1   Title: The BMP antagonist *Gremlin1* contributes to the development of cortical

2   excitatory neurons, motor balance and fear responses

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4   Running Title: *Grem1* in cortical neuron development

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21   **Key words**

22   Grem1, BMP, cortical development, excitatory neuron

23

24   **Summary statement**

25 The BMP antagonist, *Grem1,* marks neuroprogenitors that give rise to deep layer

26 glutamatergic neurons in the embryonic mouse brain. *Grem1* conditional knockout

27 mice display cortical and behavioural abnormalities.

28

Abstract

30 Bone morphogenetic protein (BMP) signaling is required for early forebrain

31 development and cortical formation. How the endogenous modulators of BMP

32 signaling regulate the structural and functional maturation of the developing brain

33 remains unclear. Here we show that expression of the BMP antagonist, *Grem1,*

34 marks a neuroprogenitor that gives rise to layer Ⅴ and Ⅵ glutamatergic neurons

35 in the embryonic mouse brain. Lineage tracing of *Grem1*-expressing cells in the

36 embryonic brain was examined by administration of tamoxifen to pregnant

37 *Grem1creERT*; *Rosa26LSLTdtomato* mice at 13.5 days post coitum (dpc), followed by

38 collection of embryos later in gestation. In addition, at 14.5 dpc, bulk mRNA seq

39 analysis of differentially expressed transcripts between FACS sorted *Grem1* positive

40 and negative cells was performed. We also generated *Emx1-cre* mediated *Grem1*

41 conditional knockout mice (*Emx1-Cre;Grem1^{flox/flox}*) in which the *Grem1* gene was

42 deleted specifically in the dorsal telencephalon. *Grem1^{Emx1cKO}* animals had reduced

43 cortical thickness, especially layers Ⅴ and Ⅵ and impaired motor balance and

44 fear sensitivity compared to littermate controls. This study has revealed new roles

45 for Grem1 in the structural and functional maturation of the developing cortex.

46

Introduction

48   During development of the nervous system, bone morphogenetic protein (BMP)

49   signaling has important roles in the promotion of dorsal identity, and the regulation

50   of cell proliferation and differentiation. Yet BMP function in the maturation of

51   neurons in vivo is poorly understood. Misregulation of BMP signaling has been

52   suggested to contribute to human neurodevelopmental conditions such as autism

53   spectrum disorders (Kumar et al., 2019), however a detailed understanding of the

54   exact role for BMP signaling in these disorders is lacking, in line with our imperfect

55   knowledge of the regulation of the BMP pathway in normal brain development.

56   The BMP ligands (BMP 2, 4, 5, 6 and 7) bind to type I (BmprIA, BmprIB, Acvr1)

57   and type II (BmprII, ActrIIA, and ActrIIB) receptors. BMP ligands are expressed

58   broadly in the telencephalon during early gestation in mice and then become more

59   tightly localized to the choroid plexus at 13.5 dpc (Furuta et al., 1997). BMPs have

60   also been derived from the mesenchyme of the brain, such as meninges and

61   endothelial cells (Imura et al., 2008, Choe and Pleasure, 2018). Both type I and II

62   BMP receptors are expressed in the telencephalon during development but this is

63   further restricted in adulthood to BmprII in the cortex and hippocampus and

64   ActrIIA/IIB in the dentate gyrus (Söderström et al., 1996). Aside from this

65   spatiotemporal regulation of receptors, the BMP signaling pathway is also regulated

66   by a family of secreted extracellular antagonists, that directly bind to the BMP

67   ligands to prevent interactions with BMP receptors both in development and disease

68   (Ali and Brazil, 2014). Antagonists such as Gremlin1 (Grem1), Noggin (Nog) and

69   Chordin (Chrd) have been shown to inhibit BMP action in a range of different cell

70   types and developmental stage-specific contexts to provide exquisite spatiotemporal

71   regulation of the pathway. The roles of Nog and Chrd have been partially elucidated:

3

72 they are required for forebrain development (Bachiller et al., 2000), as well as to

73 create a niche for adult hippocampal neurogenesis (Lim et al., 2000, Sun et al., 2007).

74 The expression and function of Grem1 in the developing brain has not yet been

75 determined. Grem1 is an extracellular secreted antagonist of BMP 2,4, and 7 that

76 signals to intestinal stem cells in the gut (Worthley et al., 2015) and plays a crucial

77 role in *Xenopus* dorsalisation (Hsu et al., 1998) and limb and kidney formation

78 (Khokha et al., 2003). Likewise the role of Grem1 in the normal adult CNS is

79 unmapped territory to date, outside of prior work in the pathogenic state of glioma

80 (Yan et al., 2014, Guan et al., 2017, Fu et al., 2018).

81   BMP signaling has been implicated in the regulation of forebrain patterning during

82 early embryogenesis, working together with other signaling pathways such as

83 fibroblast growth factor (FGF), Wnt, and Notch. High levels of BMP activity

84 suppresses anterior neural development, whereas abrogation of BMP signaling can

85 promote neural specification (Wilson and Houart, 2004, Lamb et al., 1993, Bachiller

86 et al., 2000). *In vitro* the addition of BMP 2 and 4 to mouse neural stem cell cultures

87 represses cell proliferation (Mathieu et al., 2008). BMP ligands regulate neuronal

88 differentiation as well as determination of glial cell fate, by promoting astrocyte

89 differentiation at the expense of oligodendrocytic fates (Mathieu et al., 2008, Yun et

90 al., 2004, Sun and Xu, 2010, Gomes et al., 2003).

91   Cortical neural stem cells (also known as radial glial cells, RGC) begin to divide

92 asymmetrically to start producing neurons at around 11.5 dpc in mice, signaling the

93 start of cortical development. RGC daughter cells detach from the ventricle and form

94 the first neuronal layer of the preplate by 13.5 dpc (Martynoga et al., 2012).

95 Subsequently born neurons migrate along RGCs and start to form the cortical plate,

4

96 separating the preplate into Cajal-Retzius cells in the marginal zone and subplate

97 neurons (Dwyer et al., 2016). The neocortex develops in an inside out manner, with

98 deep layers emerging first and superficial layers last. These neurons differentiate

99 into glutamatergic pyramidal neurons, whereas inhibitory interneurons are born in

100 the subcortical ganglionic eminences. Deep-layer pyramidal neurons (DLPNs, layer

101 V and VI) have both intratelenchephalic projections to superficial cortical layers and

102 extracephalic projections to other brain regions (Baker et al., 2018). Multiple

103 molecular mechanisms regulate this corticogenesis (Martynoga et al., 2012),

104 however the role of BMP signaling in cortical layer formation and functional

105 maturation of neurons has only been reported in limited studies so far. *In utero*

106 electroporation of BMP7 to murine 14.5 dpc cortical ventricular cells impaired

107 neuronal migration, suggesting BMP signaling regulates neuronal positioning and

108 migration (Choe and Pleasure, 2018). BMPs also regulate dendritogenesis and

109 neurite growth *in vitro* (Lee - Hoeflich et al., 2004, Matsuura et al., 2007). This is

110 consistent with a recent study that suggests perturbation of BMP signaling by

111 delivery of a dominant-negative version of BMP IB receptor affects migration,

112 polarity and dendritogenesis of mouse cortical neurons *in vivo* (Saxena et al., 2018).

