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ARTICLE

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Low gene expression of bone morphogenetic protein 7 in brainstem astrocytes in major depression

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Abstract

The noradrenergic locus coeruleus (LC) is the principal source of brain norepinephrine, a neurotransmitter thought to play a major role in the pathology of major depressive disorder (MDD) and in the therapeutic action of many antidepressant drugs. The goal of this study was to identify potential mediators of brain noradrenergic dysfunction in MDD. Bone morphogenetic protein 7 (BMP7), a member of the transforming growth factor-β superfamily, is a critical mediator of noradrenergic neuron differentiation during development and has neurotrophic and neuroprotective effects on mature catecholaminergic neurons. Real-time PCR of reversed transcribed RNA isolated from homogenates of LC tissue from 12 matched pairs of MDD subjects and psychiatrically normal control subjects revealed low levels of BMP7 gene expression in MDD. No differences in gene expression levels of other members of the BMP family were observed in the LC, and BMP7 gene expression was normal in the prefrontal cortex and amygdala in MDD subjects. Laser capture microdissection of noradrenergic neurons, astrocytes, and oligodendrocytes from the LC revealed that BMP7 gene expression was highest in LC astrocytes relative to the other cell types, and that the MDD-associated reduction in BMP7 gene expression was limited to astrocytes. Rats exposed to chronic social defeat exhibited a similar reduction in BMP7 gene expression in the LC. BMP7 has unique developmental and trophic actions on catecholamine neurons and these findings suggest that reduced astrocyte support for pontine LC neurons may contribute to pathology of brain noradrenergic neurons in MDD.

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Key words: Astrocyte, bone morphogenetic protein, glia, locud coeruleus, major depression, noradrenergic, suicide.

Introduction

Major depressive disorder (MDD) is a psychiatric illness affecting 13–14 million people in the USA annually (Kessler *et al.* 2003). Modern treatment for depression began in the 1950s and was followed by decades of research to develop better antidepressant drugs. Unfortunately, many newer antidepressants do not demonstrate a significantly greater therapeutic efficacy than the older drugs, although some exceptions have been noted (Montgomery *et al.* 2007; Papakostas *et al.* 2007). Inadequate or incomplete treatment of the disorder remain major health and economic issues (Thase, 2009). It is widely believed that improved pharmacological management will follow from a more thorough understanding of the pathobiology of the illness. Research dedicated to this cause has revealed a complex disorder of brain biology, including abnormalities in neurotransmitters, stress hormones, neurotrophic factors, and an involvement of glia (Banasr & Duman, 2007; Duman & Monteggia, 2006; Krishnan & Nestler, 2008; Rajkowska & Miguel-Hidalgo, 2007).

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Of neurotransmitter systems that demonstrate abnormalities in MDD, the brain noradrenergic system has received considerable attention. Short-term depletion of catecholamines by administration of α-methyl-*p*-tyrosine to psychiatrically and medically healthy subjects did not significantly affect mood (Salomon *et al.* 1997). However, rapid pharmacological depletion of catecholamines in patients taking noradrenergic antidepressants caused a rapid relapse of depression (Charney, 1998), and catecholamine depletion in unmedicated, fully remitted subjects with histories of MDD resulted in relapse into depression (Berman *et al.* 1999). These demonstrations of a prominent role of norepinephrine in the behavioural biology of depression are supported by several studies demonstrating pathology of the major neuronal source of brain norepinephrine, the locus coeruleus (LC) in depressive subjects (for review, see Ordway, 2007).

Given the body of literature suggesting that decreased expression of neurotrophic factors contribute to depression (Duman & Monteggia, 2006), we became interested in the possibility of a link between depression and trophic factors specifically involved in the development and growth of norepinephrine neurons.

Bone morphogenetic proteins (BMPs) are a family of growth factors first discovered in bone, but are also expressed in the brain where they are involved in many aspects of the differentiation and morphogenesis of neurons and glia. Of BMPs, BMP7, a member of the transforming growth factor-β (TGF-β) superfamily, is of particular interest because it plays a major role in the development of noradrenergic neurons. For example, a deletion mutation of BMP7 and BMP5 genes results in an absence of noradrenergic neurons in the LC (Tilleman *et al.* 2010). In addition, BMP7 has neurotrophic and neuroprotective effects on mature catecholaminergic neurons. For example, BMP7 induces dendritic growth of noradrenergic neurons (Lein *et al.* 1996, 2002*a*) and protects noradrenergic neurons from 6-hydroxydopamine-induced neurotoxicity (Harvey *et al.* 2004). BMP7 is down-regulated following catecholamine neuron lesions (Chen *et al.* 2003), suggesting a reciprocal regulatory link between BMP7 and catecholamines.

Because of its specific developmental and trophic effects on catecholamine neurons, it is conceivable that a deficit of BMP7 could contribute to disrupted noradrenergic signalling in MDD, or alternatively, reduced noradrenergic signalling could elicit a change in the expression of BMP7 or its receptors. Hence, in the present study we investigated the possibility that BMP7 gene expression may be altered in MDD. BMP7 gene expression levels were first measured in the noradrenergic LC dissected from post-mortem brainstems of MDD subjects and psychiatrically normal control subjects. After discovering low levels of BMP7 gene expression in MDD subjects, additional studies examined gene expression levels of several other BMPs in LC tissue, and examined whether reduced BMP7 gene expression was found in other brain regions. Laser capture microdissection (LCM) was employed to determine which type of cell in the LC exhibited a deficit in BMP7 gene expression in MDD. Finally, a social stress paradigm in rats (chronic social defeat) was used to examine the possibility that stress, a known precipitator of depression in humans (Paykel *et al.* 1969), might regulate BMP7 gene expression. The findings presented here demonstrate for the first time that astrocytes closely associated with norepinephrine neurons are abnormal in MDD, and suggest that trophic support of norepinephrine neurons by these glial cells may be deficient in MDD.

