Research Paper

Gene expression deficits in pontine locus coeruleus astrocytes in men with major depressive disorder

Michelle J. Chandley, PhD; Katalin Szebeni, MD; Attila Szebeni, PhD; Jessica Crawford, BSc; Craig A. Stockmeier, PhD; Gustavo Turecki, MD, PhD; Jose Javier Miguel-Hidalgo, PhD; Gregory A. Ordway, PhD

Chandley, K. Szebeni, A. Szebeni, Crawford, Ordway — Department of Pharmacology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tenn., USA; Stockmeier, Miguel-Hidalgo — Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, Miss., USA; Turecki — Department of Psychiatry, McGill University, Montréal, Que., Canada

Background: Norepinephrine and glutamate are among several neurotransmitters implicated in the neuropathology of major depressive disorder (MDD). Glia deficits have also been demonstrated in people with MDD, and glia are critical modulators of central glutamatergic transmission. We studied glia in men with MDD in the region of the brain (locus coeruleus; LC) where noradrenergic neuronal cell bodies reside and receive glutamatergic input. **Methods:** The expression of 3 glutamate-related genes (*SLC1A3*, *SLC1A2*, *GLUL*) concentrated in glia and a glia gene (*GFAP*) were measured in postmortem tissues from men with MDD and from paired psychiatrically healthy controls. Initial gene expression analysis of RNA isolated from homogenized tissue (n = 9–10 pairs) containing the LC were followed by detailed analysis of gene expressions in astrocytes and oligodendrocytes (n = 6–7 pairs) laser captured from the LC region. We assessed protein changes in GFAP using immunohistochemistry and immunoblotting (n = 7–14 pairs). **Results:** Astrocytes, but not oligodendrocytes, demonstrated robust reductions in the expression of *SLC1A3* and *SLC1A2,* whereas *GLUL* expression was unchanged. *GFAP* expression was lower in astrocytes, and we confirmed reduced GFAP protein in the LC using immunostaining methods. **Limitations:** Reduced expression of protein products of *SLC1A3* and *SLC1A2* could not be confirmed because of insufficient amounts of LC tissue for these assays. Whether gene expression abnormalities were associated with only MDD and not with suicide could not be confirmed because most of the decedents who had MDD died by suicide. **Conclusion:** Major depressive disorder is associated with unhealthy astrocytes in the noradrenergic LC, characterized here by a reduction in astrocyte glutamate transporter expression. These findings suggest that increased glutamatergic activity in the LC occurs in men with MDD.

Introduction

Major depressive disorder (MDD) has a lifetime prevalence of 16% in the United States.[1] Antidepressant drugs are the most used intervention for those with a diagnosis of depressive disorders, but the road to remission is long and uncertain, with 40% of patients never reaching full remission and at least 15% not experiencing any symptomatic improvements.[2] Elucidating the biological bases of MDD is likely to provide novel targets for the development of more effective drugs, or at the very least, adjunctive treatments for existing antidepressants that increase the chance of remission.

Speculation that norepinephrine plays a role in depressive disorders dates back to the early 1950s, and research since then increasingly supports this. The locus coeruleus (LC) in the pontine brainstem contains the cell bodies of the major source of norepinephrine in the brain and has been the subject of numerous investigations regarding the neuropathology of MDD.[3] The human LC is an area with very high densities of radioligand binding of antidepressant drugs to monoamine oxidase,[4] the norepinephrine transporter[5] and the serotonin transporter.[6] Numerous postmortem studies demonstrate abnormal neurochemistry of the noradrenergic LC in people with MDD and in people who died by suicide. A role of

Correspondence to: G.A. Ordway, Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, PO Box 70577, Johnson City TN 37614; Ordway@etsu.edu

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norepinephrine in depression and antidepressant drug action is supported by laboratory animal studies demonstrating neurochemical changes in the LC following chronic stress, including changes that are reversed following antidepressant administration, that resemble LC pathology in humans with MDD.[3] Collectively, laboratory animal and human findings implicate elevated activity of the noradrenergic LC in depression[7] and medication-induced reductions of LC activity as an important biological effect of antidepressant drugs.[8]

Elevated LC activity in people with MDD could be secondary to elevated excitatory glutamatergic input to the LC. Locus coeruleus activity is modulated by glutamatergic input primarily from 2 areas: the paragigantocellularis nucleus and the prefrontal cortex. Interestingly, elevated glutamate levels have been reported in human serum, the occipital cortex, the prefrontal cortex and plasma of those with a diagnosis of MDD, with a positive correlation found between symptom severity and plasma concentration of glutamate.[9–12] To our knowledge, glutamate levels have not been measured in the brainstem of people with MDD. However, chronic stress, which is associated with MDD, increases LC activity at least in part through elevated excitatory glutamatergic input.[13] Furthermore, glutamatergic abnormalities in the postmortem LC of people with MDD, including elevated *N*-methyl-D-aspartate receptor subunit expression and reduced neuronal nitric oxide synthase expression, have been observed.[14,15]

