Neuron somal size is decreased in the lateral amygdalar nucleus of subjects with bipolar disorder

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Objective: Morphometric studies of postmortem brains from subjects with mood disorders have reported altered density of glial cells in the amygdala; however, the nuclear regions have not been examined individually. **Methods:** We assessed the size and density of both neuronal and glial cells in discrete amygdalar nuclei in postmortem sections from subjects with major depressive disorder, bipolar disorder (BD) and schizophrenia and from nonpsychiatric control subjects. Three adjacent Nissl-stained sections were examined from each individual. **Results:** We report significantly decreased neuron somal size in the lateral amygdalar nucleus (LAN) and the accessory basal parvocellular nucleus (ABPC) in subjects with BD, relative to control subjects. These changes in cellular morphology were most prominent in the LAN in sections obtained from the left hemisphere. **Conclusions:** These findings add to increasing evidence for neuropathological changes in the amygdala of subjects with BD and specifically implicate the LAN and ABPC in this disorder.

Objectif : Des études morphométriques réalisées postmortem sur le cerveau de sujets atteints de troubles de l'humeur ont signalé une altération de la densité des cellules gliales des amygdales, mais les régions nucléaires n'ont pas été examinées individuellement. **Méthodes :** Nous avons évalué la taille et la densité des cellules neuronales et gliales des noyaux amygdaliens discrets dans des coupes postmortem provenant de sujets atteints de trouble dépressif majeur, de trouble bipolaire et de schizophrénie, ainsi que de sujets témoins non psychiatrisés. On a examiné trois coupes adjacentes de chaque sujet, révélées par coloration de Nissl. **Résultats :** Nous signalons une diminution importante de la taille du soma neuronal dans le noyau amygdalien latéral et le noyau parvocellulaire central accessoire chez les sujets atteints de trouble bipolaire par rapport aux sujets témoins. Ces changements de la morphologie cellulaire étaient les plus évidents dans le noyau amygdalien latéral des coupes tirées de l'hémisphère gauche. **Conclusions :** Ces constatations ajoutent aux données de plus en plus nombreuses sur les changements neuropathologiques qui surviennent dans les amygdales de sujets atteints de trouble bipolaire et mettent en cause spécifiquement le noyau amygdalien latéral et le noyau parvocellulaire central accessoire dans ce trouble.

Introduction

Bipolar disorder (BD) is a complex neurobiological disease characterized by episodes of mania and depression exhibiting increasing chronicity and severity over time. Long-term illness is associated with increasing resistance to treatment, and chronic relapse has been shown to occur in at least 80% of patients.[1,2] As such, BD is a major contributor to morbidity and mortality worldwide, as demonstrated by studies reporting increased risk of suicide ranging from 15% to 20%.[1] Understanding the pathology and pharmacotherapy of this disorder is therefore of critical importance to ensuring effective patient management leading to better long-term outcomes.

Recently, increasing attention has been focused on the role

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Medical subject headings: bipolar disorder, mood disorders, lithium, neuropsychiatry.

J Psychiatry Neurosci 2007;32(3):203-10.

Submitted July 11, 2006; Revised Oct. 12, 2006; Accepted Nov. 24, 2006

of cell death and subsequent neurogenesis in the etiology of mood disorders.[3–5] Duman and colleagues,[6] have proposed that, in depression precipitated by stress, vulnerable neurons and glia may undergo atrophy or damage caused by increased levels of glucocorticoids. These circulating glucocorticoids may alter cytoarchitectural features in specific brain regions critical to the stress response, including the amygdala and prefrontal cortex (PFC).[7] Further, studies have shown that many growth factors, including brain-derived neurotrophic factor (BDNF), are differentially expressed in various brain regions, and depending on the local environment, most can affect the viability of at least some types of neurons in vitro.[8,9] Brain imaging studies and various histological techniques have presented evidence implicating cellular loss or reduction in cell density in several key brain regions in subjects with mood disorders, including the PFC and the amygdala.[10,11]

The amygdala plays a crucial role in the formation and recollection of emotional memories and is involved in processing anxiety and fear.[12,13] These functions are particularly relevant to mood disorders, which are characterized by inappropriate emotional responses to external events. In addition, the amygdala is intimately connected to other brain regions, enabling the storage and processing of emotional stimuli in relation to other sensory events by way of interactions between the amygdala and prefrontal cognitive areas, sensory processing systems and the long-term memory system involving the hippocampus and related areas of the temporal lobe.[12,14–18] As such, dysfunction in key amygdaloid regions may lead to dysfunction of other key neuronal systems implicated in affective and behavioural regulation.

