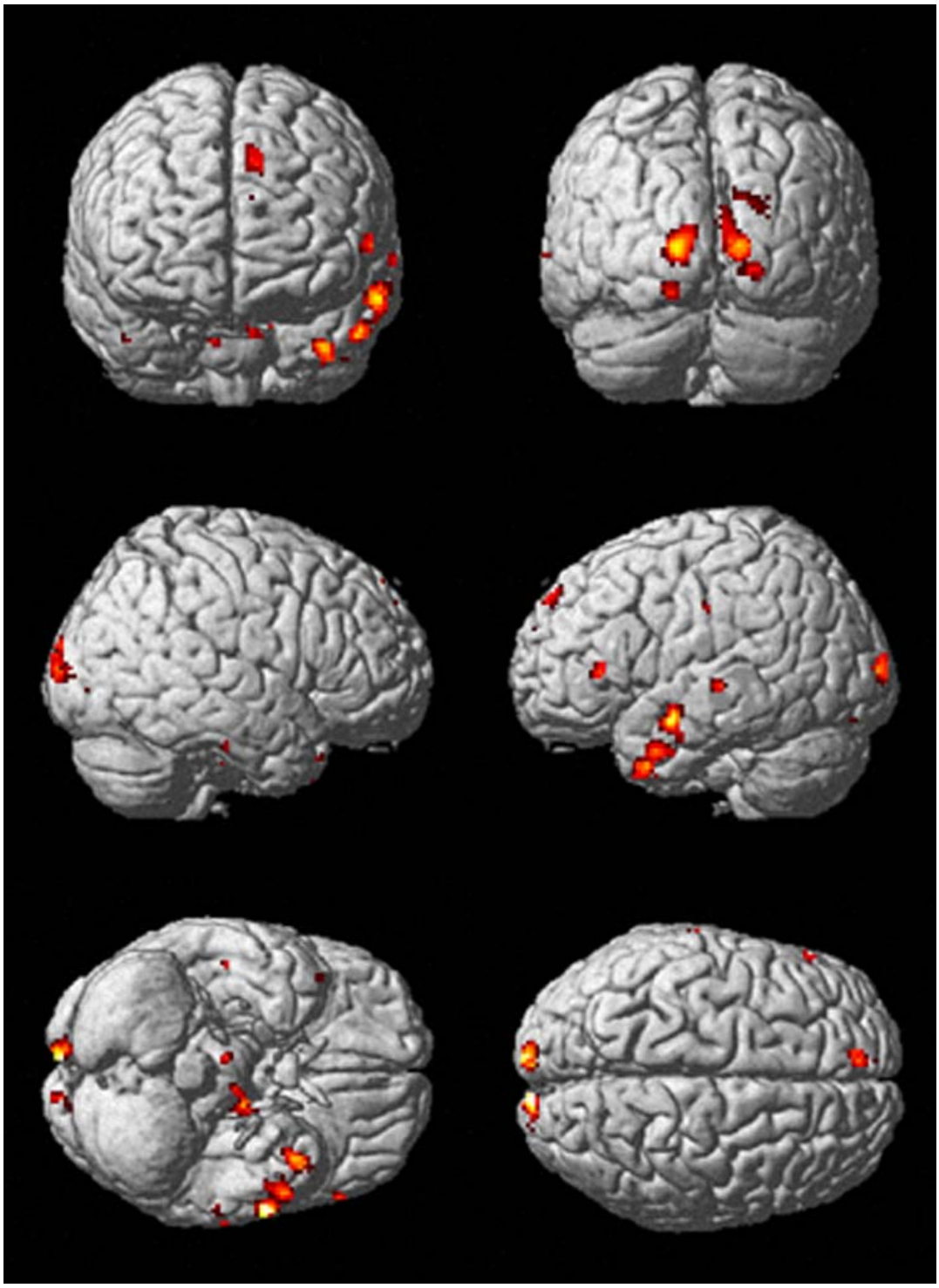

Figure 18 Surface rendering of brain regions for which language humor is greater than visual humor ($p<0.05$, uncorrected).

Table 4 a

Brain region	L/R	coordinates (x y z) of peak voxel	Z-score
inferior temporal gyrus	L	-42 10 -42	3.75
middle temporal gyrus	L	-52 4 -32	3.31
inferior temporal sulcus	L	-50 -4 -30	3.18
superior occipital gyrus	R	4 -98 20	3.53
superior occipital gyrus	R	12 -98 28	3.10
cuneus	L, R	14 -98 8	3.00
transverse occipital sulcus	L	-14 -94 -2	2.78
fourth occipital gyrus	L	-14 -86 -14	3.27
inferior frontal gyrus, pars triangularis	L	-58 32 6	3.18
superior temporal sulcus	L, R	-64 -26 0	3.10
inferior occipital gyrus	L, R	-24 -92 -22	3.07
subiculum	L	-14 -16 -20	3.03
parahippocampal gyrus	L	-10 -14 -28	2.86
short insular gyrus	L	-32 2 8	2.82

Table 4 b.