Method

Human brain tissues

Post-mortem human brain tissue was collected at autopsy at the Cuyahoga County Coroner's Office in Cleveland, Ohio, in accordance with an approved Institutional Review Board Protocol, as described previously (Karolewicz *et al.* 2005). One MDD subject was also obtained courtesy of Dr Gustavo Turecki, from the Quebec Suicide Brain Bank at the Douglas Hospital Research Center under an approval of the Douglas Hospital Research Center's Research Ethics Committee. Retrospective, informant-based psychiatric assessments were performed for all depressed and control subjects as described previously (Dumais *et al.* 2005; Stockmeier *et al.* 2002) using standardized questionnaires modified for psychological autopsy. Axis I psychopathologies were assessed and consensus diagnoses were reached in conference using information from the interview and medical records. Blood and urine samples from all subjects were examined for psychotropic medications and substances of abuse. Subjects with antidepressant or antipsychotic drugs in their post-mortem toxicology and/or with alcohol dependence (current and/or historical) were excluded from the study.

Brain tissues from a total of 19 psychiatrically normal control subjects and 19 subjects with MDD were obtained (see Table 1). Control subjects had no Axis I psychiatric diagnosis at the time of death except nicotine dependence and one subject that had a specific phobia (situational). Control subjects also had

Table 1. Subject demographics and tissue use

Group/subject	Age, yr	Sex	pH	RIN	PMI	Smoker	Toxicology	Assays	Tissue
Normal control subjects									
A-1	82	M	6.72	6.7	16	No	NDD	rtPCR	Amyg
BB1	52	M	6.28	6.4	17	No	NDD	rtPCR	LC, Ctx, Amyg
RR	37	M	6.47	7.3	17	No	NDD	rtPCR	LC, Ctx, Amyg
VV	54	M	6.52	7.7	19	Yes	Lidocaine	rtPCR	LC, Ctx
HH1	54	M	6.87	6.6	17	No; hx 10 yr[a]	Brompheniramine	rtPCR	LC, Ctx
FF1	27	M	6.88	8.4	17	Yes	NDD	rtPCR, LCM	LC, Ctx, Amyg
KS6	43	M	6.58	7.4	22	No	NDD	rtPCR	LC, Ctx
KS19	46	F	6.86	6.8	9	Yes	NDD	rtPCR	LC
KS21	48	M	6.98	7.40	9	Yes	NDD	rtPCR, LCM	LC, Ctx
KS23	58	M	6.78	7.7	21	Yes	NDD	rtPCR, LCM	LC, Ctx
KS27	74	M	6.62	6.7	21	Yes	NDD	rtPCR	LC, Ctx
KS31	59	M	6.79	7.6	6	No, hx	Lidocaine	rtPCR, LCM	LC, Ctx, Amyg
KS34	23	F	6.84	6.9	11	No	NDD	rtPCR	LC, Ctx
KS39	34	M	6.61	7.9	24	No	Ethanol (0.02)[b]	rtPCR	Amyg
KS43	67	M	6.96	8.1	24	Yes	NDD	rtPCR	Amyg
KS57	17	M	6.71	7.4	24	No	Ethanol	LCM	LC
KS59	46	M	6.95	7.0	19	No	Ethanol (0.03)	rtPCR, LCM	LC, Ctx, Amyg
KS63	18	M	6.40	6.4	31.5	Unknown	Midazolam	rtPCR	LC
KS-65	58	M	5.8	6.4	27	No	NDD	rtPCR	LC
Major depressive subjects									
H	73	M	6.59	6.6	17.5	No	Diazepam, codeine	rtPCR	Amyg
DD1	52	M	6.48	5.8	18	No	CO	rtPCR	LC, Ctx, Amyg
TT	38	M	6.52	7.2	24	No	NDD	rtPCR	LC, Ctx, Amyg
WW	65	M	6.24	6.7	30	Yes	Codeine	rtPCR	LC, Ctx
JJ1	54	M	6.24	6.3	23	No, hx 30 yr	CO	rtPCR	LC, Ctx
GG1	30	M	6.91	8.0	18	Yes	NDD	rtPCR, LCM	LC, Ctx, Amyg
KS8	42	M	6.67	6.7	44	No	NDD	rtPCR	LC, Ctx
KS17	45	F	6.84	6.6	27	Yes	NDD	rtPCR	LC
KS12	41	M	6.24	6.70	19	Yes	Chlorpheniramine	rtPCR, LCM	LC, Ctx
KS24	64	M	6.85	7.25	26	Yes	Ethanol	rtPCR, LCM	LC, Ctx
KS28	81	M	6.78	6.1	33	Yes	NDD	rtPCR	LC, Ctx
KS32	60	M	6.32	6.80	20	Yes	Ethanol	rtPCR, LCM	LC, Ctx, Amyg
KS35	36	F	6.84	7.5	25	Yes	NDD	rtPCR	LC, Ctx
KS38	38	M	6.80	7.7	20	Yes	NDD	rtPCR	Amyg
KS42	42	M	6.47	6.7	20	Unknown	NDD	rtPCR	Amyg
KS56	37	M	6.67	6.90	31	No	Ethanol	rtPCR, LCM	LC, Ctx, Amyg
KS58	18	M	6.58	6.83	27	Unknown	CO	rtPCR, LCM	LC
KS64	20	M	6.73	6.7	20	No	Diphenhydramine	rtPCR	LC
KS66	48	M	6.68	6.7	17	No	NDD	rtPCR	LC
Summary data									
Control subjects	47±4	17M/2F	6.7±0.1	7.2±0.1	19±1				
MDD subjects	47±4	17M/2F	6.6±0.1	6.8±0.1	24±2[c]				

RIN, RNA integrity number; PMI, post-mortem interval; NDD, no drugs detected; rtPCR, real-time PCR performed on punched LC tissue; LCM, end-point PCR performed on cells captured by laser capture microdissection; LC, locus coeruleus; Ctx, cortex BA 10; Amyg, basal amygdaloid nucleus.

[a] History with years since (when known).

[b] Blood ethanol in g/dl.