It is therefore not surprising that increased amygdalar activity has been reported in subjects with BD,[19] and there is some evidence to suggest that sustained activity in the amygdala during emotional processing might be associated with depression as well.[20–23] In support of these findings, volumetric changes have been observed in this region in subjects with major depressive disorder (MDD)[24–27] and BD.[28–37] Moreover, 2 recent postmortem studies report reduced glial density and glia : neuron ratio in subjects with depression who were taking antidepressant medication.[38,39] However, the inconsistencies between the findings reported in these studies remains a significant challenge, with some reporting increased and others reporting decreased amygdalar volume in subjects with mood disorders. One possible factor might be the amygdalar heterogeneity—there are significant functional and cytoarchitectural differences between the nuclei that constitute the amygdalar formation. One study[24] observed volumetric reductions associated with MDD only in the core amygdalar, consisting of the accessory basal, basal and lateral nuclei. Therefore, to extend previous findings in light of amygdalar heterogeneity, we examined changes in neuron and glial cell size and density in each of these nuclei.

Methods

Postmortem brain tissue

Formalin fixed human postmortem amygdala sections were generously provided by the Stanley Foundation Neuropathology Consortium. The family of each deceased individual was contacted by a pathologist to make a preliminary diagnosis and to request permission for donation of the brain and release of the deceased's medical records. Samples consisted of 4 adjacent 10-µm thick coronal sections from subjects with MDD, BD and schizophrenia (SCZ) and from nonpsychiatric, nonneurological comparison subjects matched for age, sex, postmortem interval (PMI), brain pH and messenger ribonucleic acid (mRNA) quality. We excluded 7 subjects from our analyses, because the levels of the amygdala sampled in these sections were outside the rostral-caudal boundaries used in the current study; the final sample size comprised 15 control subjects, 11 subjects with BD, 14 with MDD and 13 with SCZ. The demographic characteristics of these subjects are provided in Table 1. Diagnoses were independently established by 2 senior psychiatrists from past medical and psychiatric records and by telephone interviews with relatives, where necessary, using the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV)[40] criteria; a third psychiatrist was consulted in cases of disagreement. Detailed information on the composition and characteristics of this sample are available in previously published papers.[41–46]

Histochemical staining

One section from each subject was stained for myelin, using Luxol Fast Blue (Luxol Fast Blue MBSN, Solvent 38; Sigma-Aldrich Canada Ltd., Oakville, Ont.). Tissue sections were

Table 1: Subject demographics

Demographics	Group; mean (SD); and range*			
	Control subjects, *n* = 15	BD subjects, *n* = 11	MDD subjects, *n* = 14	SCZ subjects, *n* = 13
Age, yr	48.1 (10.7); 29–68	44.0 (10.2); 30–57	46.9 (9.6); 30–65	46.6 (12.9); 25–62
Sex (male/female)	9/6	7/4	8/6	8/5
Hemisphere (left/right)	8/7	6/5	8/6	8/5
Treated with lithium (+/–)†	—	4/7	2/12	—
Formalin fixation, mo	4.4 (3.9); 1–13	9.73 (4.2); 2–16	8.6 (6.8); 1–19	9.3 (7.1); 3–31
PMI, hr	23.7 (9.9); 8–42	32.9 (16.4);13–62	28.9 (9.5); 12–47	32.8 (13.1);12–61

SD = standard deviation; BD = bipolar disorder; MDD = major depressive disorder; SCZ = schizophrenia; PMI = postmortem interval.
*Unless otherwise indicated.
†+ = treated with lithium at time of death; – = not treated with lithium at time of death.

deparaffinized by exposure to xylenes and hydrated through a graded alcohol series. Sections were immediately immersed in a 0.1% solution of Luxol Fast Blue in 96% ethanol and 0.05% acetic acid. After staining overnight at 56°C, sections were washed in distilled water, followed by an additional wash in phosphate buffered saline (PBS). Sections were then incubated in 0.05% aqueous lithium carbonate followed by 70% ethanol, until staining intensity was adequate for differentiation of cells as determined by examination under an Eclipse E600 microscope at 4 × magnification (Nikon Canada, Mississauga, Ont.) and counterstained for Nissl substance before dehydration by passage through a graded alcohol series.