Brain region	L/R	coordinates (x y z) of peak voxel	Z-score
precuneus	R	6 -62 48	5.03
superior temporal sulcus, horizontal posterior segment	L, R	-38 -76 20	4.94
middle frontal gyrus	L, R	-36 26 44	4.70
inferior temporal gyrus	R	60 -48 -10	4.60
inferior frontal gyrus	L, R	-30 62 0	4.60
anterior orbital gyrus	L	-28 52 -16	3.38
superior temporal gyrus	R	48 20 -18	4.41
fronto-insula	R	38 18 -14	2.72
superior frontal sulcus	R	26 18 62	3.42
middle occipital gyrus	L	-38 -90 -4	3.87
anterior orbital gyrus	R	26 38 -20	3.70
middle frontal gyrus	R	42 24 38	3.50
inferior occipital gyrus	R	38 -86 -14	3.43
fourth occipital gyrus	R	32 -94 -14	3.39
thalamus	L	-8 -12 16	3.39
fusiform gyrus	L, R	-26 -40 -8	3.38
posterior cingulate gyrus	R	6 -48 24	3.21
lateral occipital sulcus	R	38 -90 2	3.20
lateral orbital gyrus	L	-46 46 -18	3.11

Table 4 Atlas coordinates (in MNI space) and z-scores of peak activation during the cartoon task for the interaction between the "sight gag" and "language-dependent" categories. Table 2(a) lists regions for which [language-dependent humor (funny – unfunny) > sight-gag humor (funny – unfunny)], i.e., regions of activation for which language-based humor is significantly greater than sight-gag humor. Table 2(b) lists regions for which [Sight-gag humor (funny – unfunny) > language-dependent humor (funny – unfunny)], i.e., regions more strongly activated by sight-gag humor than by language-based humor (all comparisons $p<0.005$, uncorrected, cluster>10 voxels).

Brain region	L/R	coordinates (x y z) of peak voxel	Z-score
midbrain	L	-10 -24 -12	4.61
amygdala	L/R	-28 -4 -30	4.13
hippocampus	L	-22 -24 -12	3.93
fusiform gyrus	L	-48 -56 -20	3.78
superior temporal sulcus	L/R	66 -40 10	3.54
middle temporal gyrus	L	-60 -54 2	3.39
hypothalamus	R	8 -4 -8	3.31
subiculum	R	14 -28 -6	3.19
nucleus accumbens	L	-12 4 6	2.88
inferior temporal gyrus	R	32 -6 -40	2.87
entorhinal area	R	28 0 -34	2.85
inferior frontal gyrus	L	-60 12 2	2.83

Table 5 Atlas coordinates (in MNI space) and z-scores of peak activation from a conjunction analysis of both visual humor and language based humor [(language funny – language unfunny) and (visual funny – visual unfunny)].