[c] Significantly different from control group, $p < 0.05$.

no known substance abuse disorder except nicotine dependence and one subject that had a history of mild alcohol dependence but was in sustained remission at the time of death (number of years of remission unknown). Three subjects in the control group had alcohol in their blood, but none of these subjects had a

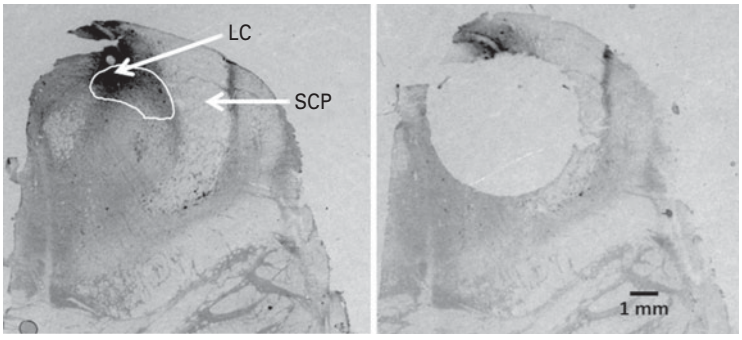

Fig. 1. Images of tyrosine hydroxylase immunostains of two adjacent sections cut through a block of tissue within the human pons illustrating the dissection of tissue containing the locus coeruleus (LC) (only the right side nucleus is shown in each image). The arrow labelled LC points to the central region of the LC. Neuromelanin-containing noradrenergic neurons were collected by LCM from the dark cell-dense region. The white line encompasses the region of the LC including its dendritic expanse where oligodendrocytes and astrocytes were collected by LCM. The right panel shows the adjacent stained section after LC tissue was punch-dissected with a 5-mm trephine for real-time PCR experiments on LC homogenates. The punched region of the LC contained the cell bodies of noradrenergic neurons, glia, and surrounding neuropil under the influence of norepinephrine, including norepinephrine released in the dendritic field of the LC (Pudovkina *et al.* 2001). SCP, Superior cerebellar peduncle.

current or historical alcohol dependence or abuse. Control subjects died as a result of heart disease (10 subjects), motor vehicle accident (three subjects), homicide (one subject), thrombophlebitis (one subject), aneurism (two subjects), bronchial asthma (one subject), and acute haemorrhagic pancreatitis (one subject). Subjects in the MDD group had no known substance abuse disorder except for nicotine dependence, two subjects who had active alcohol abuse, and one who had a history of alcohol abuse. The MDD subjects included one who had comorbid panic disorder with agoraphobia and a specific phobia (situational), one who had a comorbid learning disorder (not otherwise specified), and two who had a comorbid dysthymic disorder. Five of the 19 MDD subjects had a history of antidepressant drug prescriptions (fluoxetine, paroxetine, sertraline, trazodone) in medical records, but none of these subjects demonstrated positive toxicology for these drugs at autopsy, indicating non-compliance to medication or termination of compliance prior to death. Medication history of one of the MDD subjects was unknown. Most of the MDD subjects died by suicide (nine self-inflicted gunshot wounds, three carbon monoxide poisonings, one hanging, one drowning, one asphyxiation, one overdose with unknown substance), while the remainder died of other causes including heart disease (two subjects), and pulmonary thromboembolism (one subject). Post-mortem toxicology, age, gender, and tissue-related factors are given in Table 1. All of the demographic details of individual subjects, including causes of death and comorbidities, are not aligned in Table 1 in order to protect subject identities. The pairing of normal control and MDD subjects listed in Table 1 for each set of experiments in figures can be found in Supplementary Table S1 (available online).

Tissue preparation and sectioning

Blocks of tissue containing the pontine LC, right prefrontal cortex (Brodmann area 10), and the basal nucleus of the amygdala were frozen at autopsy and stored at $-80\,^{\circ}$C. Tissue blocks were sectioned with a cryostat microtome (Leica CM3050 S). Unmounted frozen sections containing the LC were punched with a 5-mm diameter trephine (Fig. 1) for real-time PCR (rtPCR; $3 \times 50\,\mu$m sections) experiments involving the LC. Equivalent rostro-caudal levels along the axis of the LC within a region 7–10 mm caudal to the frenulum were determined as described previously (Karolewicz *et al.* 2005). For prefrontal cortex, 10 frozen sections ($50\,\mu$m) were cut for each subject so that collected tissue contained all six layers of cortex and minimal white matter. For amygdala, a 3-mm trephine was used to punch the magnocellular portion of the basal nucleus of the amygdala from three sections ($50\,\mu$m) per subject as previously described (Xiang *et al.* 2008). Sections ($10\,\mu$m) for LCM were mounted on a room-temperature ($22\,^{\circ}$C) HistoGene® LCM microslide (Molecular Devices, USA) and placed immediately in a chilled microslide box on dry ice in a desiccator. Before sectioning and between each tissue block, the knife-holder and anti-roll plate were wiped carefully with 100% ethanol to avoid cross-contamination.

Staining

Frozen tissue sections containing LC neurons were stained using the HistoGene® LCM Frozen Section Staining kit (Molecular Devices) as described previously (Ordway *et al.* 2009). Oligodendrocytes were stained using a modified Nissl staining protocol and staining for astrocytes was performed by rapid GFAP immunocytochemistry, both as described previously (Ordway *et al.* 2009). Staining for tyrosine hydroxylase immunoreactivity was performed as described previously (Ordway *et al.* 1997).

Staining for BMP7 and BMPRII immunoreactivity was performed using formalin-fixed, paraffin-embedded pontine tissue that was sectioned onto glass slides using a microtome. Paraffin was removed from the tissue with xylene. Subsequently, tissue sections were passed through decreasing ethanol concentrations and rehydrated with water. Sections were then boiled in a 1:10 dilution of Citra Plus (BioGenex, USA; HK080–9K) in a microwave for 12 min, cooled for 20 min, washed in deionized H_2O three times for 5 min, and rinsed four times for 10 min with PBS. Endogenous peroxidase activity in the tissue was neutralized in 3% H_2O_2 in PBS (pH 7.4) for 10 min. After blocking with 10% normal goat serum in PBS with 0.3% Triton-X 100 for 30 min, sections were incubated with rabbit polyclonal anti-BMP7 antibody (1:300; Abcam, USA; ab56023) overnight at 4 °C, then for 2 h with goat polyclonal secondary antibody to rabbit IgG-H&L (1:100, HRP; Abcam; ab6721). For BMPRII immunoreactivity, after the blocking incubation, sections were incubated with goat anti-BMPRII antibody (1:100; Santa Cruz Biotechnology, USA; sc-5682) in 0.5 M Tris-buffered saline with 0.1% Triton-X 100 (pH 7.4) overnight at 4 °C, for 1 h with secondary biotinylated horse anti-goat antibody (1:200; Vector Laboratories, USA; BA-9500), and then for 1 h at 22 °C with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories).

LCM

LCM was performed on an Arcturus Veritas™ Microdissection Instrument; Model 704 (Molecular Devices) as described previously (Ordway *et al.* 2009).