113   To further establish the role of BMP signaling in forebrain development and

114 neuronal function, we focus here on the expression and function of the BMP

115 antagonist Grem1 in the developing mouse brain. We first assess *Grem1* expression

116 using transgenic *Grem1* reporter mice in the dorsal telencephalon and developing

117 neocortex. Next, to investigate the role of *Grem1* in the developing brain we use

118 transcriptomic analyses of sorted mouse *Grem1*-expressing cells and human single

119 cell RNA sequence (scRNAseq) data, combined with mouse neural stem/progenitor

120   cell (NSPC) culture *ex vivo*. Lastly, to examine the functional contribution of *Grem1*

121   to cortical development, we conditionally delete *Grem1* in the dorsal telecephalon

122   and undertake behavioural testing of mutant animals and littermate controls.

123

124   **Results**

125   ***Grem1* expressing cells are located in the dorsal telencephalon and give rise to deep**

126   **layer neocortical neurons**

127   To assess *Grem1* expression in the embryonic mouse brain we utilized transgenic

128   *Grem1creERT; Rosa26LSLTdtomato* reporter mice, in which tamoxifen treatment

129   results in expression of TdTomato in cells where the *Grem1* enhancer and promoter

130   sequences are active and the progeny of those cells (Worthley et al., 2015). Pregnant

131   *Grem1creERT; Rosa26LSLTdtomato* mice were administered tamoxifen at 11.5 dpc

132   and embryonic brains were collected 24 h later, but no TdTomato+ cells were

133   observed, suggesting that *Grem1* is not yet expressed in the brain at this early time-

134   point (**Suppl Fig.1A**). Next, we administered tamoxifen to pregnant dams at 13.5 dpc,

135   and collected embryonic brains at 14.5, 17.5 and 20.5 dpc (**Fig.1A,B**). From this

136   timepoint we observed cells expressing TdTomato 24 hours after tamoxifen

137   administration located in the lower cortical plate and subplate of the dorsal

138   telencephalon, with dendrites extending to the pia matter (**Fig.1B,G**). ISH confirmed

139   that *Grem1* RNA was detected in almost all TdTomato+ cells at 14.5 dpc, confirming

140   that the reporter line recapitulates endogenous *Grem1* expression. Six days later at

141   20.5 dpc, TdTomato+ cell somas had migrated toward the lateral ventricle with

142   dendrites extending to the pia matter, becoming layer Ⅴ pyramidal neurons

143   (**Fig.1B,C**). Layer Ⅵ neurons were TdTomato+ at 17.5 dpc and expanded in number

144   at 20.5 dpc (**Fig.1B,C**). ISH showed *Grem1* RNA was detected in most TdTomato⁺

145   layer Ⅴ pyramidal neurons, but only 22% of layer Ⅵ neurons (**Fig.1C,D**). This

146   suggests that *Grem1* is actively expressed in layer V neurons but not layer Ⅵ

147   pyramidal neurons at 20.5 dpc. Cortical *Grem1* mRNA levels dramatically decrease

148   after birth as determined by qRT-PCR analysis of total RNA isolated from mouse

149   brain cortex at birth, p10 and 4w post-birth (**Fig.S1B**).

150   To confirm that *Grem1* + cells are actively dividing in order to give rise to their

151   traced progeny, we administered the thymidine analogue BrdU concomitantly with

152   tamoxifen to pregnant *Grem1creERT; Rosa26LSLTdtomato* dams at 13.5 dpc

153   (**Fig.1A**). BrdU is incorporated into newly synthesized DNA and so marks cells that

154   are dividing. 55% of TdTomato⁺, layer Ⅴ pyramidal neurons cells are labelled with

155   BrdU at 17.5 dpc, demonstrating *Grem1* expressing cells at 13.5 dpc are progenitor

156   cells undergoing mitosis (**Fig.1E,F**). Fewer TdTomato⁺ cells (17%) in layer Ⅵ were

157   BrdU positive, suggesting lower cell proliferation over the same period (**Fig.1F**). We

158   subsequently assessed whether TdTomato⁺ cells express a marker of post-mitotic

159   mature neurons, NeuN, by immunohistochemistry. At 14.5 dpc most TdTomato⁺ cells

160   were NeuN negative and were located in the subplate, and lower NeuN positive

161   cortical plate (**Fig.1G**). In contrast, later in gestation at 20.5 dpc almost all

162   TdTomato⁺ cells in layer V and approximately half of those in layer VI express NeuN

163   (**Fig.1H**). This suggests that Grem1-lineage traced cells give rise to mature neurons

164   in the telencephalon.

165

166   **Global transcriptomic analysis defines transcript modules enriched in *Grem1-***

167   **expressing cells**

168   To further characterise *Grem1* expressing cells in the developing mouse brain, we

169   undertook mRNAseq analysis on TdTomato$^+$ and negative cells from the

170   telencephalon. Pregnant *Grem1creERT; Rosa26LSLTdtomato* mice were

171   administered tamoxifen at 13.5 dpc and embryonic brains were collected at 14.5 dpc.