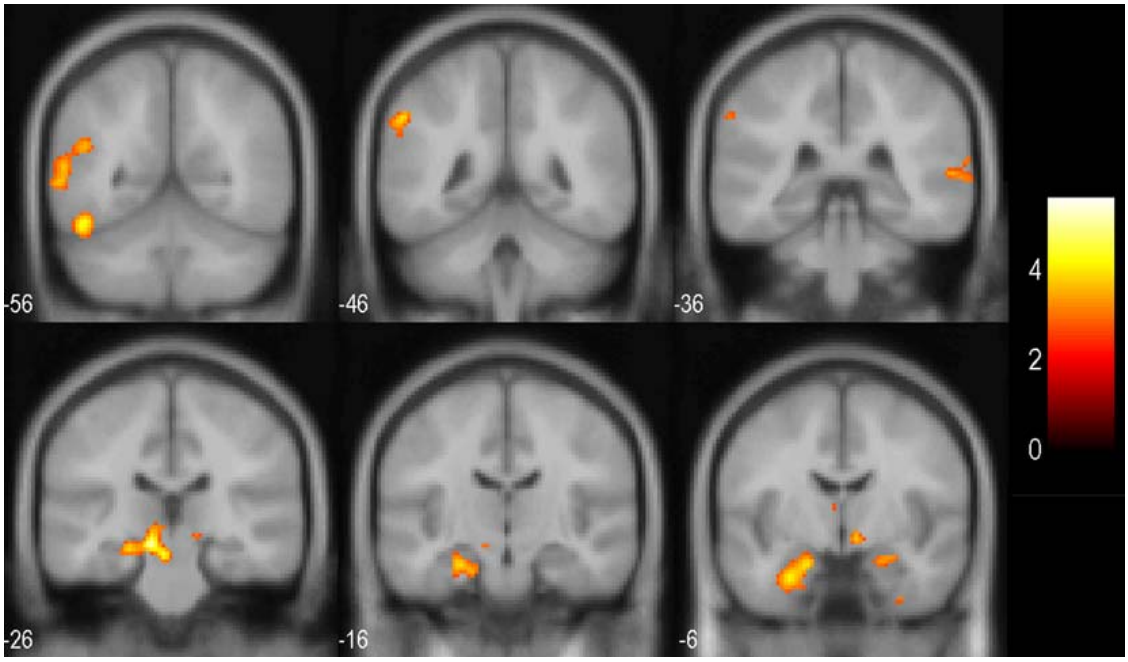

Figure 19 Coronal view of activity elicited in both language-dependent (funny – control) and visual (funny – control) humor ($p<0.005$, uncorrected, for both).

Analysis of the conjunction of the two humor types [(language humor – language controls) ∩ (visual humor – visual controls), all thresholded at $p<0.005$, cluster size > 20] revealed activity in several hedonic regions, including the midbrain and amygdala (Table 5, Figure 19).

3.5 Discussion

The results reported here demonstrate the disparate mechanisms underlying the euphoric and cognitive aspects of humor. Specifically, we show that language-dependent cartoons elicit activity in classical language areas in the left temporal lobe, while sight-gag cartoons elicit activity in higher-order visual areas. We additionally demonstrate that

both types of humor result in increased activity in reward and emotion related areas, including the nucleus accumbens and the amygdala.

The two stage model of humor consists of an initial recognition of incongruity (surprise), and the subsequent reinterpretation of the incongruent situation into a coherent whole (framework shifting) (Suls, 1972). This suggests that the details relevant to the humor require additional processing, possibly engaging feedback loops between lower level sensory areas and regions in frontal cortex associated with attention and executive function. Consistent with this model, our data show that cognitive processing during the experience of humor is domain specific, with increased activation in the modules most relevant to the element from which the humor is derived.

Sight-gag humor is dependent on visual incongruities between several elements in the cartoon. Functionally, our results show that the processing of sight-gag humor shows increased activation in higher-order visual regions bilaterally when compared to language-dependent humor, consisting of a large expanse of extrastriate regions beyond V2 (Tootell et al., 1996). Interestingly, areas V1 and V2 are not more active during the funny cartoons than they are during the non-funny cartoons, suggesting that the activation elicited by visual humor is a result of top-down modulation, rather than an increase in sensory stimulation per se. The strongest sites of activation were the precuneus and dorsolateral prefrontal cortex (BA 9/46), anatomically known as middle frontal gyrus. These two regions are associated with visual imagery (Ishai et al., 2000), contextual associations (Linden et al., 2003; Lundstrom et al., 2005; Rorie and Newsome, 2005), and conscious awareness of visual stimuli (Kjaer et al., 2001). Evidence also exists that the precuneus is active during paradigms that require varied perspective-taking (Jackson

et al., In press; Ruby and Decety, 2001) or the recruitment of theory of mind (Gallagher et al., 2000), cognitive mechanisms that are similar to the re-interpretation step that precedes "getting" a joke.

Interaction between frontal regions and stimulus-specific regions in the temporal lobe are thought to underlie recognition for faces (Haxby et al., 1994; Kanwisher et al., 1997) and objects (Riesenhuber and Poggio, 2002). Our results are consistent with this, as frontal regions and higher visual areas act reciprocally to place the cartoons' visual elements into a sensible context. This requires various inferences about spatial and conceptual relationships between objects, based on information-sparse line drawings. This cognitive effort results in the relative activation of both the parietal "where" stream as well as the temporal "what" stream of visual processing, both of which act in concert with frontal regions that integrate this processing and hold relevant information in working memory (Ungerleider and Haxby, 1994).

Activation that is present during language-dependent humor as opposed to sight-gag humor is located in left-lateralized temporal and frontal corticies. Left-hemispheric damage has long been associated with language deficits in regions associated with language processing, and the regions activated by language-dependent humor correspond strongly to classical language areas, including Broca's area, anatomically described at inferior frontal gyrus; Wernicke's area, including middle temporal gyrus and superior temporal sulcus; and the basal temporal language areas located in inferior temporal gyrus (Benson, 1993; Friederici, 2002; Just et al., 1996). Surprisingly, language-dependent humor also elicited activation increases in the region of the occipital lobe corresponding to the primary visual areas. This could arise either from increased visual input during

language humor, for example from a relatively large search pattern that includes both the caption and the picture, or from a relative suppression in primary visual activity during visual humor.