A growing body of evidence shows a loss of glia or glia dysfunction in people with MDD; results have shown reduced glia, including oligodendrocytes and GFAP-labelled astrocytes, in cerebral cortical areas and the amygdala.[16–19] Since the actions of glutamate are terminated largely by its removal from the synaptic junction by the excitatory amino acid transporter 1 (EAAT1) and excitatory amino acid transporter 2 (EAAT2) found on glia, a reduction in glia or glia health could contribute to elevated glutamate in the brain. Recently, Bernard and colleagues[20] demonstrated reductions in the levels of expression of several genes known to be enriched in glia in LC tissue from people with MDD compared with matched controls. Among these genes were *SLC1A3* (encoding EAAT1), *SLC1A2* (encoding EAAT2) and *GLUL* (encoding glutamine synthase). Reduced uptake of glutamate by LC glia, as a result of reduced *SLC1A3* and *SLC1A2* expression, could directly contribute to altered glutamatergic signalling in the LC in people with MDD, as has been postulated previously.[14,15] However, the study by Bernard and colleagues[20] did not determine whether reduced levels of expression of glia-enriched genes were in fact isolated to glia, did not determine which type of glia was affected, and ultimately could not rule out the possibility that reduced glia gene expression was a result of a reduced number of glial cells in the LC region dissected for the gene expression studies. In the present study, we investigated the cellular source of altered glutamate transporter gene expression in the LC of people with MDD using postmortem brain tissues from people with characterized MDD and from psychiatrically healthy controls. We first attempted to reproduce the findings of Bernard and colleagues.[20] Then we used laser-capture microdissection to specifically measure gene expression in LC astrocytes and oligodendrocytes. Our results provide evidence of compromised astrocytes in the LC of people with MDD, characterized here by a reduced expression of *SLC1A3, SLC1A2* and *GFAP*.

Methods

Brain tissues

Brain tissues were collected at autopsy, as previously described, at the Cuyahoga County Coroner's Office in Cleveland, Ohio, in accordance with approval from the University Hospitals Case Medical Center Institutional Review Board for Human Investigation. For the psychiatric assessment of our study sample, the next-of-kin completed the Structured Clinical Interview for DSM-IV psychiatric conditions (SCID) on behalf of the decedents, as described previously.[21,22] We also obtained brain tissues from 4 people from the Quebec Suicide Brain Bank at the Douglas Hospital Research Centre, as approved by the Douglas Hospital Research Centre's Research Ethics Committee. These samples were evaluated in a similar manner as those from the Cuyahoga County collection in that the SCID and SCID-II were completed retrospectively by the next-of-kin.[23,24] Finally, we obtained tissues from 8 people from the Brain Tissue Donation Program at the University of Pittsburgh, and these tissues were subjected to similar retrospective psychiatric evaluation techniques and evaluations of medical records.[25] For this collection, all procedures were approved by the University of Pittsburgh's Institutional Review Board and Committee for Oversight of Research Involving the Dead.

In total, brain tissues from 20 psychiatrically healthy control men and 19 men with a diagnosis of MDD were used (Table 1). Blood and urine samples from all decedents were examined for the presence of psychotropic medications and substances of abuse. All controls were void of any depressive disorder diagnosis at the time of death or in the past. Detailed information, including causes of death and comorbidities, on the control and MDD groups is provided in Appendix 1, Table S1, available at cma.ca/jpn. The samples in both groups were matched for age, pH, RNA integrity number (RIN) and nicotine history as closely as was possible with the available tissues (Table 1). Causes of death and comorbidities are not included in Table 1 to protect the identities of the decedents.

Tissue preparation and sectioning

Blocks of tissue containing the right prefrontal cortex (Brodmann area [BA] 10) and the pontine LC were cryopreserved at autopsy and stored at –80°C. Tissues were sectioned using a cryostat microtome (Leica CM3050S). For cytohistochemical staining, tissue was sectioned at 20 µm increments, desiccated overnight and stored at –80°C until use. For real-time quantitative polymerase chain reaction (qPCR) experiments using tissue homogenates, unmounted frozen sections of the prefrontal cortex and LC were punch-dissected with a 5 mm trephine. For cortex tissue, grey

matter containing all 6 neocortical layers was isolated in this manner. In the LC, equivalent rostrocaudal levels along the axis of the LC were obtained as previously described.[15] Tissue sections for laser capture microdissection (LCM) were prepared as described previously.[26] For Western blotting, frozen sections (50 μm) were cut, and tissue containing the LC was punch-dissected (10 punches isolated per tissue donor).

Laser capture microdissection

We identified oligodendrocytes and astrocytes for LCM using a modified Nissl stain and a rapid GFAP immunostain, respectively. Capture was performed on a Veritas Microdissection Instrument model 704 (Life Technologies), and the quality of cell captures was subsequently verified by PCR of cell-specific gene markers, as previously described.[27]