Quantitative PCR

Total RNA was isolated from laser-captured cells and tissue homogenates, then reverse-transcribed as described previously (Xiang *et al.* 2008). rtPCR was used to quantify transcripts in RNA from tissue homogenates as described previously (Xiang *et al.* 2008). Because of smaller amounts of RNA available, end-point PCR was used to quantify transcripts from LCM samples as described previously (Ordway *et al.* 2009). Genes and primers are listed in Table 2.

Chronic social defeat paradigm

Chronic social defeat stress is based on the resident–intruder paradigm as described previously (Koolhaas *et al.* 1997; Miczek, 1991; Rygula *et al.* 2005), with minor modifications. All animal procedures were approved by the East Tennessee State University Animal Care and Use Committee and complied with the NIH Guide for the Care and Use of Laboratory Animals. Male Fischer-344 rats (250–275 g) served as the experimental 'intruder' animal and Long–Evans rats (retired male breeders weighing 350–400 g) served as 'residents', residing with a sterile female rat for a week. Exposure of intruders was repeated four times for the first week, twice in the second and third weeks, and four times in the fourth week. The effectiveness of the defeat paradigm was evaluated by measuring sucrose consumption. On day 29, animals were euthanized and brains were dissected and frozen on dry ice. LC tissue containing neurons, glia and surrounding neuropil were dissected under a stereomicroscope from frozen slide-mounted sections cut through the brainstem at the level of the LC.

Statistical analyses

For reversed-transcribed RNA extracted from tissue homogenates from matched depressed and control subjects, cycle threshold (C_t) values of PCR-generated gene transcripts were determined and normalized by subtracting the geometric mean (Vandesompele *et al.* 2002) of the C_t values of reference gene expressions generated from the identical samples. Resulting ΔC_t values were converted to relative gene expression in fold changes comparing MDD subjects to control subjects, using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Data from rtPCR using tissue homogenate samples and end-point PCR analyses of the three cell types captured by LCM were analysed by paired Student's *t* test. Data from rtPCR analyses of tissues from social defeat rats and cage controls were analysed using an unpaired Student's *t* test. Summary statistics are reported as the mean ± S.E.M. Possible relationships between post-mortem variables and levels of gene expression were evaluated using Pearson's correlation analyses. Covariate analysis of variance was performed to determine whether demographic variables (e.g. RIN) modified the results of the statistical analyses. Statistical analyses were performed

Table 2. Primer sequences of target and reference genes

Target or reference[a]	Genbank accession number	Primer sequence	PCR product size (bp)
ACTB	NM_001101	(f) GCACCCAGCACAATGAAGATCAAG (r) TCATACTCCTGCTTGCTGATCCAC	128
BMP2	NM_001200	(f) GTGCGCAGCTTCCACCATGAA (r) CTGAGGTGATAAACTCCTCCGTGGG	122
BMP4[b]	NM_001202 NM_130850 NM_130851	(f) CCAACACCGTGAGGAGCTTCCACCA (r) GAGATCACCTCGTTCTCAGGGATGCTGC	118
BMP5	NM_021073	(f) GCCCATATGAATGCCACCAACCAC (r) CAAGGCTTTGGTACGTGGTCAGGA	80
BMP6	NM_001718	(f) AGCCTGCAGGAAGCATGAGCTGTA (r) AATTGGCAGCATAGCCCTTGGGTG	89
BMP7	BC004248	(f) AACCGCTCCAAGACGCCCAAGAACCA (r) TCTTACAGGCCTGCCTCTGGTCGCTG	91
BMP7 (rat)	XM_342591	(f) AGGGAGAGTGTGCCTTCCCTCTGAACT (r) GCTTGGGTACGGTGTCTGGGTTGATGA	102
BMP9	NM_016204	(f) GGTTCCAGAAACCTGCCCTTCTTTGT (r) TCATGGCTGATCATCTCCCTCAGCTC	98
BMP11	NM_005811	(f) CTTTGATCCCAGTGGCACAGACCT (r) TGTGTTCTCTAGGACTCGAAGCTCCA	97
BMPRII	NM_001204	(f) ACAAATCTGTGAGCCCAACAGTCAATCC (r) GGCACACGCCTATTATGTGACAGGTTG	82
BMPR1a	NM_004329	(f) TCTACCAAACTGTGCTAATGCGCCA (r) GCTGAGTCCAGGAACCTGTACCTTT	84
ACTR1b[c]	NM_004302	(f) TAAGCTGGCCTTGTCTGCTGCTAGTG (r) ATGCTCGATGAGCAATTCCAGGCTTCC	92
GAPDH	NM_002046	(f) TGCACCACCAACTGCTTAGC (r) GGCATGGACTGTGGTCATGAG	87
GAPDH (rat)	AF106860	(f) CTCCCATTCTTCCACCTTTGATGCTGGG (r) CCACCACCCTGTTGCTGTAGCCATATTC	110
UBC	NM_021009	(f) ATTTGGGTCGCGGTTCTTG (r) TGCCTTGACATTCTCGATGGT	133

[a] All genes shown are human except where noted.
[b] *BMP4* transcript variants 1, 2 and 3.
[c] *ACTR1b* (ACVR1B) transcript variants 1, 2 and 3.

using GraphPad Prism 5.0 (GraphPad Software Inc., USA) and Minitab (Minitab Inc., USA). A p value <0.05 was considered significant.

Results

Quantitative rtPCR was used to examine BMP7 gene expression levels in tissue punch-dissected from the LC region in brains from MDD subjects and matched psychiatrically normal control subjects. BMP7 gene expression in MDD subjects was approximately one third the level of expression in normal control subjects ($n=12$ pairs; Fig. 2; $p=0.001$). In this initial experiment, *ACTB* (β-actin) was used as the reference gene and tissue RNAs were exhausted from this set of samples because other work had been performed with these tissues. Further investigation below involved dissection of additional LC tissue from tissue blocks of all subjects, with three additional pairs of control and MDD subjects replacing three pairs used above from whom tissue was no longer available.