Although it is clear that a dissociation exists between the mechanisms that underlie different forms of humor, our results also emphasize the common features that characterize various types of humor. Our study replicates the results of past studies (Mobbs et al., 2003) that found heightened activity in a network of subcortical regions, including the nucleus accumbens and substantia nigra, thought to underlie the hedonic aspect of humor. For most regions, this was true not only for an investigation of the main effect of humor, but also for a parametric analysis (observing correlations of activity in these regions with varying levels of reported amusement) and for a conjunction analysis between the two different types of humor (visual and language-based). This further strengthens the evidence that humor acts similarly to primary rewards via the mesolimbic dopaminergic system. We also observed amygdala activity in both the parametric and main effects analyses, which corroborates past results (Mobbs et al., 2003; Moran et al., 2004). Recent evidence supports a role for amygdala in the processing of rewards as well as aversive events (for review, see Baxter and Murray, 2002), and animal lesion studies show that an intact amygdala is necessary to link an object to a current (as opposed to consistent) reward value. Amygdala activity may thus relate to the "re-interpretation" step in the Suls model and the associated update of the cartoon's value. Another interpretation of the amygdalar activity relates to the observation that patients with bilateral amygdala lesions fail to show normal changes in skin-conductive response (SCR) in a gambling task (Bechara et al., 1999). Changes in somatic markers such as

SCR may be concomitant with, or a crucial feature of, humor, a phenomenon that could explain the observed activity in both the amygdala and the hypothalamus

Regions of the brain highlighted in the conjunction analysis of language-based and sight-gag humor may reflect cognitive demands common to processing both types of humorous cartoons in addition to the hedonic component of humor. For example, our conjunction analysis revealed activity in the superior temporal sulcus and middle temporal lobe, regions associated with face-perception (Desimone, 1991) and with the processing of social informational cues such as the assessment of gaze and head direction (O'Doherty et al., 2003b). Inferior temporal gyrus is known to be associated with the semantic retrieval processes that occur when viewing line drawings (Mazard et al., 2005), and the hippocampus is also postulated to have a role in semantic processing under conditions of lexico-semantic ambiguity (Hoenig and Scheef, 2005). In all of these cases, it is likely that we are seeing heightened processing of relevant stimuli in the funny cartoons in comparison with the non-humorous control cartoons, analogous to the increased activity we report in domain-specific areas during the processing of language-dependent or sight-gag cartoons.

We also report in this study that humorous cartoons activate the two regions in the human brain known to have Von Economo cells (von Economo and Koskinas, 1929), a specialization in neuronal morphology that has evolved in the last 15 million years (Allman et al., 2002; Allman et al., 2005; Nimchinsky et al., 1999). Furthermore, we show that the BOLD response in these two regions, anterior cingulate cortex (ACC) and fronto-insula cortex (FI), is correlated with the subjective rating of funniness (see Figure 13). Humor involves both uncertainty (during the initial appraisal of the humorous

situation) and sociality (via laughter or other social signals), both of which have been shown to elicit activity in ACC and FI (Bartels and Zeki, 2004; Critchley et al., 2001; O'Doherty et al., 2003a; Shin et al., 2000; Singer et al., 2004a; Singer et al., 2004b). We propose that the ability to appreciate humor is related to the ability to make rapid, intuitive assessments, a skill that would be particularly adaptive during the complex social interactions typical of the hominoids, and that the von Economo cells are a phylogenetic specialization in the circuitry that underlies such fast and intuitive decisions. It is the convergence of this fast intuition with a slower, deliberative assessment that creates the cognitive mismatch upon which humor is based. A listener "gets" a joke the moment that the initial intuitive interpretation is updated, thus providing the input required to "re-calibrate" ACC and FI. We propose that a similar mechanism enables fluent social interaction. This is consistent with a recent study using a placebo paradigm, which suggests that the ACC and orbitofrontal cortex modulate expectation in a top-down manner (Petrovic et al., 2005). Another interpretation involves the regions' roles in mediating the autonomic changes that are likely to be induced by humor (Critchley, 2002; Critchley et al., 2001). Again, this is consistent with the activity we observed in the amygdala and hypothalamus, both of which have descending projections to autonomic output nuclei. Critchley suggests that these two regions play a primary role in mediating autonomic changes. These various explanations are not mutually exclusive, since the changes in expectation that occur during humor are likely to be associated with fluctuations in anticipatory arousal states. This could be the physiological correlate of the "release of tension" humor mechanism proposed by Freud (Freud, 1960).