Three additional adjacent sections from each subject were stained for total cell number with the Nissl method. Briefly, tissue sections were deparaffinized by exposure to xylenes and hydrated through a graded alcohol series. Subsequently, sections were washed in PBS for 5 minutes and incubated in 0.1% thionin (Sigma-Aldrich Canada Ltd., Oakville, Ont.)/0.1 M sodium acetate for 30 minutes at room temperature. Sections were then incubated in 95% ethanol until staining intensity was adequate for differentiation of cells as determined by examination under an Eclipse E600 microscope at 4 × magnification (Nikon Canada, Mississauga, Ont.) and dehydrated by passage through a graded alcohol series. Subsequently, slides were mounted with cover slips using VECTASHIELD® mounting medium (Vector Laboratories, Burlington, Ont.).

Delineation of amygdalar nuclei

The amygdala consists of several nuclei that can be distinguished on the basis of cell size and configuration and the tracts of myelinated fibres that run between them. Therefore, to accurately delineate amygdalar nuclei, myelin/Nissl stained sections were examined for myelinated tracts and cellular architecture under a dissecting microscope. With reference to criteria and illustrations presented in various brain atlases[47] and using the same amygdalar regions identified in a previous study conducted by our laboratory in this subject sample,[48] we delineated 4 selected nuclei: accessory basal parvocellular (ABPC) and accessory basal magnocellular (ABMC), basal and lateral nuclei (see Fig. 1). The resulting nuclear boundaries were overlaid on each of the 3 adjacent Nissl-stained sections for each subject and further refined based on cellular architecture observed under a light microscope with a 4 × objective lens. We confirmed these boundaries, using a series of acetylcholinesterase-stained sections encompassing the full rostral-caudal extent of the amygdala (generously provided by Dr. Gregory Ordway). The anatomic position of each slide was established relative to this series to ensure comparison of equivalent levels in the amygdala; all samples were obtained from a level approximately 10 mm–11 mm caudal from the rostral pole of the amygdalar complex.

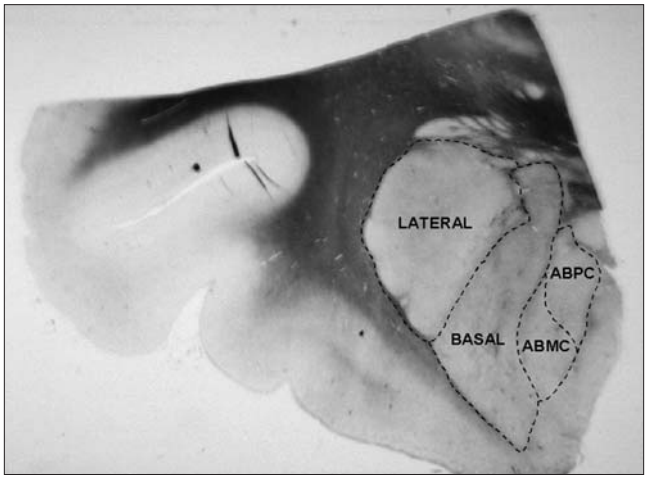

Fig. 1: A representative image of a section of postmortem brain stained for myelinated fibres (Luxol Fast-Blue) and Nissl substance (Thionin). Myelinated tracts and cellular architecture were compared with criteria and illustrations presented in various brain atlases, which allowed us to delineate the amygdalar nuclei, as demonstrated by dotted lines. The nuclei included the accessory basal magnocellular and parvicellular (accessory basal parvocellular [ABPC] and accessory basal magnocellular [ABMC], respectively) basal and lateral nuclei.