Given that BMP7 is one of a family of proteins, the gene expression of other BMPs was examined using mRNA isolated from punched-dissected LC tissue. Since a different set of samples were used for this further analysis, BMP7 gene expression was analysed again to confirm the finding above. Furthermore, three reference genes were used for this analysis *ACTB*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ubiquitin C (*UBC*) to increase the level of scrutiny

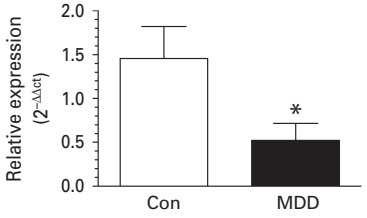

Fig. 2. Initial real-time PCR analysis of BMP7 gene expression in punch-dissected LC tissue from 12 matched pairs of MDD subjects (■) and psychiatrically normal control subjects (Con, □). Relative gene expression levels are expressed as fold changes using the method of Livak & Schmittgen (2001). Target gene expression was normalized with *ACTB*. The asterisk indicates statistical significance ($p = 0.001$).

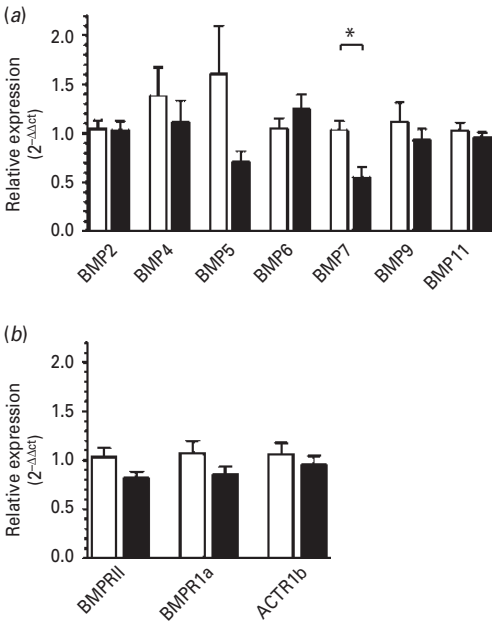

Fig. 3. Real-time PCR analysis of the gene expression of several BMPs (*a*) and associated receptors (*b*) in punched-dissected locus coeruleus (LC) tissues from 12 matched MDD (■) and psychiatrically normal control (□) subjects. Some of the subjects used in this set of experiments were different than those used to generate data in Fig. 2 as noted in Results. Target gene expression was normalized with the geometric mean of three reference genes (*GAPDH, UBC* and *ACTB*) as described in the Methods section. Relative gene expression levels were computed as fold changes using the method of Livak & Schmittgen (2001). The line above the bars indicates statistical significance for the specified comparison ($p = 0.0018$). No other comparisons between control and MDD subjects reached statistical significance.

of the study. Gene expression levels for BMP2, BMP4, BMP5, BMP6, BMP9, and BMP11 genes were similar in control and MDD subjects (Fig. 3*a*). In contrast, BMP7 gene expression was significantly lower ($p = 0.0018$, Fig. 3*a*) in MDD subjects, confirming the original finding above in this separate set of samples.

Levels of gene expression of three receptors that mediate BMP signalling were also investigated using the same samples, including BMPRII, BMPR1a, and ActR1b. These receptors were chosen (amongst other BMP receptors) based on demonstrable gene expression levels previously reported for the mouse LC (Allen Brain Atlas Resources, 2009). No significant differences in levels of BMP receptor gene expression were found comparing normal control and MDD subjects (Fig. 3*b*).

To determine whether low BMP7 gene expression in MDD is widespread throughout the brain, quantitative rtPCR was used to probe the expression of BMP7 in two other brain regions, prefrontal cortex (12 pairs of control and MDD subjects) and amygdala (eight pairs of control and MDD subjects). Not all of the subjects used for these studies were the same as were used for the study of LC tissues (see Supplementary Table S1), as indicated in Table 1, because cortical and amygdala tissues were not available for all of the same cases. In contrast to the LC, no differences in the levels of gene expression of BMP7 in cortical grey or white matter were observed comparing MDD to control subjects (Fig. 4). Similarly, no differences in BMP7 gene expression were observed in the basal nucleus of the amygdala (Fig. 4).

To determine which cell type was responsible for the reduced BMP7 gene expression, LCM was employed to separately capture noradrenergic neurons, astrocytes, and oligodendrocytes from sections cut through the LC from six pairs of normal control and MDD subjects (Table 1). The method used to capture each cell type has been previously validated by examining gene expression profiles of cell-type specific markers, i.e. tyrosine hydroxylase for noradrenergic neurons, glial acidic fibrillary protein (*GFAP*) for astrocytes, and myelin oligodendrocyte glycoprotein (*MOG*) for oligodendrocytes (Ordway *et al.* 2009). Data collected were normalized to three reference genes (*GAPDH, UBC* and *ACTB*) and also separately to the number of cells collected (Ordway *et al.* 2009). Of the three cell types, astrocytes had the largest level of BMP7 gene expression, being nearly 4-fold higher than that in noradrenergic neurons, whether expressed relative to reference genes (Fig. 5*a*) or relative to the number of cells captured (Fig. 5*b*). A robust reduction of BMP7 gene expression was

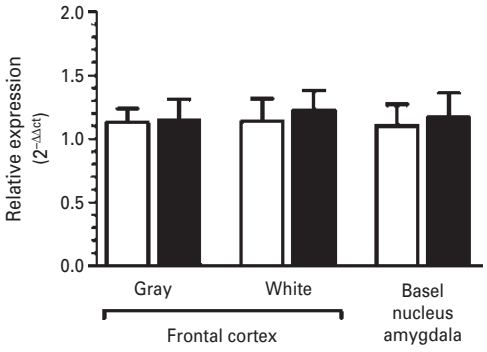

Fig. 4. Real-time PCR analysis of the gene expression of BMP7 in frontal cortical (BA 10) and amygdala tissues from carefully matched MDD (■) and psychiatrically normal control (□) subjects. BMP7 gene expression was normalized with the geometric mean of the gene expression of ubiquitin C and TATA box binding protein (cortical tissues) or with GAPDH (amygdala) gene expression as described in the Methods section. Relative gene expression levels were computed as fold changes using the method of Livak & Schmittgen (2001). Sample sizes were 12 matched pairs of subjects for cortical tissues and eight matched pairs of subjects for basal amygdala.

observed in astrocytes, but not in neurons, from major depressive subjects as compared to normal control subjects ($p = 0.0058$, Fig. 5*a*, *b*). Oligodendrocytes showed a smaller reduction in BMP7 gene expression that was only statistically significant when gene expression levels were normalized by the number of cells captured ($p = 0.042$, Fig. 5*b*) rather than by reference gene expressions. Given that levels of BMPRII gene expression were highest amongst the BMP receptors studied in the LC punches above, BMPRII gene expression was evaluated in the three cell types captured by LCM. BMPRII gene expression was highest in neurons relative to the other two cell types (Fig. 5*c*, *d*). No significant differences in BMPRII gene expression were observed in any of the three LC cell types comparing control and MDD subjects.