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4 Summary and Reflections

4.1 Summary of results

In the above body of work, I describe the anatomy of the von Economo neurons. I have shown that the VENs in fronto-insula and anterior cingulate cortex form a single population when characterized on the basis of their dendritic architecture, and that, using this same criteria, this population is distinct from pyramidal neurons. In particular, I have shown that a typical VEN has a sparse dendritic tree, with less than half the total dendritic length of a typical pyramidal neuron.

I have additionally shown that the VENs express a rich array of surface receptors, many of which implicate these cells in the mediation of social decision making (see below). For example, I found that most VENs strongly express the D3 receptor, whereas only about half of the layer 5 pyramidal cells do, and that this expression is dense on the soma and on the apical dendrites. Other notable discoveries include the VEN expression of the 5HT-1b receptor, and the 5HT2b receptor, the latter of which is the first described occurrence of this receptor on cells in the human brain.

These results lend themselves to a hypothesis supporting the role of the VENs in fast decision making during uncertain circumstances, particularly in social contexts. We probed this hypothesis functionally by doing an fMRI study of humor, which activated both FI and ACC.

4.2 The social cognition hypothesis

In light of the above evidence, we hypothesize that the recently evolved von Economo neurons are a functional specialization of a circuit involved in making appropriate responses during quickly changing, ambiguous circumstances (Allman et al., 2005). Links between the von Economo cells and interoception – including, literally, "gut feelings" – could provide the basis for their role in fast decision making in the absence of explicit reasoning. In apes and humans, complex social interactions between conspecifics provide a forum in which this cognitive capacity would prove to be particularly useful. This is because participants must rapidly synthesize an enormous number of relevant but often ephemeral informational cues in order to act appropriately. We thus propose that von Economo cells mediate the rapid assessments and behavioral modifications required for the successful navigation of social interactions.

4.3 Future directions

As with any body of research, more work needs to be done. The receptor immunohistochemistry done in this paper is by no means exhaustive, and new antibodies are developed every year, increasing opportunities for exploration. Double labeling of various receptors will indicate if they are co-expressed; for example, V1a vasopressin (see Appendix) and D3 colocalization would further implicate these cells in mediating the rewarding aspects of social bonding. We can also further explore the role of the GTF2i protein that is absent in William's syndrome patients and upregulated in humans compared to other primates (see Appendix).

There are also additional, basic questions about the VENs that could be addressed in the future. For example, are the VENs inhibitory or excitatory? Certainly all of the available evidence suggests that they are excitatory – for example, they are projection neurons, and have a receptor profile similar to other layer 5 pyramidal neurons – but with the successful application of an antibody that recognizes GAD or EEAC, this question may be definitively answered. Another basic question that I was unable to explain during my tenure as a graduate student was the origin of the axon in these cells. In many Nissl-stained VENs, the axon appears to sprout from the side of the soma. However, confirming this will require either electron micrographs or the colocalization of axon-specific markers with somatic marker. Given the confluence of axons in the grey matter, this is not a straightforward task, and may require the application of an antibody specific to the axon hillock itself. Finally, I am extremely interested to see the results of the computational models of the von Economo Golgi stains. Will the VENs have a distinctive physiological "fingerprint" as a result of their unusual dendritic morphology? And if not, what else might have driven the evolution of a new cell shape so late in phylogeny?

There are additional ways to test the social cognition hypothesis (with respect to VENs) in addition to immunohistochemical and Golgi methods. Stereological counts of the VENs will be illuminating, particularly performed on brains of donors who had pathological disorders involving social dysfunction: autism, William's syndrome, acallosal agenesis, and fronto-temporal dementia. New and imaginative fMRI studies will bolster (or debunk) the hypothesis regarding FI and ACC coactivation during social

interactions. Finally, lesion studies will permit us to assess whether damage to FI results in a selective deficit in social intelligence.

5 Appendix A – V1a Receptor and GTF-2ii in the VENs

Functional imaging paradigms associated with social behavior reliably activate both VE cell regions. For example, both ACC and FI are active during the act of lying (telling untruths), and they are both active when a subject receives an unfair offer while playing the Ultimatum game(Sanfey *et al.*, 2003; Spence *et al.*, 2004). Studies by Bartels and Zeki show both regions are active when subjects view the face of their love partner or child (Bartels and Zeki, 2000; Bartels and Zeki, 2004). Singer and colleagues showed in 2004 that both VE cell regions are active when a person feels empathy for pain, that is, when they know that their loved one, outside of the scanner, is being delivered an electric shock (Singer et al., 2004b). Interestingly, the extent of activation an individual shows under these conditions is directly correlated to that individual's score on a trait measurement for empathy. Finally, in a separate study, Singer and colleagues demonstrated that left FI is specifically active when subjects view faces of individuals who are reported to behave in a trustworthy fashion (Singer et al., 2004a).