Table 1: Brain tissue information and assays

Group; ID	Age, yr	Sex	pH	RIN	PMI	Smoker	Toxicology	Assays	Tissue
Controls									
FF1	27	M	6.88	8.4	17	Yes	No drug detected	rtPCR, LCM, IHC, WB	LC, Cx
KS6	43	M	6.58	7.4	22	No	No drug detected	rtPCR	LC, Cx
KS15	43	M	5.99	6.9	17	No	Carbon monoxide	WB	LC
KS21	48	M	6.98	7.4	9	Yes	No drug detected	rtPCR, LCM, IHC, WB	LC, Cx
KS27	74	M	6.62	6.7	21	Yes	No drug detected	rtPCR, WB	LC, Cx
KS31	59	M	6.79	7.6	6	No, history	Lidocaine	rtPCR, LCM, IHC, WB	LC, Cx
KS53	30	M	6.98	7.2	19	Occasional	No drug detected	WB	LC
KS57	17	M	6.71	7.4	24	No	Ethanol	rtPCR, LCM, IHC, WB	LC,
KS59	46	M	6.95	6.8	19	No	No drug detected	rtPCR, LCM, IHC, WB	LC, Cx
KS61	31	M	6.86	3.6	28	No information	No drug detected	WB	LC
KS63	18	M	6.40	6.4	32	Unknown	Midazolam	rtPCR, LCM, IHC, WB	LC
KS65	58	M	5.80	6.4	27	No	No drug detected	rtPCR, LCM, IHC, WB	LC
KS74	72	M	6.30	7.3	16	No	Diltiazem	LCM	LC
KS76	42	M	6.70	7.4	24	No	Butalbital	LCM	LC
KS78	18	M	6.60	7.9	16	Unknown	No drug detected	LCM	LC
KS80	18	M	7.00	8.7	15	No	Diazepam	LCM	LC
KS82	47	M	6.10	8.3	25	No	Propoxyphane	WB	LC
RR	37	M	6.47	7.3	17	No	No drug detected	rtPCR, WB	LC, Cx
VV	54	M	6.52	7.7	19	Yes	Lidocaine	rtPCR, WB	LC, Cx
KS67	54	M	6.50	6.5	26	No	Morphine	IHC	LC
Mean	42		6.59	7.2	20				
SEM	4		0.08	0.2	1				
MDD									
GG1	30	M	6.91	8.0	18	Yes	No drug detected	rtPCR, LCM, IHC, WB	LC, Cx
KS8	42	M	6.67	6.7	44	No	No drug detected	rtPCR	LC, Cx
DD	52	M	6.48	5.8	18	No	Carbon monoxide	WB	LC
KS12	41	M	6.24	6.7	19	Yes	Chlorpheniramine	rtPCR, LCM, IHC, WB	LC, Cx
KS28	81	M	6.78	6.1	33	Yes	No drug detected	rtPCR, WB	LC, Cx
KS32	60	M	6.32	6.8	20	Yes	Ethanol	rtPCR, LCM, IHC, WB	LC, Cx
KS54	40	M	6.78	6.8	22	Unknown	No drug detected	WB	LC
KS58	18	M	6.58	6.8	27	Unknown	Carbon monoxide	rtPCR, LCM, IHC, WB	LC,
KS56	37	M	6.67	6.9	31	No	Ethanol	rtPCR, LCM, IHC, WB	LC, Cx
KS62	26	M	6.77	7.1	7	Unknown	Unknown	WB	LC
KS64	20	M	6.73	6.7	20	No	Diphenhydramine	rtPCR, LCM, IHC, WB	LC
KS66	48	M	6.68	6.7	17	No	No drug detected	rtPCR, LCM, IHC, WB	LC
KS75	77	M	NA	6.7	20	No	Carbon monoxide, diazepam, temazepam	LCM	LC
KS77	38	M	6.70	8.7	19	No	No drug detected	LCM	LC
KS79	25	M	6.90	7.6	13	Yes	No drug detected	LCM	LC
KS81	18	M	7.00	8.5	10	No	Ethanol	LCM	LC
JJ1	54	M	6.24	6.3	23	No, 30-yr history	Carbon monoxide	WB	LC
TT	38	M	6.52	7.2	24	No	No drug detected	rtPCR, WB	LC, Cx
WW	65	M	6.24	6.7	30	Yes	Codeine	rtPCR, WB	LC, Cx
Mean	43		6.62	7.0	22				
SEM	4		0.06	0.2	2				

Cx = prefrontal cortex (Brodmann area 10); IHC = immunohistochemistry performed on tissue sections; LC = locus coeruleus; LCM = laser-capture microdissected cells used in end-point polymerase chain reaction assays; M = male; MDD = major depressive disorder; NA = not available; PMI = postmortem interval; RIN = RNA integrity number; rtPCR = real-time polymerase chain reaction; SEM = standard error of the mean; WB = Western blot.

Polymerase chain reaction

Total RNA was isolated and then reverse transcribed (rt) to complementary DNA from both punch-dissected tissue and laser-captured cells, as previously described.[26] We used quantitative rtPCR to assess transcript levels in tissue homogenates, as described previously; briefly, target genes were normalized to the geometric mean of *GAPDH*, *ACTB* and *UBC*.[28] All qPCR products were single, individual peaks identical to a synthesized oligonucleotide standard incorporating the complementary sequences to each primer set. We used semiquantitative end-point PCR to quantify messenger RNA levels from laser-captured samples, where levels of expression of target genes were normalized with the geometric mean of expression levels of reference genes, as used in qPCR.[27] All semiquantitative end-point PCRs were performed in triplicate, where every replicate was verified using microcapillary electrophoresis (Agilent Bioanalyzer), indicating that each reaction was a single amplified product at the appropriate nucleotide length. All primer sets and genes are listed in Appendix 1, Table S2.