Quantitative analyses

Nissl-stained sections were examined blind to experimental groups with a Retiga camera (Q-Imaging Corporation, Burnaby, BC) attached to an Eclipse E600 microscope fitted with an Optiscan motorized stage (Prior Scientific, Cambridge, UK). Using a computer running Histometrix 6.0 imaging software (Medical Solutions PLC, Nottingham, UK), the surface area of each amygdalar nucleus was determined and overlaid with a uniform random grid. At each intercept, an unbiased sampling frame (USF) corresponding to 65% of the field of view was observed through a 100 × objective lens; the number of USFs for each region of interest was determined, such that the corresponding coefficient of error was no greater than 0.1. Nissl stained cells were identified as neurons based on the presence of a clearly visible nucleolus and stained cytoplasm; glial cells were identified by their relatively smaller nucleus and the absence of nucleoli and visible cytoplasm. Only neurons with clearly visible nucleoli and glial nuclei were counted, contingent on the occurrence of these structures in the XY inclusion frame of the USF. The cross-sectional area in micrometers of each cell was determined by the nucleator probe; this consisted of 3 isotropic lines drawn through the nucleolus (neurons) or nucleus (glia), upon which were marked the points of intersection with the visible margin of the cell soma (see Fig. 2). Results were averaged across all 3 sections for each subject.

Data and statistical analyses

We included differences in demographic characteristics between diagnostic groups in age, formalin fixation time and PMI by analyses of variance (ANOVAs); we assessed differences in sex and hemisphere with Pearson's chi-square analy-

sis ($p < 0.05$ in all cases). Changes in measures of cell size or density were expressed as the mean, standard error of the mean (SEM) for each amygdalar subregion. We used independent-sample Student's t tests to assess the influence of sex and we used multiple correlation analyses to examine the effects of age, formalin fixation time and PMI on neuronal and glial cell densities and soma sizes ($p < 0.05$). Differences in individual parameters were analyzed with separate repeated-measures (nuclei) analyses of covariance (ANCOVAs), with age, sex, formalin fixation time and PMI as covariates. Because all comparisons were made with the CTL group, the critical value was adjusted with Dunnett's post hoc test; this value was further adjusted by the Bonferroni procedure to reflect the number of nuclei examined (diagnosis: $p < 0.0042$, 4 nuclei, CTL vs. BD, MDD, SCZ; treatment: $p < 0.0063$, 4 nuclei, CTL vs. treatment). Analyses were repeated on subjects stratified by brain hemisphere.

Results

There were no differences between diagnostic groups in age ($F_{0.302} = 3.52$, $p = 0.824$), formalin fixation time ($F_{2.623} = 3.52$, $p = 0.61$), PMI ($F_{1.575} = 3.52$, $p = 0.207$), sex ($\chi^2_{3.52} = 0.118$, $p = 0.990$) or hemisphere ($\chi^2_{3.52} = 0.215$; $p = 0.975$). Further, there were no significant differences in neuronal or glial size or density across sex and no significant correlations were observed between neuronal or glial size or density and age, formalin fixation time or PMI in any amygdalar nucleus (Table 2).

As described above, the full extent of the amygdala was unavailable for analysis. Within the available sections, we found no differences between diagnostic groups in the surface area of any amygdalar nucleus (data not shown). In addition, we found no differences between diagnostic groups in either glial density, glial size, or neuron density (Fig. 3 a-c). However, we did observe a significant effect of diagnosis on neuron somal size across nuclei ($F_{3.46} = 3.489$, $p = 0.023$). Specifically, although no changes were observed between the control and either the MDD or SCZ groups, somal size was significantly decreased (29.7%) in the lateral amygdalar nucleus (LAN) in subjects with BD compared with control subjects ($p = 0.003$) and decreased (28.3%) in the ABPC nucleus ($p = 0.009$; Fig. 3 d). However, we found no effect of treatment with lithium, antidepressant drugs, or anticonvul-

Table 2: Correlation of age and postmortem interval with measures of cellular morphology in each subnuclear region

Measure	Region	Pearson correlation		
		Age	Formalin	PMI
Neuron size	ABMC	–0.077	–0.175	0.111
	ABPC	–0.003	0.189	–0.116
	Basal	0.121	0.057	–0.033
	Lateral	0.036	0.166	0.06
Neuron density	ABMC	–0.001	–0.107	–0.088
	ABPC	0.027	0.021	–0.198
	Basal	0.045	0.081	–0.032
	Lateral	0.039	–0.012	–0.114
Glial size	ABMC	–0.101	–0.223	–0.001
	ABPC	0.086	0.124	–0.215
	Basal	0.102	0.005	–0.066
	Lateral	0.031	0.047	–0.14
Glial density	ABMC	0.026	–0.087	–0.102
	ABPC	0.06	0.144	–0.203
	Basal	0.025	–0.076	–0.035
	Lateral	0.144	0.252	–0.076

PMI = postmortem interval; ABMC = accessory basal magnocellular; ABPC = accessory basal parvocellular.