5.1 Vasopressin V1a

Figure 12 VEN from ACC labeled with a V1a receptor antibody.

Fortunately, there is an excellent molecular model that allows us to specifically implicate the von Economo neurons in these various social behaviors. A body of work by Insel and Young indicates that the oxytocin and vasopressin V1a receptors mediate social bonding (Insel et al., 1998; Lim et al., 2004; Young et al., 2001). Insel and colleagues also suggest that these molecules may interact with dopamine to impart the rewarding aspects of social bonding (Insel *et al.*, 1998). I tested adult human ACC and FI tissue for reactivity to antibodies raised against the vasopressin V1a, V1b, V2 receptors and oxytocin receptor. My results show that the antibodies specific for the V1a receptor label a subpopulation of VE cells, as well as pyramidal neurons in layers 2/3 and 5 of ACC and FI (Figure 20). V1b receptors, while apparent on a subpopulation of large pyramidal cells in layer 5 of ACC, did not label the VENs. However, the pattern of labeling was interesting in that the apical dendrites

labeled with the V1b receptor antibody formed columns that spanned layer 5 to layer 1. The vasopressin V2 and oxytocin receptor antibodies did not reveal any specific labeling.

5.2 GTF-2iRD1

One of the most remarkable immunocytochemical findings for the VENs is their strong dendritic staining with the antibody to a gene product for the gene GTF2iRD1 (see Figure 21). This finding is the result of collaboration between the Korenberg and Allman labs. GTF2iRD1 together with GTF2i are duplicated genes which are part of the set of genes that are deleted in William's syndrome (Pérez Jurado et al, 1998). The loss of this duplicated pair is associated with poor visuospatial abilities and possibly hypersocial behavior in this syndrome (Hirota et al, 2003; Korenberg, personal communication).

GTF21RD1's duplicate GTF2i is among the 25 most upregulated genes in an array of 7645 genes tested in a comparison between humans and chimpanzees (Preuss et al 2004). GTF2i expression is 2.5 to 4.2 times greater in humans than in chimpanzees. The gene products for GTF2i and GTF2iRD1 function both as transcription factors in the cell nucleus and signal transducers in the cytoplasm (Roy, 2001).

In the VENs, the gene products extend far out into the dendrites where they may mediate interactions between the dendritic periphery and gene transcription in the nucleus (Figures 21 and 22). This cytoplasmic labeling is constrained to layer 5 in humans and does not occur at all in monkeys. In monkey tissue, the antibody for this gene product labels cell nuclei only, without layer specificity (Figure 21).