Immunohistochemical analysis

Sections used were immunohistochemically labelled with GFAP, as previously described.[27] Two-dimensional analysis was carried out using Image J software to calculate both areal fraction and density of cells. To determine the amount of GFAP immunoreactivity in a given area, we obtained greyscale images of GFAP-labelled LC tissue. The GFAP immunoreactive staining was determined by assigning a fixed threshold above background (defined on slides lacking primary antibody) and by generating a binary image. We calculated fractional area by dividing the GFAP-positive area (μm^2) in the binary image by the total area of the image frame. A total of 10 randomized frames were analyzed in the LC region for each slide, and 3 nonadjacent slides cut at anatomically equivalent levels were analyzed per tissue sample donor (total of 30 images averaged per donor). To estimate the 2-dimensional density of GFAP-positive cells, we used an approach similar to that for the areal fraction. The GFAP-positive cells lying within the sampling area and not touching borders were counted for each of the 10 randomized images per slide (the same 3 nonadjacent slides per donor as those used for area fraction calculations).

Western blotting

Punches were homogenized in ice-cold Tris lysis buffer (20 mM, pH 8) supplemented with ethylenediaminetetraacetic acid (0.2 mM), NP40 (0.5%), NaCl (150 mM), sodium dodecyl sulfate (0.1%), sodium deoxycholate (0.5%) and protease inhibitor cocktail (Thermo Scientific). Homogenates were sonicated twice, each time for 20 seconds, then divided and stored at –80°C until use. Proteins (2 µg) were separated using a 10% Bis/Tris gel with a Hoefer SE250 gel electrophoresis unit and then transferred to nitrocellulose membranes. Membranes were blocked with 5% instant milk at 22°C, incubated with mouse anti-GFAP (1:1000) for 3 hours at 22°C (Sigma), washed at 22°C 3 times for 10 minutes each time, and incubated with anti-mouse IgG (GE Healthcare) for 1 hour. We identified protein banding using horseradish peroxidase conjugated to the mouse IgG, which was then subjected to a chemiluminescent reaction (PerkinElmer). We quantified bands using MCID 7.0 software (InterFocus Imaging) with standardized parameters for all membranes. Each membrane blot consisted of samples from matched pairs so that experimental conditions were identical. We determined relative protein expression by calculating a ratio of GFAP to α-tubulin immunoreactivity (NB100–690; Novus Biologicals) determined on the same blot. All samples were analyzed in triplicate.

Statistical analysis

All data were subjected to Student *t* tests for paired comparisons. We paired control and MDD samples according to the matching criteria mentioned previously (pairs were also processed simultaneously through all experimentation). Statistical significance for the multiple comparisons of levels of *SLC1A2*, *SLC1A3*, *GLUL* and *GFAP* expressions was adjusted by Bonferroni correction to $p = 0.012$ to reduce chances of a type I error. We considered comparisons of GFAP immunoreactivity levels and cell counts to be significant at $p = 0.05$, uncorrected. All data were graphed and statistically analyzed using GraphPad Prism version 5.0 (GraphPad Software). All results of our statistical analyses appear in Appendix 1, Tables S3 and S4.

Results

The expression levels of 4 glia-related genes, *SCL1A3*, *SCL1A2*, *GLUL* and *GFAP*, were first analyzed by real-time PCR of rtRNA isolated from homogenized tissue that was punch-dissected from the LC region from decedents who had MDD and from matched controls (Fig. 1). *SCL1A3* expression levels

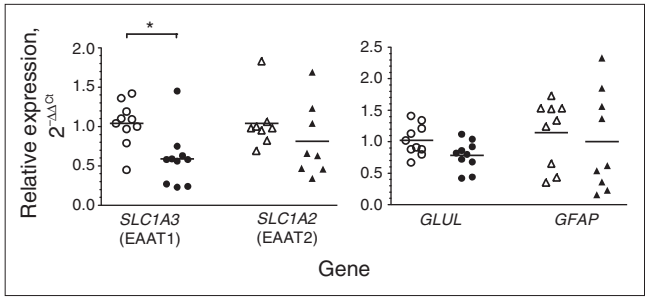

Fig. 1: Real-time quantitative polymerase chain reaction analysis of *SLC1A3* ($n = 10$), *SLC1A2* ($n = 9$), *GLUL* ($n = 10$) and *GFAP* ($n = 9$) in punch-dissected locus coeruleus tissue from matched pairs of tissue samples from psychiatrically healthy controls (open symbols) and men with major depressive disorder (closed symbols). Target gene expression was normalized with the geometric mean of 3 reference genes (*GAPDH*, *UBC* and *ACTB*). Relative gene expression levels are shown as fold changes using the method by Livak and Schmittgen.[29] *$p = 0.001$.

were significantly lower (–43%, $p = 0.001$) in the MDD than the control group. In contrast, levels of *SCL1A2* expression were not significantly different between the MDD and control groups ($p = 0.36$). *GLUL* expression was modestly lower (–23%, $p = 0.02$) in the MDD than in the control group. *GFAP* expression in the LC was similar between the groups ($p = 0.66$).

Gene expression data collected from tissue homogenates contain an admixture of cell types, including different types of glia, neurons, surrounding neuropil and glial processes, and vascular tissue. A change in the number of a specific type of cell within a dissected brain region could contribute to an observed gene expression change if the change is a gene expressed in a specific cell. With regard to glia themselves, both astrocytes and oligodendrocytes express genes related to glutamate uptake and metabolism.[30] Hence, we performed LCM of individual cell types within the LC to examine expression levels of the glia-related genes in the MDD and control groups.