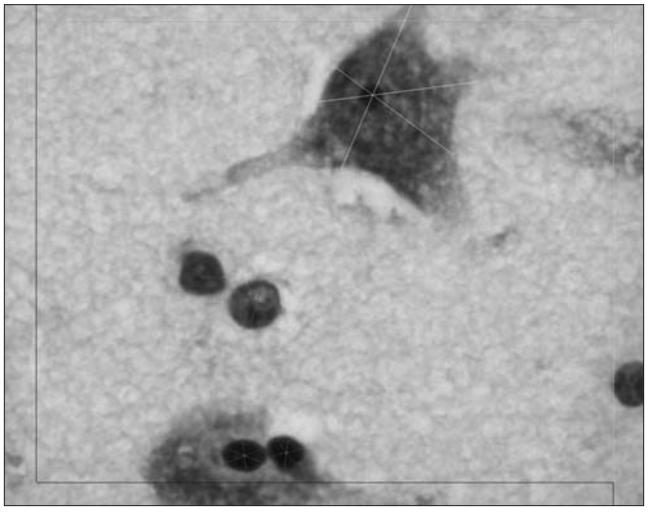

Fig. 2: A representative image acquired from a Nissl-stained section of postmortem brain showing a typical unbiased sampling frame (UFS) at 100 × magnification. Neurons are identified by a clearly visible darkly stained nucleolus in a lighter stained nucleus, the presence of an axon hillock or dendrites, or both, and their larger size; glial cells are smaller, with generally little visible cytoplasm.

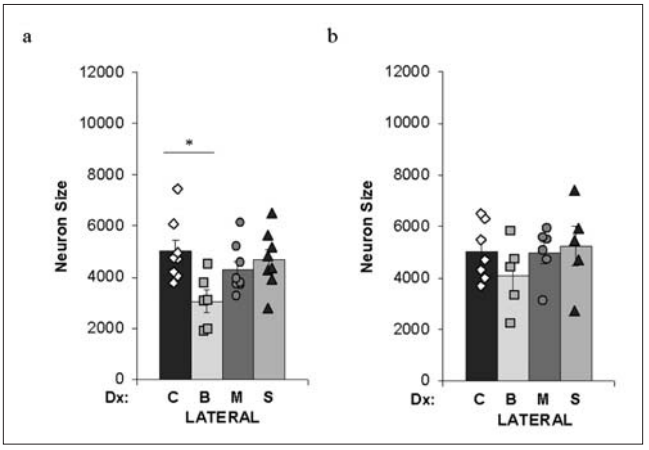

Fig. 4: Changes in neuron somal size in the amygdala from psychiatric subjects compared with control subjects, stratified by hemisphere. Graphs of neuron somal size in the left (a) and right (b) hemispheres from nonpsychiatric control subjects (C) and subjects with bipolar disorder (B), major depressive disorder (M) and schizophrenia (S) in the lateral amygdalar nucleus. Each symbol represents 1 subject. Bar graphs indicate the average, with the standard error of the mean for each group. *$p < 0.0042$ (Bonferroni adjusted Dunnett's post hoc tests).

sants (i.e., valproate or carbamazepine) at the time of death on measures of cellular pathology in any of the 4 amygdaloid nuclei examined (data not shown). We were unable to observe an association between any measure and family history of BD or MDD, compared with control subjects (data not shown). This is an important variable in some earlier findings using this same subject sample.[49] Similarly, there where no discernable effects of suicide as a cause of death (data not shown).