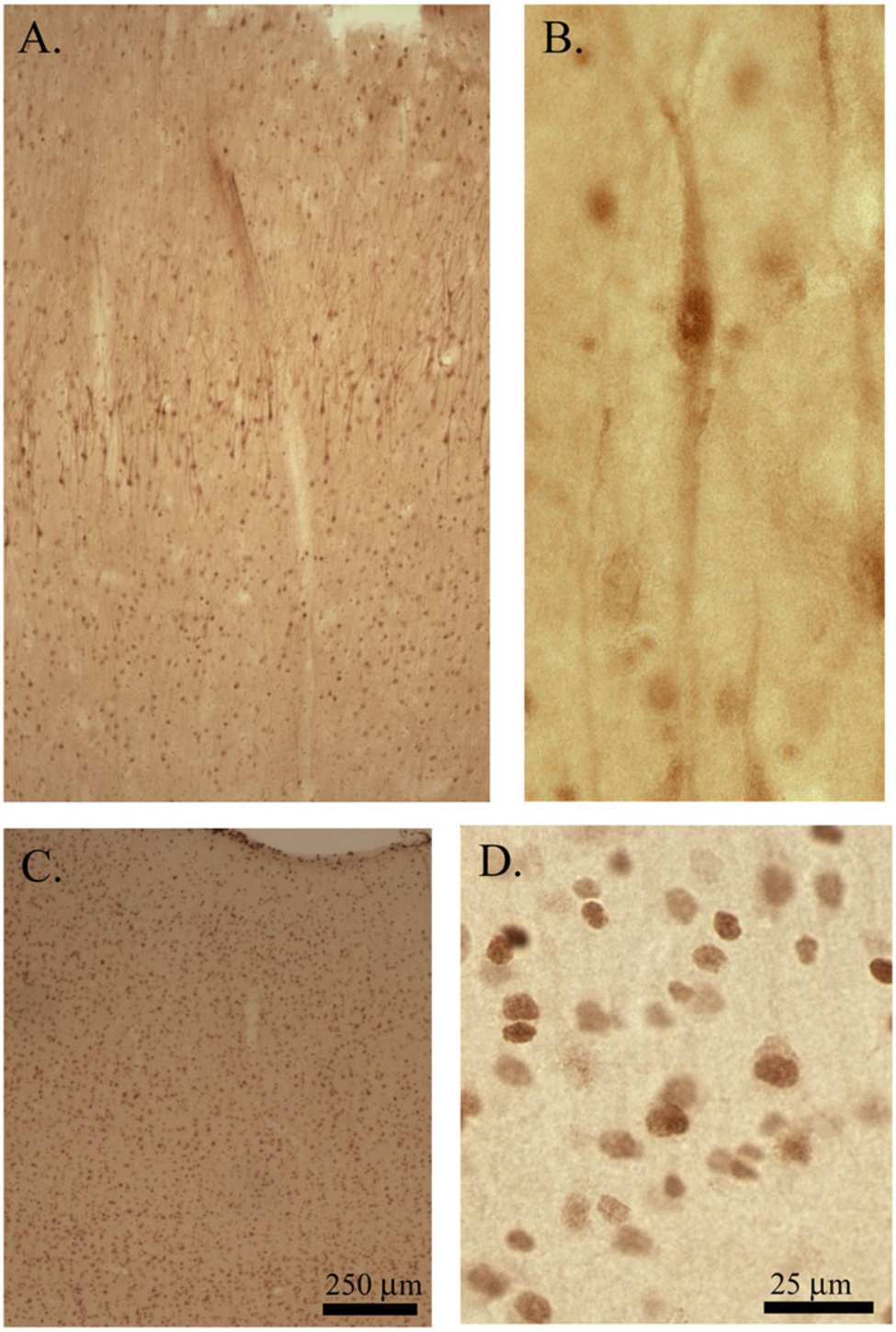

Figure 13. Labeling for the protein product of GTF2i-RD1, a gene that is deleted in William's syndrome. (A) Low power photomicrograph of human FI (16 year old male). Note extensive cytoplasmic labeling in layer 5. (B) High power image of a labeled von Economo neuron from the same specimen as in (A). (C) Low power photomicrogaph of macaque frontal cortex labeled with the same antibody as in (A) and (B). Note non specific nuclear labeling. Scale bar applies to both (A) and (C). (D). High power photomicrograph of neurons from (C). Scale bar applies to both (D) and (B).