Levels of *SLC1A3* expression were robustly lower in astrocytes from decedents with MDD than in samples from matched controls when normalized to reference genes (–53%, $p = 0.006$, Fig. 2A) or to the number of cells (–58%, $p = 0.003$, Fig. 2C). Likewise, *SLC1A2* expression in astrocytes was significantly lower in MDD than control samples normalized to reference genes (–29%, $p = 0.003$, Fig. 2A) or to number of cells (–39%, $p < 0.001$, Fig. 2C). *GLUL* expression in astrocytes was not significantly different between the groups (Fig. 2B and 2D). *GFAP* expression normalized to reference genes was modestly reduced (–19%, $p = 0.04$, Fig. 2B) in astrocytes of MDD tissues, although this did not reach statistical significance. However, normalization of GFAP gene expression to the number of cells captured showed a significantly lower expression in astrocytes from MDD samples than control samples (–27%, $p = 0.005$, Fig. 2D). In marked contrast to astrocytes, there were no significant differences in the levels of expression of *SCLA3*, *SCLA2* and *GLUL* in oligodendrocytes between the groups (Fig. 3A–D). *GFAP* expression was below the limits of detection in oligodendrocytes.

It is worth noting through examination of Figures 2 and 3 that levels of *SLC1A3* expression were highest in astrocytes, with expression in oligodendrocytes being about 20% of that in astrocytes in the control and MDD groups. *SLC1A2* expression was higher in astrocytes than oligodendrocytes, although expression in oligodendrocytes was about 60% of that in astrocytes. The level of *GLUL* expression in oligodendrocytes was nearly 200% of *GLUL* expression in astrocytes.

To determine whether reductions in glutamate transporter genes were common to other brain regions, gene expression was measured in homogenates of the prefrontal cortex (BA 10). In contrast to LC homogenates, no statistically significant differences were observed for *SLC1A3*, *SLC1A2*, *GLUL* or *GFAP* expression in the prefrontal cortex (Fig. 4).

To determine whether levels of glutamate transporter protein were altered in the LC in the MDD group, we attempted Western blotting, but the amount of tissue required for these assays exceeded available tissue. Only frozen blocks of pontine tissue containing the LC were available, and immunostains of

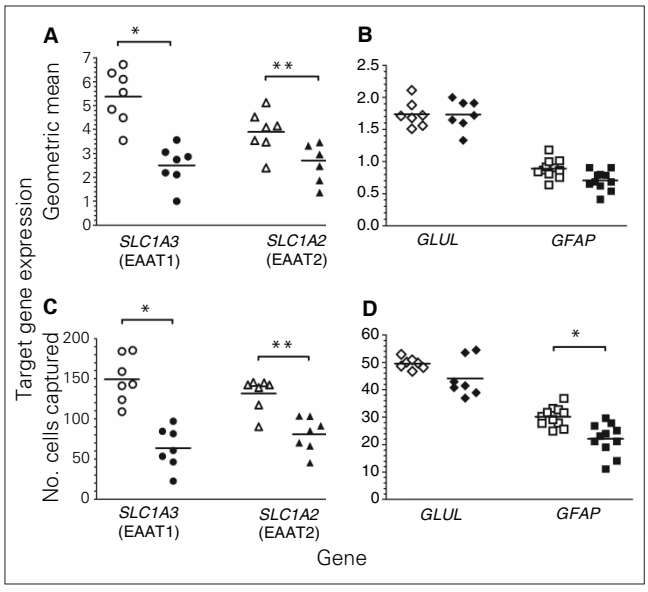

Fig. 2: Gene expression using quantitative end-point polymerase chain reaction conducted with laser-captured astrocytes from locus coeruleus tissue from matched pairs of psychiatrically healthy controls (open symbols) and men with major depressive disorder (closed symbols). Target gene expression of *SCLA3* ($n = 7$), *SCLA2* ($n = 7$), *GLUL* ($n = 7$) and *GFAP* ($n = 11$) was normalized using the geometric mean of the gene expression of 3 reference genes (*GAPDH*, *UBC* and *ACTB*; **A** and **B**) and normalized to the number of cells captured (**C** and **D**). *$p < 0.01$, **$p < 0.001$.

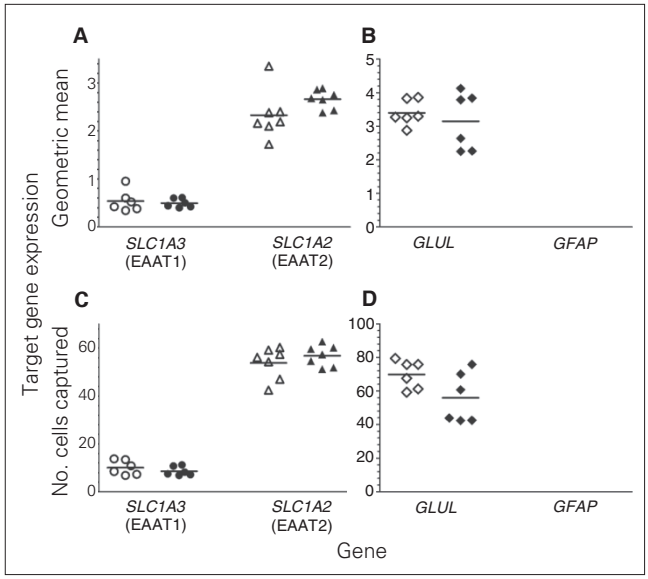

Fig. 3: Gene expression using quantitative end-point polymerase chain reaction conducted with laser-captured oligodendrocytes from locus coeruleus tissue from matched pairs of psychiatrically healthy controls (open symbols) and men with major depressive disorder (closed symbols). Target gene expression of *SCLA3* ($n = 6$), *SCLA2* ($n = 7$) and *GLUL* ($n = 6$) was normalized using the geometric mean of the gene expression of 3 reference genes (*GAPDH*, *UBC* and *ACTB*; **A** and **B**) and normalized to the number of cells captured (**C** and **D**). No significant changes in gene expression were found. *GFAP* gene expression was undetectable.