Given the importance of laterality in previous studies of the amygdala,[38] we repeated our analyses on subjects stratified by hemisphere. Diagnosis had a significant effect on neuron somal size in the left hemisphere ($F_{3,23} = 3.485$, $p = 0.033$), with smaller soma in the LAN of BD subjects ($n = 6$) relative to control subjects ($n = 8$; average decrease 39%; $p = 0.003$; Fig. 4a); no significant effects of any other factor were observed. Moreover, no significant changes were observed in any measure in the right hemisphere (Fig. 4 b).

Discussion

This is, to our knowledge, the first study to examine changes in cellular morphology in discrete amygdalar nuclei. We report decreased neuron somal size in the LAN and ABPC regions — findings that add to evidence linking BD with cellular changes in the amygdala. Decreased somal size is suggestive of reduced axodendritic arbours or aberrant synaptic connections.[50,51] Thus, our observations suggest that neurons in certain amygdalar regions may have less extensive arborisation or decreased afferent or efferent (or both) connectivity in people with BD. This may result from altered expression of neurotrophins or postreceptor signalling molecules. Alternatively, these changes may occur subsequent to a loss of input from other brain regions; this is especially relevant given reports of abnormal cellular morphology in the prefrontal cortex and anterior cingulate cortex,[10] both of which project to the basolateral complex (basal, lateral and AB nuclei) of the