172   At this time-point almost all TdTomato$^+$ cells express endogenous *Grem1* RNA

173   (**Fig.1C**). Dissociated brain cells were flow sorted and live, TdTomato$^+$ and

174   TdTomato$^-$ cells were collected for transcriptomic analysis. TdTomato$^+$ cells

175   accounted for $5.1 \pm 2.1\%$ of all live cells (n=5) (**Fig.2A**). Bulk mRNA sequencing of

176   TdTomato$^+$ and TdTomato$^-$ populations and analysis of 1845 DEG between the two

177   cell populations revealed that *Grem1* was significantly upregulated in TdTomato$^+$

178   cells and BMP transcriptional target genes (Id1, Id3, Id4) were significantly

179   downregulated (adjusted P value (FDR) ≤ 0.05 (**Fig.2B**). We next undertook a

180   correlation analysis to identify modules of the 1845 DEGs that are coordinately

181   regulated in TdTomato$^+$ cells (**Fig.S2**). Shared membership of a module can suggest

182   genes that together perform a particular function. The clustering tree depicts the

183   topological distance between different modules, i.e. how similar or different the

184   expression of transcripts within the module are from other modules and highlighted

185   one module that contained 288 genes with particularly tightly correlated transcripts

186   (light green module, **Fig.S2**). Network visualization of the correlation analysis

187   showed that the majority of transcripts within this tightly correlated module are

188   upregulated in TdTomato$^+$ cells (magenta square, **Fig.2C**). We investigated the

189   known function of genes within this module using a hypogeometric test for gene set

190   enrichment. Significantly enriched pathways (adjusted P value (FDR) ≤ 0.001) were

191   related to neuronal differentiation and projection, calcium signaling (possibly

192   associated with synapse functions) and axons (**Fig.2D**). The BMP target gene, *Id1*,

193   was identified as one of the central hub genes in this cluster and its expression was

194   significantly associated with other transcripts that have functions in neuronal

195   maturation (such as Lrrtm3, Ryr3) (**Fig.2E**). This suggests that neuronal functions

196   are upregulated in the *Grem1*-expressing TdTomato$^+$ cells of the developing

197   telencephalon, and that this biological function is linked, at least in part, to BMP

198   signaling.

199

200   ***Grem1/GREM1* is enriched in excitatory neuronal lineage cells during brain**

201   **development**

202   To further characterize the *Grem1*-expressing TdTomato$^+$ cell population we

203   undertook a candidate gene profiling approach using the bulk mRNAseq data. Gene

204   expression patterns of neuronal markers from a series of differentiation stages are

205   shown in **Fig.3A**. Immature neuronal markers, such as *Dcx, Ncam1, Neurod 2/6,*

206   *Tbr1*, were significantly upregulated at the RNA level in TdTomato$^+$ cells in

207   comparison to the TdTomato$^-$ cells at 14.5 dpc, while neural stem cell, radial glia,

208   and intermediate progenitor transcripts were significantly under-represented

209   ($p<0.05$). When RGCs (marked by co-expression of Sox2 and Pax6) generate

210   neocortical neurons, the expression of specific transcription factors can be used to

211   identify neurons within particular cortical layers(Martynoga et al., 2012). Our bulk

212   RNAseq analysis showed that transcripts encoding the layer Ⅴ marker, *Fezf2*, and

213   Ⅵ markers, *Tbr1 / Sox5*, were significantly upregulated in *Grem1*-expressing

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214   TdTomato⁺ cells, whereas the RGC markers, *Sox2* and *Pax6* were significantly

215   downregulated (absolute value of log2 fold change≥2.0, FDR<0.05) and did not

216   colocalize with Tdtomato at 14.5 dpc by immunohistochemistry (**Fig.S3**).

217   To assist with identification of the neuronal subtype likely generated from *Grem1-*

218   expressing TdTomato⁺ cells, we also performed GSEA on DEG transcripts between

219   TdTomato⁺ and TdTomato⁻ cells. We found a significant enrichment of a glutamate

220   secretion gene set in the DEGs (NES = 2.16 p = 0.0049) (**Fig.3B**), whereas GABAergic

221   and dopaminergic gene sets were not enriched (NES = -0.93 p = 0.75, NES = -0.69 p

222   = 0.87 respectively) (**Fig.S4A**). Consistent with the GSEA, glutamatergic neuron

223   markers, such as Slc17a7 (vGlut1), Grin1 and Grin2b were significantly upregulated

224   in Grem1 expressing cells at 14.5 dpc using a candidate gene approach, whereas

225   gabaergic and dopaminergic neuron markers, such as Slc6a1 (GABA transporter 1),

226   Gad1, Gad2, and Th (Tyrosine hydroxylase) were significantly downregulated

227   (p<0.05, **Fig.S4B**). Tbr1 plays a central role in the development of early-born cortical

228   excitatory neurons and regulates the connectivity of layer Ⅵ neurons (Hevner et al.,

229   2001). To confirm that Grem1 cells generate excitatory neuronal lineages, we

230   undertook immunohistochemical staining with Tbr1. This revealed *Grem1-*

231   expressing TdTomato⁺ cells at 14.5 dpc expressed very low to no Tbr1, however they

232   did give rise to Tbr1⁺ neurons in cortical layer Ⅵ at 20.5 dpc (**Fig.3C,D**).

233   To understand the relevance of our mouse focused study to the human setting, we

234   reanalyzed publicly available human scRNAseq data generated from human brain

235   at mid-gestation (22-23 weeks post-conception) (Fan et al., 2018). We categorized

236   each human cell in the dataset as either *GREM1⁺* or *GREM1⁻* based on the presence

237 or absence of *GREM1* RNAseq counts for each cell. Next, we compared the

238 transcriptional profiles of our mouse embryonic brain *Grem1*-expressing TdTomato[+]

239 cells and TdTomato^{-} cells, with the human mid-gestational brain *GREM1*^{+} and

240 *GREM1*^{-} cells using a multidimensional scaling plot (**Fig.S4C**). Samples within the

241 same group cluster tightly together, dimension 2 separates the samples along species

242 lines, while dimension 1 clearly shows a similar separation of samples based on

243 altered expression profiles depending on *Grem1/GREM1* status in mouse and human

244 developing brain (**Fig.S4C**). By mapping the *GREM1*^{+} cells onto a tSNE plot

245 generated from the human scRNAseq data, this revealed that most *GREM1*-

246 expressing cells accumulated in the excitatory neuron cluster within the human mid-

247 gestational cortex (**Fig.3E**). Lastly, we analysed the expression of BMP-signaling

248 components in the human scRNAseq dataset (**Fig.S4D,S5**). Interestingly, of the

249 pathway antagonists and similar to *GREM1, Sclerostin domain-containing protein*

250 *1* (*SOSTDC1)* transcripts were also highly enriched in the excitatory neuron cluster,

251 with *CHRD, NOG, BMP Binding Endothelial Regulator* (*BMPER)* and *Follistatin*

252 (*FST)* transcripts also enriched in specific cell types (**Fig.S4D**). Transcripts encoding

253 BMP2,4,7 that are antagonized by GREM1 were detected in various cell populations,

254 however expression of *Bmp2,7* was inversely correlated with *Grem1* expression in

255 the excitatory neuron cell population suggesting paracrine pathway regulation

256 (**Fig.S5**). Expression of the BMP target genes, *Id1,3,4* was also inversely correlated

257 with *Grem1*-expression in excitatory neurons, consistent with our developmental

258 mouse brain data (Fig 2B). This highlights the complexity of regulation of BMP

259 signaling during development of the cortex.