frozen postfixed tissue sections for EAAT1 and EAAT2 were very light and of relatively poor quality despite repeated attempts. In contrast, we used GFAP immunoreactivity to identify astrocytes for LCM and staining of Western blots, and tissue sections were of sufficient quality and signal intensity for quantitation. Western blots revealed a strikingly wide range of levels of GFAP immunoreactivity among control and MDD samples (Fig. 5A). Nevertheless, levels of GFAP immunoreactivity were significantly lower (–62%, all $p < 0.05$) in MDD than in matched control samples. GFAP immmunoreactivity measured in tissue sections also demonstrated significantly lower levels (–52%, all $p < 0.05$) in MDD than in matched control samples (Figs. 5B, 5D, 5E). Finally, the density of GFAP-labelled astrocytes in MDD samples was significantly lower (–46%, $p < 0.05$) than that in control samples (Fig. 5C).

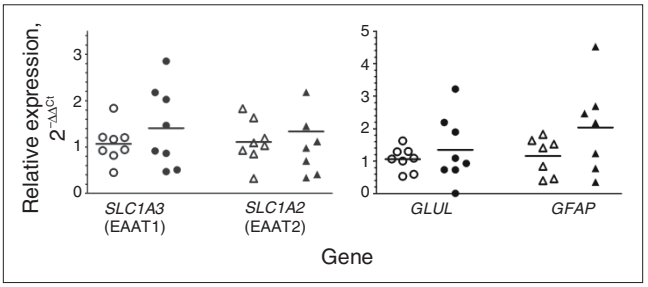

Fig. 4: Quantitative polymerase chain reaction analysis of *SLC1A3* (n = 8), *SLC1A2* (n = 8), *GLUL* (n = 8) and *GFAP* (n = 7) using tissue homogenates from the frontal cortex (Brodmann area 10) from pairs of matched psychiatrically healthy controls (open symbols) and men with major depressive disorder (closed symbols). Target gene expression was normalized to the geometric means of gene expression for *GAPDH*, *UBC* and *ACTB*. Gene expression data are displayed as fold changes using the method of Livak and Schmittgen.[29] There were no significant differences between any of the groups.

Reference genes and demographic variables

As a result of our careful matching of control and MDD decedents, there were no significant differences between the groups in terms of age, postmortem interval, RIN and pH values (Table 1). In addition, there were no significant differences between the MDD and control groups in the expression levels of the 3 reference genes in RNA isolated from homogenized tissues or from laser-captured astrocytes and oligodendrocytes (data not shown). There were no significant correlations between age and the expression of any of the reference or target genes, or between age and levels of GFAP immunoreactivity. With regard to GFAP immunoreactivity, it should be noted that this study was not designed to specifically address age and GFAP protein levels. That is, tissues from decedents across the range of ages were not run on the same blot. Instead, tissues from paired MDD and age-matched control