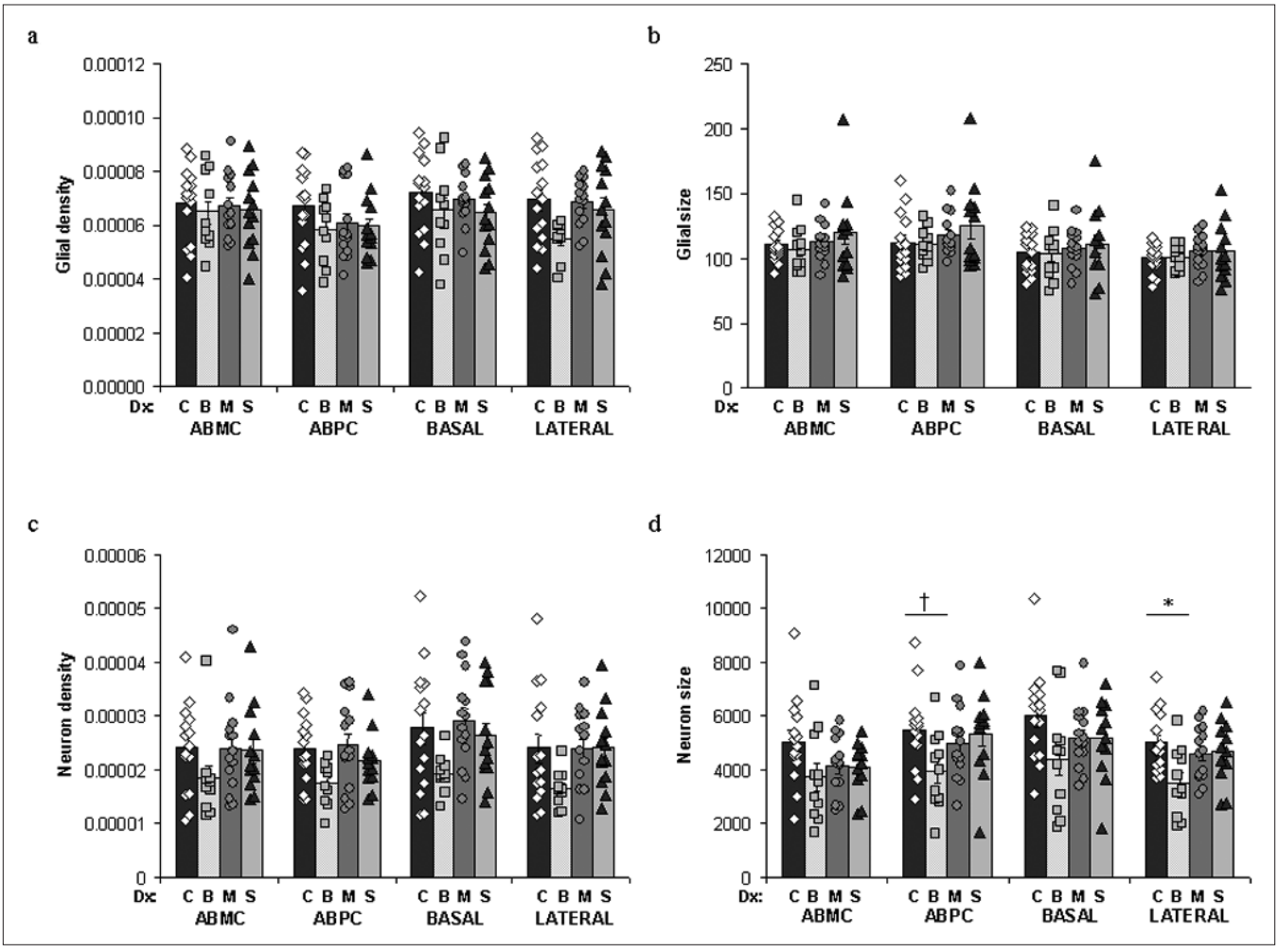

Fig. 3: Measures of cellular pathology in amygdala from psychiatric subjects, compared with control subjects. Graphs of glial cell density (a), glial cell size (b), neuron density (c), and neuron somal size (d) in nonpsychiatric control subjects (C) and subjects with bipolar disorder (B), major depressive disorder (M) and schizophrenia (S) in each subnuclear region, including the accessory basal magnocellular and parvicellular (ABMC and ABPC, respectively), basal and lateral nuclei. Each symbol represents 1 subject. Bar graphs indicate the average, with the standard error of the mean for each group. *$p < 0.0042$, †$p < 0.01$ (Bonferroni adjusted Dunnett's post hoc tests).

amygdala.[18] As such, the current findings suggest a possible cellular basis for the volumetric changes observed in subjects with mood disorders.

From a clinical perspective, these results are especially interesting given the well-documented role of the amygdala in regulating emotional responses. The LAN in particular is thought to link cortical brain regions involved in processing sensory stimuli with structures responsible for eliciting emotional responses to these stimuli.[18] As lesions to this region eliminate conditioning to aversive stimuli[52] and lead to inappropriate responses to threatening situations,[12] the aberrant neuronal morphology observed here may be particularly relevant to understanding the increased risk-taking behaviour and poor judgement demonstrated by subjects in the manic state.

Although several postmortem cytoarchitectural studies have been published showing neuronal and glial pathology in the prefrontal cortex, anterior cingulate and thalamus, only 2 other such studies have been conducted to date in the amygdala, and neither of these assessed measures of cell size.[38,39] Consistent with these studies, we were unable to detect any significant changes in neuronal or glial density in the amygdala of BD subjects. Our results are at variance with prior observations of decreased glial density and glia:neuron ratio in BD subjects not treated with lithium, although the previous studies included only 2 subjects who were not treated with lithium. Likewise, reports of decreased glial cell density in subjects with MDD were not confirmed in our sample; however, Hamidi and colleagues[39] recently reported that the reduction in glial cell density in subjects with MDD may be primarily due to changes in oligodendrocytes. We have not assessed these cells directly in our sample, indicating that further experimentation is required. In addition, the average age of MDD subjects in both studies (77.7 yr, range 59–90) was markedly older than that of our sample (46.9 yr, range 30–65). This suggests that age, or perhaps the duration or severity of the illness or both, may play a role in abnormal glial measures in MDD.

Interestingly, 2 recent postmortem studies of the amygdala, although they reported decreased glial density in subjects with MDD, observed left-lateralized cellular abnormalities.[38] In the current study, stratification of the sample by hemisphere led to the observation that neuron somal size is decreased predominantly in the left hemisphere. Despite the smaller sample size resulting from this stratification, these findings indicate that abnormalities in neuron cell size may be left-lateralized in subjects with BD and suggest that further investigation is warranted. Moreover, several imaging studies in the amygdala from patients with BD have also reported left-lateralized abnormalities in both metabolic activity and volume.[28,34–37,53] There are also studies reporting bilateral changes,[29–33] and at least one study has shown right-lateralized changes in metabolic activity in the amygdala of BD patients.[19] The difficulty in accurately delineating amygdalar nuclei with brain imaging techniques and the differences in cellular composition and morphology between discrete amygdalar nuclei may in part account for the discrepancies between these findings.