260

261 **Grem1 promotes proliferation and neural differentiation in neural stem / progenitor**

262 **cells *ex vivo***

263   Next we collected NSPCs from embryonic brains of *Grem1$^{flox/flox}$* mice at 14.5 dpc

264   and transduced the cells ex vivo with control, *Cre*-expressing or *Grem1*-expressing

265   lentivirus to generate control *Grem1$^{flox/flox}$, Grem1$^{-/-}$* and *Grem1* over-expressing

266   (*Grem1$^{O/E}$*) primary cultures. Endogenous Grem1 was detected in *Grem1$^{flox/flox}$*

267   control cultures by western blot, with absent or elevated protein levels in *Grem1$^{-/-}$*

268   and *Grem1$^{O/E}$* cultures, respectively (**Fig.4A**). Control *Grem1$^{flox/flox}$* cultures were

269   responsive to BMP pathway induction as determined by increased BRE-luciferase

270   reporter activity and expression of the BMP target genes, *Id1/2/3/4*, following

271   addition of recombinant human BMP2 (**Fig.4B, Fig.S6A**). This induction of BRE-

272   reporter activity and increase in BMP target gene transcript levels was significantly

273   enhanced in *Grem1$^{-/-}$* cells and attenuated in *Grem1$^{O/E}$* cells (**Fig.4B, Fig.S6A**). This

274   confirmed that Grem1 acts as an antagonist of BMP2 and suppresses downstream

275   transcriptional targets in embryonic NSPCs.

276   We next employed neurosphere assays to determine the effect of Grem1 modulation

277   on proliferation of embryonic NSPCs. Only mitogen responsive cells proliferate to

278   form clusters termed neurospheres, where sphere size correlates with proliferative

279   ability (Mori et al., 2006, Reynolds and Rietze, 2005). *Grem1$^{O/E}$* cells form

280   significantly more and larger neurospheres than control *Grem1$^{flox/flox}$* cells, whereas

281   *Grem1$^{-/-}$* cells form significantly fewer (**Fig.4D,E**). To understand this phenomenon

282   from the aspect of cell viability, we evaluated the proliferation rates of *Grem1$^{flox/flox}$,*

283   *Grem1$^{-/-}$* and *Grem1$^{O/E}$* cultures. Over-expression of *Grem1* significantly increased

284   the rate of NSPC proliferation compared to control *Grem1$^{flox/flox}$* NSPCs, while

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285   conversely the number of viable $Grem1^{-/-}$ cells did not increase over the 72h time

286   course analysed (**Fig.4F**). These results suggested that Grem1 contributes to the

287   proliferation of NSPCs. Following on from this observation, we wanted to assess the

288   role of Grem1 in the differentiation of NSPCs to neurons, astrocytes and

289   oligodendrocytes. When cultured in recombinant BMP2-containing differentiation

290   media, addition of 1ng/ml recombinant GREM1 significantly increased the number

291   of NSPCs that differentiated into Tuj1$^+$ neurons and O4$^+$ oligodendrocyte lineage

292   cells, and decreased the number of GFAP$^+$ astrocytes, in comparison to vehicle

293   treated control NPSCs (**Fig.4G,H**). This suggests that Grem1 may regulate the

294   differentiation potential of NPSCs.

295

296   ***Grem1* is required for normal cortical development**

297   In order to assess the functional role of *Grem1* in mouse forebrain development in

298   vivo, we generated tissue-specific *Grem1* conditional knockout mice using the *empty*

299   *spiracles homeobox 1* (*Emx1*)-*cre* driver. We first verified that *Emx1*-*cre* generates

300   efficient cre-mediated recombination in the dorsal telencephalon at 14.5 dpc by

301   visualizing TdTomato$^+$ cells in reporter *Emx1*-*cre ; Rosa26LSLTdtomato* mouse

302   brains (**Fig.S7A**). Next we confirmed that *Emx1*-*cre ; Grem1$^{flox/flox}$* (*Grem1*

303   conditional knockout, *Grem1^{cKO}*) mice had a significant reduction in *Grem1* RNA in

304   the developing forebrain compared with cre negative littermate controls by ISH at

305   14.5 dpc (**Fig.5A**) and real time RT-PCR at postnatal day0 (**Fig.5B**). *Grem1^{cKO}* mice

306   were viable and fertile. To examine the morphological consequences of conditional

307   *Grem1* loss in the developing mouse brain we performed Nissl staining on tissue

308   samples from *Grem1^{cKO}* and cre negative littermate controls. At 10 weeks of age,

309 total cortical thickness was significantly reduced in *Grem1cKO* mice in comparison

310 with *Grem1flox/flox* littermate controls both in males and females, due to significantly

311 thinner cortical layers Ⅴ and Ⅵ (**Fig.5C, Fig.S7B**). The position and thickness of

312 each layer was confirmed by immunohistochemistry using markers specific for each

313 layer, i.e. CDP for layer Ⅱ-Ⅳ, Ctip2 for layer Ⅴ and Ⅵ and Foxp2 for layer Ⅵ

314 (**Fig.5D,E**). Cellular density was also more sparse in *Grem1cKO* mice in comparison

315 with *Grem1flox/flox* littermate controls (**Fig.S7C,D**). Conversely, the other predominant

316 region of *Emx1-cre* driver activity, the hippocampus, that has important functions

317 in memory for navigation pertinent to our behavioural testing, displayed normal

318 morphology and cell density in *Grem1cKO* mice (**Fig.S7E**).

319   Our earlier transcriptional network analysis from bulk RNAseq data identified an

320 *Id1* associated gene cluster that was differentially regulated in the *Grem1-*

321 expressing TdTomato⁺ cells from the cortex at 14.5 dpc (**Fig.2E**). From this cluster,

322 *Lrrtm3* and *Ryr3* were selected for further analysis because of their known functions

323 in synapse development (Balschun et al., 1999, Um et al., 2016, Roppongi et al., 2017,

324 Futagi and Kitano, 2015, Linhoff et al., 2009). The expression of these two

325 transcripts were significantly downregulated in the cortex of *Grem1cKO* mice

326 compared with littermate controls at postnatal day 10 (**Fig.S7F**). This provides

327 further evidence of the relationship between *Grem1* expression and these genes.

328

329 **Motor coordination and fear responses were impaired in *Grem1cKO* mice**

330   To assess the functional consequences of embryonic *Grem1* deletion in the mouse

331 forebrain, we undertook behavioural testing with *Grem1cKO* mice in comparison with

14

332   *Grem1^flox/flox* littermate controls. We compared motor balance using the Rotarod test.