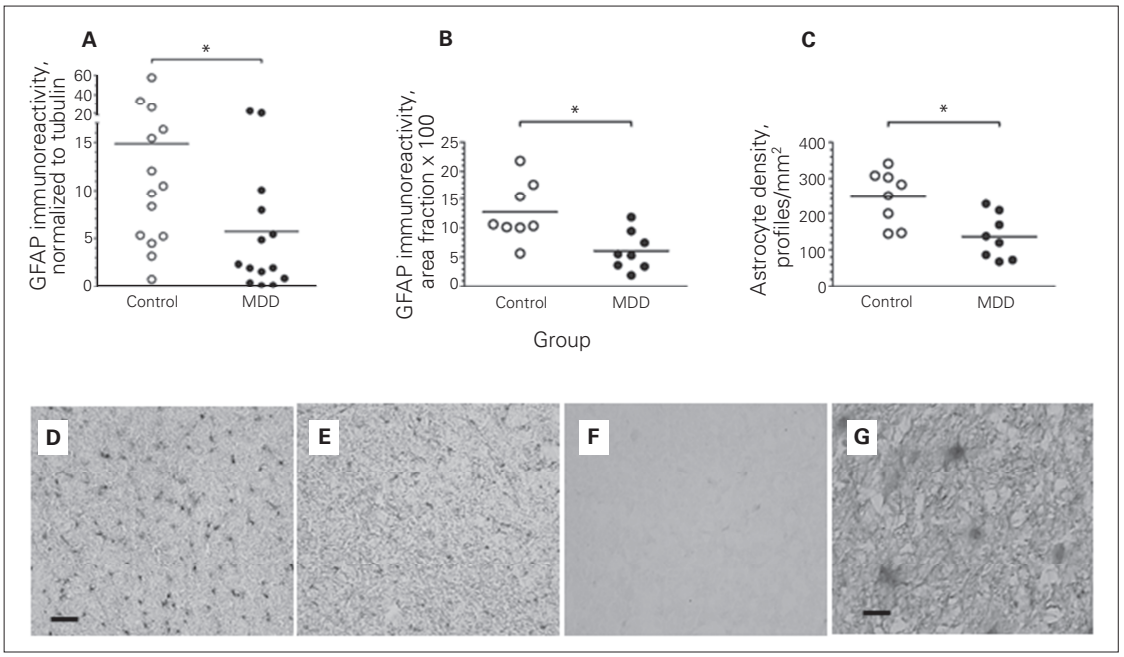

Fig. 5: GFAP immunolabelling in locus coeruleus (LC) tissue from matched pairs of psychiatrically healthy controls (open circles) and men with major depressive disorder (MDD; closed circles). Panel **A** shows data from Western blots of homogenized punch-dissected LC tissue normalized to tubulin immunoreactivity on the same blots (n = 14). Panel **B** shows data from pseudoquantitative analyses of images of GFAP immunostained sections (n = 8). Panel **C** shows estimates of astrocyte densities computed from 2-dimensional analysis of GFAP-stained cell profiles in the same tissue sections used in panel **B** (n = 8). The lower images are representative GFAP-immunostained sections from a control (**D**) and MDD (**E**) decedent and background staining (**F**) generated by the secondary antibody in the absence of the primary antibody. Panel **G** shows GFAP-stained cells (magnetization ×60) from the control donor (**D**). The bars in the lower left corner of panels **D** and **G** indicate a scale of 50 µm and 10 µm, respectively. *$p < 0.05$.

decedents were run on the same blot. There were no significant correlations between RIN or pH values and the levels of expression of target or reference genes. Primers were designed to generate small amplimers (about 100 nucleotides; Appendix 1, Table S2), reducing the potential impact of RNA degradation on PCR product levels.[27,31]

Finally, 4 of the 19 decedents with MDD had a history of antidepressant prescriptions, and 2 of them died as a result of heart disease rather than suicide or overdose of unknown cause. Unfortunately, these small subgroups were not of sufficient size within a given set of experiments to statistically evaluate the effects of antidepressant drug history or nonsuicide on gene or protein expression levels. For comparisons where more than 1 control/MDD pair could be evaluated, the following observations are noted. Individual comparisons of *SLC1A3* gene expression in LC tissue homogenates from matched control–MDD pairs in which the decedents with MDD had a history of antidepressants indicated fold changes of 0.42, 0.47 and 0.79 compared to paired controls (mean change of 0.56). The mean change of the remaining control–MDD pairs in which the decedents with MDD had no history of antidepressants was 0.41. Three decedents with MDD whose tissues were used in our Western blotting analysis had previous antidepressant use, and levels of GFAP immunoreactivity in these samples were reduced by 97%, 9% and 89% (mean reduction of 65%) relative to their paired control. The mean reduction of GFAP in decedents with MDD without prior antidepressant drug use was 48%. We used brain tissues from 3 different brain collections, leaving the possibility that population, tissue collection or other differences may have impacted the data. Too few samples were used from the Quebec Suicide Brain Bank to evaluate separately. Four donor pairs from the Pittsburgh collection were used to generate the GFAP gene expression data from LCM-captured cells. The average percentage reduction of GFAP gene expression in astrocytes from MDD samples relative to matched controls was 15% for the Pittsburgh samples and 18% for Cuyahoga County samples.

Discussion

The results of the present study demonstrate pathology of astrocytes in the region of the noradrenergic LC. Evidence of dysfunction of astrocytes in the LC region in individuals with MDD include reduced expression of *SLC1A3, SLC1A2* and *GFAP*; lower GFAP protein levels; and reduced density of GFAP-positive astrocytes. Altered expression of glutamate transporter genes has been reported previously in a number of studies of neurodegenerative and psychiatric diseases,[32–34] including MDD.[20,35] The present study provides direct evidence of astrocyte pathology in the cell body region of a monoamine neurotransmitter, indicating that glia cell abnormalities reported in more superior/rostral brain regions in MDD[16,19] extend to the brainstem and may contribute to pathology of monoamine systems.

Animal studies demonstrating the effects of manipulations of glutamate transporters provide direct evidence that glutamate transporters play a major role in the pathophysiology of depressive behaviour. Blockade of Glt-1 (rodent *Slc1a2*, homologue of human EAAT2) transporters with dihydrokainic acid in rats induces depressive-like behaviours and impairs spatial memory.[36] Whereas Glt-1 knockout mice do not survive, Glast-knockout mice (mouse *Slc1a3*, homologue of human EAAT1) survive but show signs of social withdrawal and impaired learning.[37] If increased synaptic glutamate is a contributing factor to MDD due to etiological dysfunction of glutamate transporters, then medications that increase glutamate uptake in astrocytes would be expected to have beneficial therapeutic effects in the treatment of depression. In fact, treatment with the antibiotic ceftriaxone, which induces the expression of EAAT2 in primary human astrocytes,[38] reduces depressive behaviours in animal models.[39] Riluzole, which increases glutamate uptake in vitro,[40] has been shown in animal models to reverse glial dysfunction induced by chronic stress.[41] Collectively, these findings indicate that medications enhancing the glutamate transporter may reverse the effects of reduced glutamate transporter expression in individuals with MDD.