As in subjects with BD, imaging studies of the amygdala in patients with SCZ exhibit a great deal of variability. Meta-analyses of early imaging studies generally report slight but significant volume reductions in this region in subjects with SCZ.[54–58] However, many of these studies considered the hippocampus and the amygdala jointly, and their findings may not be attributable to the amygdala specifically. Studies that have attempted to evaluate the amygdala independently tend to report no significant differences in amygdalar volumes between people with SCZ and control subjects[30,59–61]; reduced amygdalar volumes have been reported in some studies.[62,63] With respect to investigations conducted in postmortem brain tissue, 1 early study reported a reduction in amygdalar volume in subjects with SCZ,[64] but more recent studies have reported no statistically significant changes in the volume of this structure between people with SCZ and control subjects.[65,66] Moreover, Pakkenberg,[67] who examined neuron and glial cell number in the basolateral nucleus of the amygdala, was also unable to observe changes in these measures in postmortem brain tissue from people with SCZ. This study supports and extends these latter findings, further suggesting that SCZ is not characterized by overt changes in neuronal or glial morphology or density in the constituent nuclei of the amygdala.

There are several potential limitations to this study. First, the thickness of the tissue sections did not allow for the use of an optical dissector method of cell counting with defined upper and lower guard zones; thus, our results may be biased as a result of overcounting. We did, however, conduct our analyses in 3 adjacent sections to ensure the intersample reliability of our data and used nucleoli as discrete counting units for neurons to minimize these effects. Second, we did not have access to the entire rostral-caudal extent of the amygdala. Because random sampling of an entire structure is necessary to determine total cell number, we can only present data on neuronal and glial density for a specific anatomic level corresponding to 10 mm–11 mm caudal from the rostral pole of the amygdalar complex. The anatomic position of each section was confirmed by comparison with a complete series of acetylcholinesterase-stained amygdalar sections. Third, decreased neuron somal size in the LA and ABPC regions in BD may be secondary to the effects of medication used by these patients. However, the number of patients treated with multiple drugs in this study complicates attempts to definitively segregate the individual contribution of these medications to the current findings. Similarly, sufficient data on each subject's medication history, dosage and length of time ill were not available, given the retrospective nature of each subject's clinical assessment entailed by the use of postmortem samples. In the current sample, only 2 subjects with BD were both never treated and negative for a history of substance abuse, which precluded focused analyses on these subjects. Therefore, although we report no significant medication effects, further postmortem studies in subjects for whom a detailed medical history is available are needed. Finally, we present data indicating a lateralization of changes in neuron somal size to the left hemisphere, which is concordant with several investigations.[28,34–37,53] Although these findings are interesting, their

confirmation in a larger sample, and in subjects from which both hemispheres are available, is required.

Conclusion

We observed a marked reduction in neuron somal size in the LAN and ABPC nuclei of the amygdala of subjects with BD; these changes are especially pronounced in sections obtained from the left hemisphere. Since somal size is considered a marker for axodendritic outgrowth,[50] this finding extends previous reports of neuronal pathology in several brain regions previously associated with BD, including the prefrontal cortex, anterior cingulate and hippocampus, in addition to the amygdala.[11] The present study emphasizes the need to recognize the heterogeneous nature of the amygdala, as reflected in the cytoarchitectonic composition of different amygdalar nuclei.

Acknowledgements: Thanks to the Stanley Foundation Brain Bank and Drs. M. Knable, E.F. Torrey, M. Webster and R. Yolken for providing the postmortem brain tissue. This research was funded by Canadian Institutes for Health Research grant MOP-14998 and the Stanley Medical Research Institute. Dr. Bezchlibnyk is supported by an Ontario Graduate Scholarship.

Competing interests: None declared.

Contributors: Drs. Bezchlibnyk, Wang, MacQueen, McEwen and Young designed the study. Drs. Bezchlibnyk and Sun aquired the data, and Dr. Bezchlibnyk analyzed it. Dr. Bezchlibnyk wrote the article, and Drs. Sun, Wang, MacQueen, McEwen and Young revised it. All authors gave final approval for the article to be published.

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