BMP7 protein immunoreactivity in the LC

To determine whether BMP7 gene expression changes were paralleled by similar protein expression changes in the LC, we attempted to measure BMP7 immunoreactivity using quantitative Western blotting and ELISA. However, the quantities of frozen LC tissue that were required to perform these studies exceeded available tissues. It seemed important to at least establish the presence of BMP7 protein in the region of the LC. To do this, the general distribution of BMP7 and BMPRII immunoreactivity in the region of the human LC was examined using immunocytochemistry of fixed human LC tissue. BMP7 immunoreactivity was apparent in glia and noradrenergic neurons in the region of the LC (Fig. 6, left panel). For BMP7, glia were darkly stained, although stained glial processes were not obvious. Noradrenergic neurons and proximal processes also demonstrated BMP7 immunoreactivity. BMP7 immunoreactivity was more intensely stained in the epithelial layer at the floor of fourth ventricle and in cerebellar cortical neurons and glia (not shown). BMPRII immunoreactivity was most robust in LC neurons, with minimal staining of glia (Fig. 6, right panel).

Consideration of potentially confounding issues

The possibility that variables unrelated to psychiatric status contributed to gene expression differences in the LC between the two groups of subjects was extensively considered. For the PCR analyses of the punched-dissected LC tissues, levels of gene expression of each reference gene in control and MDD subjects were normalized using the other two reference genes. No significant differences were observed comparing control and MDD subjects for *GAPDH*, *UBC* or *ACTB* (Fig. 7). However, it is noteworthy that β-actin gene expression in the LC was modestly lower in MDD subjects relative to control subjects ($p = 0.059$). Given this tendency, levels of expression for all BMP genes were recalculated using only GAPDH and UBC for normalization. BMP7 gene expression remained significantly lower in MDD subjects ($p = 0.0009$; data not shown). The results of the statistical analyses of the other BMPs using just two reference genes were unaffected, except for the observation of a modest but significantly lower gene expression for BMPIIR ($p = 0.01$).

Levels of reference genes in laser captured cells from the two study groups were also evaluated. Levels of each reference gene expressed relative to the mean of the other two were analysed for each cell type. No differences in the levels of gene expression of GAPDH, UBC or ACTB were observed in noradrenergic neurons, astrocytes or oligodendrocytes comparing control to MDD subjects (see Supplementary Table S2, online).

MDD and normal control subjects were carefully matched for several parameters to reduce the influence of potentially confounding issues. However, not all variables can be matched exactly in post-mortem studies because of the number of variables and the

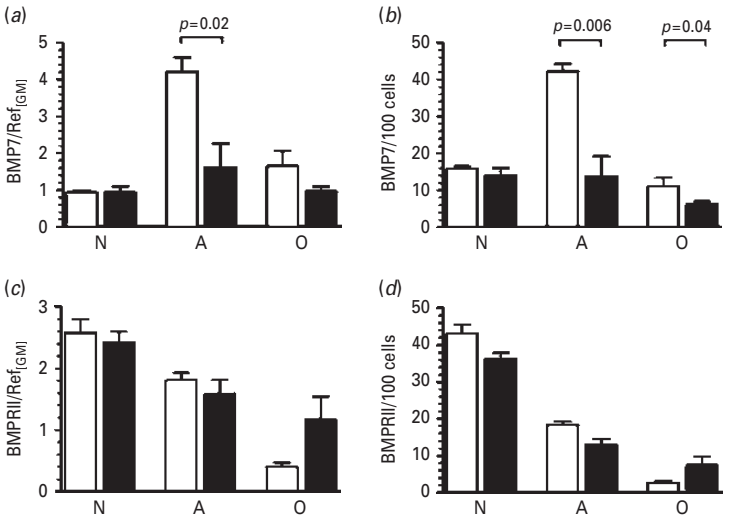

Fig. 5. Gene expression of BMP7 and BMPIIR in neurons (N), astrocytes (A) and oligodendrocytes (O) captured by laser capture microdissection from tissue sections cut through the locus coeruleus (LC) from six matched pairs of MDD (■) and psychiatrically normal control (□) subjects. Target gene expression was normalized with the geometric mean of the gene expression of GAPDH, UBC and ACTB (*a*, *c*), and was also normalized with the number of cells captured (*b*,*d*). The lines above the bars indicate statistical significance for the specified comparison. No other comparisons reached statistical significance.

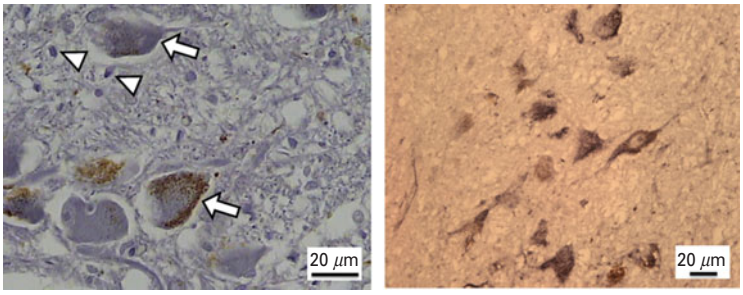

Fig. 6. Sections cut through the human locus coeruleus showing BMP7-immunoreactivity (left panel, 40 ×) and BMPRII-immunoreactivity (right panel, 20 ×). BMP7-immunoreactivity is obvious in large noradrenergic neuronal cell bodies (arrows) containing neuromelanin and in smaller glia (carets) in the immediate area surrounding neurons. BMPIIR-immunoreactivity is primarily evident in the large darkly stained noradrenergic neurons.