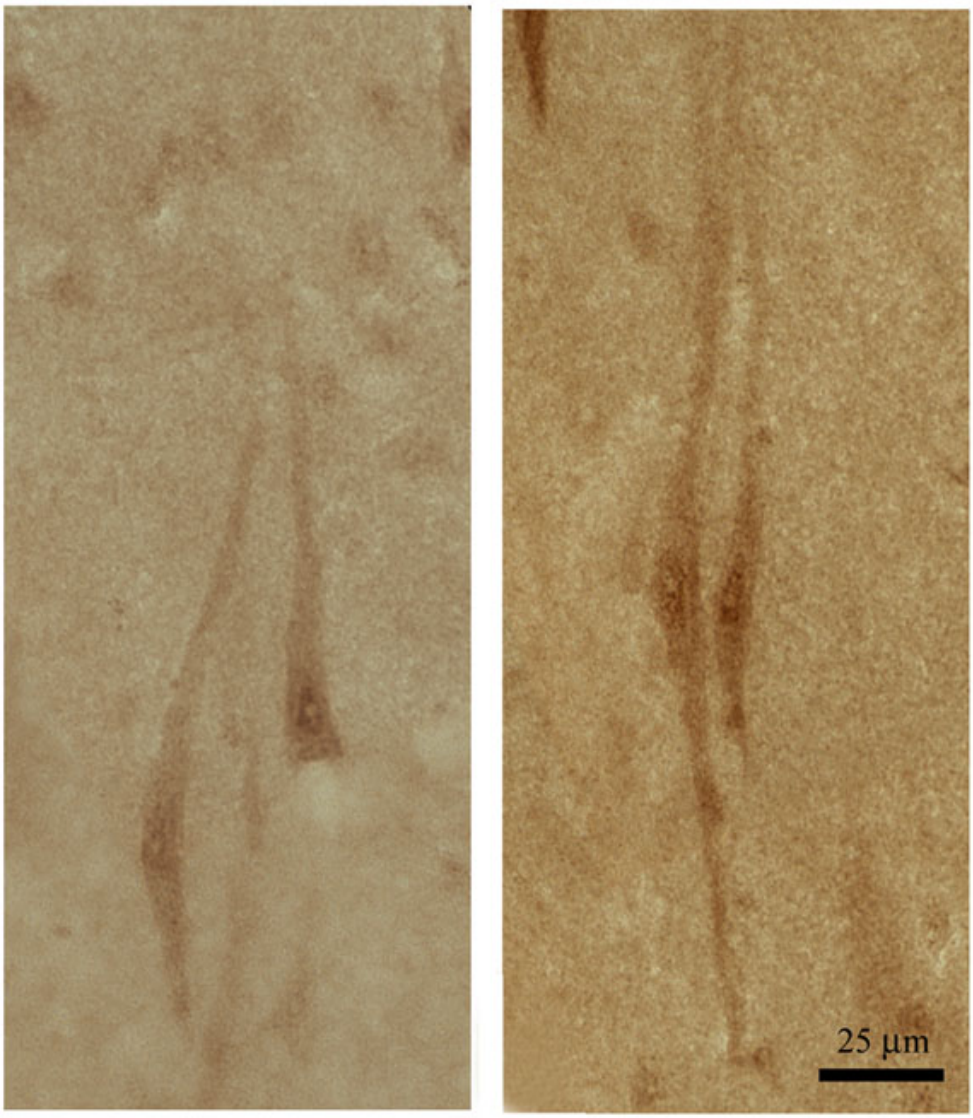

Figure 14 VENS and a pyramidal cell in ACC labeled with an antibody against the protein product of GTF2iRD1. Scale bar applies to both images.

6 Appendix B – Table of Immunohistological Results

Immunohistochemistry on human tissue is subject to inconsistencies that arise from variations in postmortem interval, fixation length, and postfixation storage time, not to mention all of the vagaries inherent in the art. For this reason, the following table should be taken with a grain of salt. For example, purely negative results, labeled "no labeling," may not necessarily indicate that absence of that particular molecule, but merely that the antibody did not recognize it. Non-specific results – labeling of everything, including extracellular space – are also denoted by "no labeling." Negative results are reportable only when a cell population that excludes the von Economo neurons is distinctly labeled by a particular antibody – for example, those for calbinden, calretinen, and parvalbumin. In some cases, the labeling profile does not lend itself to identification of the labeled elements by virtue of morphology. For example, the serotonin transporter antibody labels elements throughout the grey matter, but it is impossible to say whether the VENs are included in this labeling without a cytoplasmic or nissl counterstain. Use of fluorescent chromophores would be the best approach in these cases, for I tried in several instances to do double labeling with immunoprecipitation chromagens (i.e., DAB, TMB, and others), without satisfactory results.

antigen	VENs labeled?	comments
5HT-1b R	yes	Labels pyramids only in layer 2/3, neurons and fibers in layer 5, and fibers only in layer 6. Also labels pyramids in human BA 47, 6, 32, 9, and 10 and macaque frontal cortex.
5HT-2a R	faint	Non specific; labels all pyramids and VENs, similar to macaque results described by Goldman-Rakic.
5HT-2b R	yes	Layer 5 specific in ACC and FI. In macaque, labels frontal cortex with region specific profile.
5HT-2c R	no	No labeling
5HT-3 R	no	No labeling
β-3 adrenergic R	yes	Pyramids and VENs in layer 5 ACC; FI not tested
Calbindin	no	Layer 2/3 pyramids, glial cells in ACC
Calretinin	no	Small round bipolar cells in layer 2/3
Caspase-3	no	Pyramids, a few VENs
DAT	yes	Soma and apical of VENs, somas of layer 3 and 5 pyrs, punctate labeling throughout extracell space and white matter
GABAb R	yes	Deep layer labeling of pyramids and VENs. Most prominent on basal part of soma.
GAD	–	Labeled nucleoli only (?)
GAT-1	–	Labeled "cartridges" as reported previously, but was unable to determine whether they are apposed to VENs
GluR1	yes	Pyramids and VENs in layer 5 ACC; FI not tested
GluR2	yes	Pyramids and VENs in layer 5 ACC; FI not tested
HR1 R	no	No labeling
Kappa opioid R	no	No labeling
Map-2	yes	All neurons
Mu opoid R	no	No labeling
NMDAr1	yes	Pyramids and VENs in layer 5 ACC; FI not tested
Non-phosphoylated neurofilament	yes	Large pyramids in all layers and VENs
OxytocinR	no	No labeling
Phosphorylated neurofilament	–	Every axon
Parvalbumin	no	Multipolar non-spiny interneurons
Prolactin R	no	No labeling
Serotonin transporter	–	small punctate clusters in deep layers, many agains blood vessels
Tau	–	All fibers
Trk-b	yes	Somas and apical dendrites of VENs and layer 5 pyramids in ACC; FI not tested
Tryptophan hydroxylase	no	No labeling
Vasopressin R V1a	yes	Somatic, all pyramids and VENs
Vasopressin R V1b	no	Long apical dendrite labeling from Layer 5 pyramids up to Layer 1

Table 6 Table of immunohistochemical results. R = receptor.

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