We previously reported evidence of astrocyte pathology in the LC region in individuals with MDD.[26] The present study provides further evidence of astrocyte dysfunction in the noradrenergic LC region. Reduced *SLC1A3* and *SLC1A2* expression on astrocytes would be expected to elevate synaptic glutamate and increase excitatory glutamatergic input to LC neurons found in human MDD. These astrocyte pathologies led us to further explore a more general indicator of astrocyte function, GFAP, and the glial marker glutamine synthase (encoded by *GLUL* and also expressed by oligodendrocytes). While *GLUL* expression was similar in control and MDD tissues, reduced *GFAP* expression was found in astrocytes from MDD samples, along with a modest, yet significant, reduction in GFAP immunoreactivity, as demonstrated by Western blotting and in GFAP-immunostained tissue sections. In addition, we found reduced density of GFAP-immunostained astrocytes in the LC region in the MDD samples compared with matched control samples. Reduced GFAP immunoreactivity in MDD has been reported for anterior cingulate cortex white matter,[42] dorsolateral prefrontal cortex deep layers,[43,44] the amygdala[16] and the cerebellum.[45] Interestingly, Wistar-Kyoto rats (a strain often used as a model of depression and stress sensitivity) exhibit lower levels of GFAP immunoreactivity in the cerebral cortex and amygdala and a reduced number of GFAP-immunoreactive astrocytes compared with Sprague-Dawley rats.[46] However, to our knowledge, no difference in the number of S100β-labelled astrocytes has been reported between these 2 strains of rat. While we observed a lower density of GFAP-immunoreactive astrocytes in the LC region in MDD samples than control samples, we cannot assume that this reflects fewer astrocytes in individuals with MDD. Reduced *GFAP* expression and lower GFAP protein levels (as verified in Western blots) would likely result in fewer astrocytes being identified using cell counting methods. This has also been noted by other authors.[16] Hence, it is reasonable to suspect that the reduction in GFAP immunoreactivity in the present study may be a regulatory event in existing astrocytes.

While factors such as inflammation and oxidative stress induce *GFAP* expression, little is known about factors that reduce it. Since inflammation increases *GFAP* expression, and

since MDD has been associated with elevated indices of inflammation,[47] a possible interpretation of the present findings is that brainstem astrocytes are generally deficient in their inflammatory response. In fact, the multiple deficiencies in gene expression in these cells in MDD (reduced *BMP7*,[26] *SLC1A2*, *SLC1A3, GFAP*) are consistent with the postulate that LC astrocytes are unhealthy in individuals with MDD. A possible cause of these gene expression changes in astrocytes in individuals with depression is that they occur after prolonged changes in noradrenergic transmission. We and others have reported several indices of abnormal noradrenergic activity in individuals with MDD.[3] Astrocytes express noradrenergic receptors,[48] and norepinephrine can regulate glutamate uptake in cultured glia cells.[49,50] Hence, elevated noradrenergic activity associated with MDD[3] may alter astrocyte function. Of course, an alternative hypothesis could also underlie LC pathology: astrocyte deficits may contribute to noradrenergic neuron dysfunction, since these cells liberate supportive neurotrophic factors.[26]

Limitations

There are weaknesses in the present study that temper conclusions regarding our interpretation. Overall, small sample numbers may have masked statistical differences. Likewise, the small numbers of comorbid conditions (Appendix 1, Table S1) hampered evaluation of the impact of individual comorbidities on the findings. We were unable to verify that reduced *SLC1A3* and *SLC1A2* expressions were accompanied by reduced EAAT1 and EAAT2 protein levels because of a lack of sufficient amounts of LC tissue for those assays. Although we were unable to detect gene expression changes in BA 10, these experiments were performed using an admixture of cell types, not with LCM, leaving the possibility that alterations in astrocytes were masked by a lack of change in other cell types in this tissue. It is noteworthy, however, that we observed significant reductions of glutamate transporter gene expression in homogenates of LC tissue before extending the study to laborious laser capture experiments to determine the cellular source of the changes. Also, a lack of change in BA 10 does not imply that other cortical areas do not have glutamate transporter gene expression changes. Probably the most important index of transporter function is uptake, which cannot be performed reliably in postmortem human tissue. We were unable to untangle pathology associated with suicide from that associated with MDD, since most decedents with MDD died by suicide. This shortcoming cannot be addressed currently because of a lack of tissues from people with MDD who died a natural death. Finally, only astrocytes that express GFAP were studied. Hence, we note that the results of this study indicate dysfunction in only a subpopulation of astrocytes in individuals with MDD.

Conclusion

The present study, showing evidence of astrocyte dysfunction in the noradrenergic LC region in men with MDD, strongly implicates a deficit in the regulation of glutamate action in this region. Excitatory glutamatergic input to the LC is a major modulator of LC activity.[51] Stress increases glutamate release in the LC,[13,52] and MDD is commonly precipitated by stress. Hence, it seems reasonable to suggest that dysfunctional glutamate uptake by unhealthy astrocytes in the LC could amplify potential detrimental effects of elevated stress-evoked glutamate release in the LC at the level of neurons as well as astrocytes. In fact, findings from postmortem studies of the LC in individuals who had MDD demonstrate a number of characteristics suggesting that people with MDD experience chronic elevated LC activity.[3] Many recent studies demonstrate the importance of astrocytes in neurotransmission[53] and even demonstrate that destruction of astrocytes can induce depression-like symptoms in rats.[54] Future research on the role of glia in depression has great promise to yield new approaches to the management of this disorder.

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