limitation of the availability of brain tissue from subjects that meet inclusion criteria. Post-mortem intervals, ages, pH values, and RNA integrity number (RIN) values and their averages appear in Table 1. There were no statistically significant differences between the two study groups comparing ages, pH values, or RIN values. The post-mortem interval (PMI) values of MDD subjects were significantly longer than control subjects. However, as we have observed previously (Xiang *et al.* 2008), the length of the PMI did not correlate with the quality of RNA extracted from tissues, either for the control group (Pearson's $r = -0.085$, $p = 0.72$), the MDD group (Pearson $r = -0.13$, $p = 0.60$), or for the combination of all control and MDD subjects (Pearson $r = -0.21$, $p = 0.19$). Also, PMI did not significantly correlate with expression levels of any of the genes studied. The longer PMI values of the MDD subjects is typical in our hands and is due to the fact that most of the MDD subjects died by suicide, which in most cases is committed in isolation making discovery of the deceased longer. RIN values were lower than those typically observed with fresh brain tissues from animal studies, but were typical for post-mortem brain tissue in our hands. There were no significant correlations between RIN values and PCR data for any of the target or reference

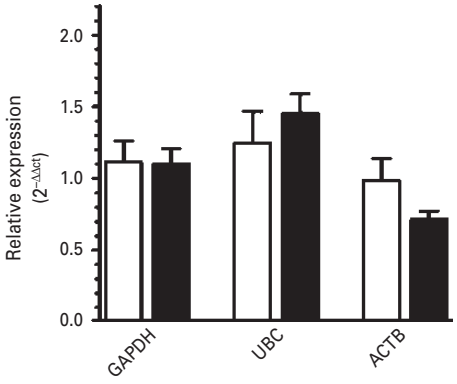

Fig. 7. Real-time PCR analysis of the expression of reference genes used in the analyses of BMPs in punched-dissected locus coeruleus tissues from 12 carefully matched MDD (■) and psychiatrically normal control (□) subjects. Each reference was normalized with the geometric mean of the expression of the other two reference genes. Relative gene expression levels were computed as fold changes using the method of Livak & Schmittgen (2001). There were no statistically significant differences, although there was a trend for reduced ACTB gene expression in MDD subjects.

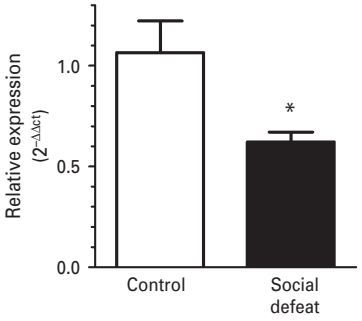

Fig. 8. Real-time PCR analyses of the gene expression of BMP7 in locus coeruleus tissue dissected from rats exposed to social defeat ($n=6$) stress and cage controls ($n=6$). BMP7 gene expression was normalized with GAPDH gene expression in the same tissues. Relative gene expression levels were computed as fold changes using the method of Livak & Schmittgen (2001). The asterisk indicates statistical significance comparing defeated rats to control rats ($p=0.022$). The social defeat paradigm was associated with significant reductions in sucrose consumption in defeated rats (see Supplementary Fig. S1).

genes. Furthermore, the outcome of the statistical analyses did not change when RIN was used as a covariate. The lack of an impact of RIN values in the range of those obtained here on PCR product amounts is similar to that described by others (Schroeder *et al.* 2006), likely at least in part because primers were deliberately designed to amplify small products, i.e. 80–133 bases (see Table 2). Levels of PCR products of this length are generally independent of RNA quality (Fleige & Pfaffl, 2006).

Finally, four MDD subjects had a history of antidepressant drug prescriptions and two MDD subjects died of non-suicide causes in the study of LC BMP7 gene expression. These sample sizes were too small to evaluate statistically. Individual comparisons of BMP7 gene expression levels of MDD subjects with antidepressant prescriptions to their matched control subjects revealed the following fold changes: 0.32, 0.32, 0.72, and 1.06. Hence, three out of four MDD subjects demonstrated lower BMP7 gene expression than their matched controls, implying no major effect of historical antidepressant exposure on BMP7 gene expression. Individual comparisons of BMP7 gene expression levels of the non-suicide MDD subjects to matched control subjects revealed fold changes of 0.38 and 0.32, suggesting that reduced BMP7 gene expression is not specific to suicide but observed in all MDD subjects.

Effect of chronic stress on BMP7 gene expression in rats

Experiments were performed to determine whether BMP7 changes in MDD are mimicked in the rat social defeat paradigm, a rodent stress model producing symptoms analogous to depression in humans (Rygula *et al.* 2005). Statistically significant reductions in sucrose consumption in the defeated rats were observed in weeks 1–4 of the paradigm (see Supplementary Fig. S1, online) compared to the control rats, confirming the effectiveness of the defeat paradigm. Gene expression was measured by rtPCR using RNA isolated from homogenates of dissected LC tissue. BMP7 gene expression was significantly lower in defeated rats as compared to control rats ($p=0.022$, Fig. 8).

Discussion

Bone morphogenetic proteins are a family of multifunctional proteins produced by a number of different body tissues, including the brain where they play a major role in the developing nervous system. Besides directing differentiation of glia (Gross *et al.* 1996; Mabie *et al.* 1997), BMP7, like BMP5 and BMP6, promote catecholamine neurite outgrowth (Guo *et al.* 1998; Jordan *et al.* 1997). In addition, BMP7 promotes catecholaminergic neuronal differentiation (Brederlau *et al.* 2002) and disruption of BMP7 function results in a loss of the catecholamine phenotype (Schneider *et al.* 1999; Tilleman *et al.* 2010). Noradrenergic actions of

BMP7 drew our intense interest given evidence of noradrenergic pathology in MDD (Ordway, 2007), as well as numerous reports of deficits in other neurotrophic factors in MDD. The results of the present investigation revealed that the BMP7 gene is expressed in catecholamine neurons and surrounding astrocytes and oligodendrocytes in the human pontine brainstem, and its expression is significantly lower in LC astrocytes from MDD subjects compared to matched control subjects.

Evaluating the neurobiological impact of a BMP7 deficit in MDD is difficult because there is limited information about the regulation and function of BMP7 in the adult nervous system. Experimental stroke models in adult rats demonstrate elevations of brain BMP7 (Chang *et al.* 2003), while infusion BMP7 reduces infarct size (Perides *et al.* 1995) and enhances recovery in stroke rats (Chang *et al.* 2003; Kawamata *et al.* 1998). Lesions of midbrain dopamine neurons in adult rats results in a robust reduction in the gene expression levels of several BMPs, including BMP7, and their receptors in the substantia nigra (Chen *et al.* 2003). Conversely, exogenous administration of BMP7 reduces the damaging effects of 6-hydroxydopamine on catecholamine neuronal indices and improves catecholamine-mediated behaviour in adult rats (Harvey *et al.* 2004), demonstrating neuroprotective and possibly neuro-reparative effects of BMP7. Collectively, these studies demonstrate that BMP7 is capable of exerting robust and important biological effects in the adult mammalian CNS.