333   Latency to fall was significantly shorter in *Grem1^cKO* mice both for males and females,

334   suggesting an impaired motor balance with loss of *Grem1* (**Fig.5G**). During the open

335   field test used to assess exploratory behaviours, both male and female *Grem1^cKO*

336   mice spent significantly more time in the central area away from the walls than

337   littermate controls (**Fig.5G**). The total distance moved and velocity of movement were

338   similar between *Grem1^cKO* and littermate controls (data not shown). Behavioral

339   testing using an elevated plus maze also indicated that female *Grem1^cKO* mice spent

340   significantly more time in the open maze arms and entered into the open arms more

341   frequently than littermate controls (**Fig.5G**). Both of the exploratory behavior tests

342   indicate that conditional loss of *Grem1* leads to reduced anxiety-like behavior. Lastly,

343   we used the Y maze test, to assess the short-term memory of *Grem1^cKO* and

344   littermate controls. We observed no difference in the number of entries made to the

345   novel arm of the Y maze between groups (**Fig.S8**), suggesting that *Grem1* expression

346   is not required for short-term memory function.

347

348   **Discussion**

349   The spatiotemporal regulation of BMP signaling in brain development is poorly

350   understood. Here we focus on the expression and function of the BMP antagonist

351   Grem1 during brain development, using transgenic lineage tracing, gene expression,

352   in vitro culture and conditional knock out approaches.

353   In the developing mouse brain *Grem1* is first expressed at 13.5 dpc in the actively

354   dividing dorsal telencephalon cells that retain mitotic potential (**Fig.1E,F**). *Grem1-*

355   expression identifies neuroprogenitors committed to the NeuN+ layer Ⅴ and Tbr1+

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356   VI neurons. Transcriptomic profiling using our mRNAseq data from embryonic

357   *Grem1*-reporter mouse brains and publicly available human developmental brain

358   scRNAseq data suggests that *Grem1/GREM1* expression is primarily associated with

359   markers of glutamatergic excitatory neuronal lineages, rather than GABAergic or

360   dopaminergic neurons or other cell types of the developing brain (**Fig.3B,E**,

361   **Fig.S4A,B**). Consistent with this, *Grem1* was not expressed in the ganglionic

362   eminences, from which GABAergic interneurons originate; nor were GABAergic

363   transcripts upregulated or enriched by GSEA in *Grem1*-expressing TdTomato⁺ cells

364   in the dorsal telencephalon using bulk RNAseq analysis (**Fig.S4A, B**). Likewise

365   Grem1-lineage traced cortical neurons expressed the excitatory marker, Tbr1

366   (**Fig.3C**). *Grem1*-expression labels and generates important excitatory neuronal

367   lineages, i.e. pyramidal neurons, that play high-level cognitive functions in the

368   neocortex. As Grem1 expression is required for the correct development of early-born

369   excitatory neurons of the neocortex (**Fig.1G**), misregulation of BMP signaling likely

370   affects the balance between excitatory and inhibitory neuronal activities, which has

371   been implicated in neurodevelopmental disorders (Dani et al., 2005).

372   Our analysis of scRNAseq data from human midgestational cortex (22-23 weeks

373   post-conception) suggests that there is coordinated regulation of BMP agonists and

374   antagonists at this developmental stage in humans, but that there may be also be

375   functional redundancies (**Fig.S4D**). Noggin is the most extensively studied of the

376   BMP antagonists in the CNS, where it is a recognized neural inducer during

377   gastrulation (Lamb et al., 1993). Addition of recombinant Noggin to neural stem cells

378   promotes neuronal and suppresses astrocytic differentiation *in vitro* (Mikawa and

379   Sato, 2011). In our study, we confirmed that Grem1 acts as a BMP antagonist in

16

380   embryonic NSPC cultures, suppressing downstream BMP transcriptional targets

381   (**Fig.4,S6**). Similar to Noggin, we determined that Grem1 promotes proliferation and

382   neural differentiation, at the expense of astrocytic lineages *in vitro* (**Fig.4**). BMP

383   transcriptional targets were significantly downregulated in *Grem1*-expressing

384   TdTomato$^+$ cells also in vivo (**Fig.2B**) and *Grem1$^+$* excitatory neurons in the human

385   embryonic cortex (**Fig.S5**). Together these results suggest that Grem1 expressing

386   cells are fated to become neurons via antagonism of potential paracrine BMP signals

387   (**Fig.S4D,S5**) (Furuta et al., 1997, Imura et al., 2008, Choe and Pleasure, 2018).

388   Our histological analyses identified that the cortex (particularly neocortical layers

389   Ⅴ and Ⅵ) of *Grem1^{cKO}* animals is significantly thinner, with less cells, in

390   comparison to littermate controls, with no obvious morphological changes in the

391   hippocampus. Our in vitro experiments confirmed that Grem1 drives proliferation

392   and neuronal differentiation in NSPC, thus this cortical phenotype is likely due to

393   reduced proliferation of embryonic NSPCs in *Grem1^{cKO}* mice. Layer Ⅴ neurons

394   target the spinal cord, cerebellum, striatum and the thalamus (Shipp, 2007) and play

395   roles in movement preparation, movement guidance, and the execution of well-timed

396   movements (Baker et al., 2018, Li et al., 2015). The observed decrease in layer Ⅴ

397   neurons would lead to impaired preparation for and coordination of movement in

398   *Grem1^{cKO}* animals and explain the motor balance defect in these animals in

399   comparison to litter mate controls. Emerging evidence suggests that layer Ⅵ

400   neurons play a central role in modulating thalamic and cortical neurons during

401   sensory processing (Wang et al., 2016). Our behavioural testing confirmed that loss

402   of Grem1 expression significantly impaired fear sensitivity in *Grem1^{cKO}* animals,

17

403   compared to littermate controls, consistent with a deficiency in layer VI neurons

404   with roles in sensory connections. BMP signaling is known to promote

405   synaptogenesis (Xiao et al., 2013, Shen et al., 2004) and altered synaptogenesis can

406   affect both fear sensitivity and motor abilities (Ha et al., 2016, Wood and Shepherd,

407   2010). Network analysis of DEG between *Grem1*-expressing TdTomato$^+$ and

408   TdTomato$^-$ cells from the developing mouse dorsal telencephalon identified a gene

409   cluster of inter-related transcripts in which the BMP target gene, *Id1*, acts as a hub.

410   Genes in the cluster play roles in neuron morphogenesis, synapse and axon

411   maturation (**Fig.2D,E**). For example, *Leucine-rich-repeat transmembrane neuronal*

412   *proteins 3/4* (*Lrrtm3/4*) genes encode synapse organizing proteins critical for the

413   development and function of excitatory synapses (Um et al., 2016, Roppongi et al.,

414   2017, Linhoff et al., 2009). Likewise, the *Ryanodine receptor3* (*Ryr3*) gene encodes a

415   member of a family of receptors that shape synaptic transmissions(Balschun et al.,

416   1999) by amplifying spike-driven calcium signals in presynaptic terminals, and

417   consequently enhancing the efficacy of transmitter release (Futagi and Kitano, 2015).

418   In *Grem1^{cKO}* animals, the mRNA expression of *Lrrtm3* and *Ryr3* was significantly

419   decreased in comparison to littermate controls (**Fig.S7F**). These results suggest

420   *Grem1* may contribute to synapse formation and function by inhibiting BMP

421   signaling and expression of associated gene networks, the subject of further studies.

422   In summary, this is the first study to reveal the important function of Grem1 in

423   cortical development, particularly as a marker, and maker, of excitatory

424   neuroprogenitors. In the future, Grem1 may hold value beyond understanding the

425   cellular biology of brain development and function as we develop new approaches to

426   help tackle complex neurodevelopmental and neurological diseases.

427

Materials and Methods

429

Mice

431   *Grem1-CreERT* transgenic mice (Worthley et al., 2015) were crossed with *R26-*

432   *LSLTdTomato* mice (B6.Cg-*Gt(ROSA)26Sor^tm14(CAG-tdTomato)Hze*/J, JAX 007914) to

433   generate a tamoxifen-induced *Grem1* reporter line. Pregnant dams were

434   administered 6mg of tamoxifen by oral gavage to induce embryonic *Grem1*-tracing,

435   with some dams bearing 13.5 dpc embryos also intraperitoneally injected with 5-

436   Bromodeoxyuridine (BrdU, Roche, 40 mg/kg) to track cell division. *Grem1^flox/flox*

437   mice (Gazzerro et al., 2007) were crossed with *Emx1-cre* mice (B6.129S2-

438   *Emx1^tm1(cre)Krj*/J, JAX 005628) to generate *Emx1-cre* mediated *Grem1* conditional

439   knockout mice (*Emx1-cKO*). The line was maintained by crossing *Emx1^-cre/+* ;

440   *Grem1^flox/flox* mice with *Grem1^flox/flox* mice.

441   All mice were on the C57BL6/J background and experimentation was conducted

442   following approval by SAHMRI Animal Ethics Committee (approval number

443   SAM284) in accordance with the Australian code for the care and use of animals for

444   scientific purposes, 8th edition.

445

Preparation of single cell suspensions and flow cytometry

447   Pregnant *Grem1creERT; Rosa26LSLTdtomato* mice were administered tamoxifen at

448   13.5 dpc. At 14.5 dpc, dorsal telencephalons of the embryos were dissected in cold

449   phosphate-buffered saline (PBS) and the meningeal membranes were removed.

450   Neurocult Enzymatic Dissociation kit (Stemcell Technologies) was used for cell

451   dissociation according to the manufacturer's protocol. Dissociated cells were

452   resuspended in FACS buffer containing DAPI (0.5µg/ml). Sorting and analyses were

453   carried out on a FACS Fusion flow cytometer (Becton-Dickinson). Dead cells were

454   excluded by gating on forward and side scatter and by eliminating DAPI-positive

455   events. The cells harvested from *cre* negative littermate control mice were used to

456   set background fluorescence levels. Viable cells were sorted into FACS buffer,

457   collected via centrifugation and resuspended in Trizol (Invitrogen) for RNA

458   extraction.

459

460   **RNA extraction and mRNA seq**

461   RNA was extracted from sorted cells in Trizol according to the manufacturer's

462   protocol with the exception of addition of glycogen (20µg/µl) and isopropanol

463   precipitation overnight at -80℃ to maximize yield. RNA quality and quantity were

464   analyzed using a NanoDrop and TapeStation. Total RNA was converted to strand-

465   specific Illumina compatible sequencing libraries using the Nugen Universal Plus

466   mRNA-Seq library kit from Tecan (Mannedorf) as per the manufacturer's

467   instructions (MO1442 v2). Briefly, 500ng of total RNA was polyA selected and the

468   mRNA fragmented prior to reverse transcription and second strand cDNA synthesis

469   using dUTP. The resultant cDNA was end repaired before the ligation of Illumina-

470   compatible barcoded sequencing adapters. The cDNA libraries were strand selected

471   and PCR amplified for 12 cycles prior to assessment by Agilent Tapestation for

472   quality and Qubit fluorescence assay for quantity. Sequencing pools were generated

473   by mixing equimolar amounts of compatible sample libraries based on the Qubit

474   measurements. Sequencing of the library pool was performed with an Illumina

475   Nextseq 500 using single read 75bp (v2.0) sequencing chemistry.

476

477   **Bioinformatic analysis**

478   **RNAseq data processing**

479   Fastq files from the sequencing run were firstly subjected to quality controls with

480   FastQC version 0.11.3. Raw reads with low quality were removed using Trim Galore

481   alignment (Phred score less than 28 and/or reads contains adaptor sequences). After

482   trimming, all bases with low quality scores and adaptor sequences were removed.

483   The trimmed reads were mapped to Ensembl mouse genome (GRCm38) with STAR

484   2.4.2a. No more than 1 base mismatch was allowed. Only uniquely mapped reads

485   were retained. Option -quantMode was enabled to generate gene level quantification.

486   The counts files were then merged into an expression table for downstream

487   differential expression analysis.

488

489   **Differential expression analysis**

490   Differentially expressed genes (DEG) between the *Tdtomato*⁺ (*Grem1* positive) and

491   *Tdtomato*⁻ (*Grem1* negative) populations were analyzed with edgeR packages in R

492   version 3.6.0 (Robinson et al., 2010) . Reads were inspected through

493   multidimensional scaling plot and outliers were removed. TMM normalized counts

494   were log2-transformed and counts per million (CPM) obtained. Paired comparisons

495   between the *Tdtomato*⁺ (*Grem1* positive) and *Tdtomato*⁻ (*Grem1* negative)

496   populations were performed. DEG reported as significant were selected by requiring

497   both adjusted P value (FDR) $\leq$ 0.05 and absolute value of log2 fold change $\geq$ 2.0.