BMP7, as well as other related BMPs, signal through several different heterodimeric serine/threonine kinase receptors, including BMPIIR, BMPR1a, BMPR1b, and activin receptors (Attisano & Wrana, 2002; Canalis *et al.* 2003). Levels of gene expression of BMP7 and its receptors vary by brain region in the rodent (Charytoniuk *et al.* 2000) and human forebrain (Allen Brain Atlas Resources, 2009). The results presented here are the first examination of BMP7 and BMP receptor gene and/or protein expression in the human pontine region, including the LC, and demonstrate that both neurons and glia in the brainstem express BMP7. BMPRII gene expression was robust in human LC neurons, consistent with levels reported previously in mouse LC (Allen Brain Atlas Resources, 2009) BMP receptor gene expression showed a tendency for reduced levels in MDD subjects, but further study of BMP receptors and signalling pathways in MDD is needed before firm conclusions can be made.

Although the present study is the first to demonstrate a potential role of BMPs in MDD, there are other studies demonstrating an association of BMP or BMP-related signalling molecules with antidepressant drug action. Phosphorylated Smad proteins, intracellular mediators of activated BMP receptors, are upregulated by repeated treatment of rats with antidepressant drugs (Dow *et al.* 2005). Also, direct infusion of activin, which activates some of the same receptors as BMPs, into the hippocampus produces an antidepressant behavioural response (Dow *et al.* 2005). These findings suggest that increasing BMP signaling may have antidepressant effects. Interestingly, a single nucleotide polymorphism upstream of the BMP7 gene has been reported to be less frequent in subjects associated with response and remission following administration of the antidepressant citalopram (Garriock *et al.* 2010). Four of the 16 MDD subjects for whom the LC was studied presently had been prescribed antidepressants sometime prior to committing suicide. If the BMP7 polymorphism is associated with reduced promoter activity and thereby reduced BMP7 gene expression, then it is intriguing to speculate that this polymorphism could ultimately contribute to suicide because of a lack of antidepressant efficacy.

Several neurochemical and morphological pathologies of the LC from major depressive subjects or victims of suicide have been described previously. These include abnormalities in levels of several noradrenergic proteins, altered levels of proteins reflecting regulatory inputs to the LC, and abnormalities in neuronal morphology (Bernard *et al.* 2011; Gos *et al.* 2008; Shibata *et al.* 2007, 2008; see Ordway, 2007). The present study extends these previous observations to glial pathology in the region of the LC. Pathologies of glia (Bowley *et al.* 2002; Cotter *et al.* 2001; Fatemi *et al.* 2004; Ongur *et al.* 1998; Rajkowska *et al.* 1999; Uranova *et al.* 2004) have been demonstrated previously in several brain regions, but the present study is the first to demonstrate specific involvement of astrocyte pathology in the brainstem, in particular low levels BMP7 in LC astrocytes in MDD subjects. It is interesting that glia-derived BMP7 stimulates dendritic growth in co-cultures of noradrenergic neurons and glia (Lein *et al.* 2002*b*). Based on the cellular distribution of BMP7 and at least one of its receptors (BMPIIR) as demonstrated here for the human LC, it is conceivable that BMP7 liberated from astrocytes that are in close proximity to LC neuronal dendrites serves a paracrine role in the maintenance or growth of LC neuronal dendrites. If like BMP7 gene expression, BMP7 protein levels are low in MDD subjects, then deficient glia-derived trophic support to noradrenergic LC neurons may result.

There are a number of weaknesses of the present study. A majority ($n=16$) of the 19 MDD subjects died by suicide. With such a small number of non-suicide MDD subjects, it was not possible to statistically analyse the potential confounding effect of suicide on the data. Although it is intriguing to consider the possibility that reduced BMP7 gene expression in MDD is accompanied by a reduction in BMP7 protein, a reduction in protein does not always parallel less gene expression. Unfortunately, attempts to measure BMP7 protein in frozen human LC failed. BMP7 gene expression changes were not observed in homogenates of the prefrontal cortex or in the basal nucleus of the amygdala. It is possible that a change in BMP7 gene expression was missed because gene expression was not analysed at the level of single cell types in these two brain regions. However, it is worth noting that reduced BMP7 gene expression was observed in the LC in both homogenates of LC tissue as well as in LCM-captured LC astrocytes. BMP7 gene expression may be reduced in other regions not explored as well, such as in other brainstem catecholamine cell groups. The action of BMPs is modulated by a number of extracellular proteins that sequester BMPs and thereby inhibit their action at BMP receptors. These include noggin, follistatin, chordin and other cysteine knot proteins (Canalis *et al.* 2003). These additional targets need to be studied to gain a complete understanding of BMP signalling alterations in MDD.

Low levels of BMP7 gene expression in LC astrocytes may contribute to noradrenergic neuronal pathology that has been previously associated with MDD. It is equally possible that pathology of noradrenergic signaling within the LC may contribute to local glial dysfunction, since astrocytes express receptors for norepinephrine and respond to norepinephrine signaling (Bekar *et al.* 2008; Hertz *et al.* 2004; Porter & McCarthy, 1997). Translating these clinical findings will require basic studies to elucidate how BMP7 gene expression is regulated, and how BMP7 regulates the function of adult noradrenergic neurons. Initial studies here demonstrate that social defeat stress in rats produces a reduction in BMP7 gene expression in the LC. Based on the known actions of BMP7, it seems possible that a stress-induced reduction in BMP7 could contribute detrimentally to other catecholaminergic disruptions that accompany chronic stress and depression (Ordway, 2007; Ordway *et al.* 2002). Previous studies (Harvey *et al.* 2004, 2005) have implicated potential therapeutic utilities of BMP7 in neurodegenerative disorders and traumatic brain injury. The present study suggests that BMP7 plays a role in the catecholamine pathology of major depression and implies that therapeutic strategies to increase BMP7 signalling may have pro-antidepressant effects.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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