498

Supervised Weighted Gene Correlation Network Analysis (WGCNA)

Only DEGs were used for subsequent network analysis. The gene network was constructed using the R package WGCNA following the procedure as described (Langfelder and Horvath, 2008). After the low expression genes (FPKM < 1) had been filtered out from all gene expression libraries, Pearson's correlation based adjacency was calculated on the basis of pairwise correlations of gene expression within *Tdtomato*[+] (*Grem1* positive) samples. Topological overlap of the correlations was used to weight the edges of the correlation network. The higher the weight, the stronger the interaction between two genes. Connectivity for a single gene was calculated as the sum of weights relative to the rest of the genes, and the top 5% of genes with the highest connectivity in the network defined as hub genes. For visualisation, a heat map was generated using the TOMplot() function in WGCNA R package (version 1.68), with dissimilarity topological overlap (1 - topological overlap), employed for hierarchical clustering. To generate the network plot, the weights of the network were cut off at 0.1. Hypergeometric enrichment tests for each module-defined gene list were performed with the enricher() function, as part of clusterProfiler R package version 3.13.0 (Yu et al., 2012). Bonferroni adjustment for p value was used, only gene sets with an adjusted p value less than 0.05 were considered for interpretation of the biological function modules.

Gene set enrichment analysis (GSEA)

To utilize the Molecular signatures database (MSigDB), we accessed all gene sets (.gmt file, version 6.2) from the Broad Institute and chose a subset for further analyses including BioCarta, Hallmark, Gene Ontology (GO), Kyoto Encyclopedia of

523   Genes and Genomes (KEGG), Pathway Interaction Database, and the Reactome

524   Pathway Database. To enable cross species comparisons, mouse gene ensembl IDs

525   were converted to human orthologous gene symbols using biomaRt R package,

526   version 2.41.7 (Durinck et al., 2009). Humanised gene lists for the whole

527   transcriptome were ranked based on logfc values by comparing TdTomato$^+$ and

528   TdTomato- cells. The R package clusterProfiler version 3.13.0 (Yu et al., 2012) was

529   used to scan through all the gene sets mentioned above, using Benjamini & Hochberg

530   adjusted p values.

531

532   **Analysis of publicly available single cell RNA seq dataset**

533   Single cell RNAseq raw expression data was accessed for human mid-gestational

534   brain (22 and 23 weeks post-conception) cortex samples from GSE103723 (Fan et al.,

535   2018). The cells from two individual samples were grouped together, with the sum

536   of reads calculated for each gene to represent the data structure of bulky

537   transcriptomics. The limma package(Ritchie et al., 2015) was used to remove batch

538   effects with removeBatchEffect() and to generate multidimensional scaling plots. To

539   understand the expression pattern of human *GREM1* and its possible contribution

540   to neural differentiation, normalized counts and tSNE coordinates were employed

541   for visualization using plots generated with ggplot2 package in R environment

542   (Wickham, 2009). To investigate cell types expressing BMP antagonists, pearson's

543   Chi-Square test of independence was performed in the R environment with

544   chisq.test() function. Testing variables were gene expression categorised into high

545   and low by median expression, and using cell type identifiers from the original

546   scRNAseq study (Fan et al., 2018). Scaled standardised residuals for each gene were

23

547   used to plot the heatmap with ComplexHeatmap package (Gu et al., 2016).

548

549   ### *In situ* hybridization (ISH)

550   All ISH analyses were performed on formalin-fixed and paraffin-embedded mouse

551   tissue samples using RNAscope technology (RNAscope 2.5 HD Detection Kit,

552   RNAscope Multiplex Fluorescent Reagent Kit v2, Advanced Cell Diagnostics)

553   following the manufacturer's instructions. Briefly, tissue sections were baked in a

554   dry oven (HybEZ II Hybridization System, Advanced Cell Diagnostics) at 60°C for 1

555   h and deparaffinized, followed by incubation with an H_2O_2 solution (Pretreat 1

556   buffer) for 10 min at RT. Slides were boiled in a target retrieval solution (Pretreat 2

557   buffer) for 15 min, followed by incubation with a protease solution (Pretreat 3 buffer)

558   for 30 min at 40°C. Slides were incubated with a mouse *Grem1* probe (NM_011824.4,

559   region 398-1359, catalogue number 314741) for 2 h at 40°C, followed by successive

560   incubations with signal amplification reagents. Staining was visualized with DAB

561   or TSA Plus Cyanine 5 (NEL745001KT, PerkinElmer;1:750). Combined ISH and

562   immunofluorescence (IF)/immunohistochemistry (IHC) was undertaken by first

563   performing ISH, followed by IF/IHC. For IF following ISH, the sections were blocked

564   with blocking buffer (X0909, Dako) and then incubated with a primary antibody

565   (rabbit polyclonal anti-RFP, Rockland, 600-401-379) overnight at 4℃. The sections

566   were washed in 1x T-PBS 3 times and then incubated with Alexa Fluor 555-

567   conjugated secondary antibody (Thermo Fisher Scientific) for 60 min at room

568   temperature. The sections were mounted with ProLong Gold antifade reagent

569   containing 4'6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific), and

570   fluorescence was examined using an inverse immunofluorescence microscope BZ-

24

571 X710 (Keyence, Japan) with optical sectioning. For IHC following ISH, after

572 inactivation of endogenous alkaline phosphatase with alkaline phosphatase blocking

573 solution for 10 minutes (SP-6000, Vector Laboratories), the slides were blocked with

574 blocking buffer (X0909, Dako) and incubated with a primary antibody (rabbit

575 polyclonal anti-RFP, Rockland, 600-401-379) at 4 °C overnight. The sections were

576 washed with PBS three times, followed by incubation with a horse anti-rabbit IgG

577 polymer (MP-5401, Vector Laboratories) for 30 min at room temperature. The slides

578 were washed with TBS three times, and signal developed using an alkaline

579 phosphatase substrate (SK-5105, Vector Laboratories). The reaction was stopped by

580 washing in TBS four times, and slides were counterstained with hematoxylin,

581 dehydrated in EtOH, cleared in xylene, and coverslipped. Cells expressing more than

582 1 ISH signal were regarded as positive for Grem1 RNA.

583

584 **Real time RT-PCR**

585 RNeasy mini kits (Qiagen) were used to isolate RNA from snap frozen mouse brain

586 tissues. RNA was reverse-transcribed into cDNA with cDNA master (Sigma). PCR

587 amplification was performed with Kappa Sybr qPCR mix or Kappa Probe qPCR mix

588 using QuantStudio7 (Applied Biosystems). The mouse GAPDH gene was used as an

589 endogenous control. The following primers were used:

590 TaqMan probes and primers, Gremlin1 (IDT, Mm.PT.58.11631114), Id1

591 (IDT,Mm.PT.58.6622645.g), Id2 (IDT, Mm.PT.58.13116812.g), Id3 (IDT,

592 Mm.PT.58.29482466.g), Id4 (IDT, Mm.PT.58.6851535)

593 Sybr primers, Gapdh-forward AAGGTCATCCCAGAGCTGAA, Gapdh-reverse

594 CTGCTTCACCACCTTCTTGA Ryr3-forward TGCTGTCGCTTCCTTTGCTA, Ryr3-

25

595 reverse   CATCGATGGGGACGCTAGAC,   Lrrtm3-forward

596 TAGCAAATCAGGCTCCAGGG, Lrrtm3-reverse GAGTTCATGATGGACCCCACA

597

598 **Immunohistochemistry**

599 To collect 14.5 dpc, 17.5 dpc and 20.5 dpc embryonic brains, euthanized pregnant

600 dams immediately underwent cardiac perfusion with 4% PFA. Whole embryos at

601 14.5 dpc, or dissected embryonic brains for 17.5 and 20.5 dpc were fixed in 4% PFA

602 at 4℃ overnight. Embryos and brains were then cryoprotected in 30% sucrose and

603 frozen in OCT embedding medium. 16 μm sections were cut using a Leica CM1900

604 cryostat. To make paraffin sections, samples were post fixed in 10% neutral buffered

605 formalin overnight and processed. 5 μm sections were cut using a Leica HM325

606 microtome. Sections were blocked with Protein Block Serum-Free (Dako) for 1 hour

607 at room temperature, incubated overnight with first antibody at 4℃, secondary

608 antibody for 1 hour at room temperature, and coverslipped with Vectashield Antifade

609 Mounting Medium (Vector laboratories). The following antibodies were used: Anti-

610 TBR1(Abcam, ab183032, 1:400), Anti-NeuN (Abcam, ab104225, 1:500), Anti-β Ⅲ

611 tubulin (Sigma, T5076, 1:400), Anti-O4 (R & D systems, MAB1326, 1:500), Anti-

612 GFAP (Dako, Z0334, 1:250), Anti-FOXP2 (Abcam, ab16046, 1:10000), Anti-Ctip2

613 (Abcam, ab18465, 1:800), Anti-CDP (Santa Cruz, sc-13024, 1:400), Anti-BrdU

614 (Abcam, ab6326, 1:600), Anti-RFP (Rockland, 600-401-379, 1:1000), Anti-Sox2

615 (Millipore, AB5603, 1:400), Anti-Pax6 (Millipore, AB2237, 1:100)

616 Images were acquired on a Leica SP5 spectral scanning confocal microscope.

617

26

618 Neural stem cell and progenitor cell culture

619 Before seeding cells, tissue culture dishes were coated with PDL (100μg/ml) and

620 laminin (10μg/ml). Embryonic neural stem and progenitor cells were isolated from

621 pregnant mice at 14.5 dpc. Dorsal telencephalons were dissected from each embryo

622 in PBS, meningeal membranes removed and the tissue triturated to a single cell

623 suspension. Cells were cultured in NeuroCult™ Proliferation Medium containing 20

624 ng/ml epidermal growth factor (EGF) (Stemcell Technologies). For differentiation

625 assays, cells were seeded onto 4 well chamber slides (Thermofisher #NUN177399) in

626 Neurocult Differentiation medium (Stemcell Technologies). To induce recombination

627 or overexpression of *Grem1*, cells collected from Grem1$^{flox/flox}$ embryos were infected

628 with plenti-EF1-*Cre-2a-sfGFP-2a-puro*, plenti-EF1-*Grem1-2a-sfGFP-2a-puro,* or

629 plenti-EF1-*2a-sfGFP-2a-puro* lentivirus, and transduced cells were selected using

630 puromycin for 5 days and used for experiments before passage 5. Lentivirus plasmids,

631 psPAX2 and MD2.G were transfected to 293T cells to generate lentivirus, and viral

632 supernatant was concentrated using Amicon-Ultra 100k spin columns. To assess

633 neurosphere forming ability, cells were seeded in 6-well uncoated plates (1.6×10^5

634 cells per well) and cultured for 5 days. The number of neurospheres sized 50-100 μm

635 and >100 μm was counted. For cell viability assays, neural stem / progenitor cells

636 were seeded in 96-well coated plates (1×10^4 cells per well) and RealTime-Glo™ MT

637 Cell Viability Assay (Promega) was used with the continuous-read protocol at 0, 24,

638 52 and 72 hours. Recombinant human BMP2 (Prospec) was added to the

639 differentiation medium.

640

641 Luciferase assay

27

642   BMP pathway activity was measured using the BMP response element luciferase

643   reporter pGL3 BRE luciferase (addgene #45126), internal control pRL/TK-luciferase

644   reporter and the dual luciferase reporter assay kit (Promega). Neural stem /

645   progenitor cells were seeded in coated 24-well plates (1.6x10^5 cells per well) and

646   transfected with the reporter plasmids using xTremeGENE HP DNA transfection

647   reagent (Roche). Cells were collected 48hr after transfection using Passive lysis

648   buffer (Promega). The pGL3 empty vector was used as a control for BMP-

649   independent changes in reporter activity.

650

651   **Western blot**

652   Cell lysates were solubilized with M-PER Mammalian Protein Extraction Reagent

653   containing complete protease and phosphatase inhibitors. Lysates were separated

654   by SDSPAGE and transferred to PVDF membranes (Millipore). After blocking with

655   5% nonfat skim milk in PBST for 30 min at room temperature, the membranes were

656   incubated overnight at 4℃ with anti-Gremlin1 antibody (R & D systems, AF956,

657   1:1000) or anti-ßActin (Santa Cruz, sc-47778, 1:1000) in 0.5% nonfat skim milk in

658   PBST. Membranes were washed with PBST and incubated with alkaline

659   phosphatase-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG,

660   GE healthcare life sciences, 1:10000) for 1 hr at room temperature. Finally, the blot

661   was visualized with Immobilon HRP substrate (Millipore) using a Chemi Doc XRS1

662   (Bio-Rad).

663

664   **Behavioral tests**

665   Mice were submitted to Rotarod, open field, elevated plus maze, and Y maze tests at

666 the age of 7-10 weeks. Mice while in their home cages were acclimatized to the

667 behavior suite for at least 30 min prior to testing. The data was acquired blindly to

668 the genotype.

669 Rotarod. Animals were placed on the rotarod (Panlab/Harvard Apparatus) that

670 linearly increased rotation speed from 4 to 40 rpm during a 120 second period. An

671 accelerating protocol was employed to eliminate the need for habituation to the

672 rotarod. This procedure was repeated for a total of three trials per mouse, separated

673 by 15min inter-trial intervals and the mean latency to fall from the rotarod in

674 seconds was compared between wild type littermate controls and $Grem1^{cKO}$ mice to

675 assess motor coordination.

676 Open field test. The open field test was conducted in four identical square arenas

677 (50x50x50cm) surrounded by walls. Mice were individually placed in a corner of a

678 clean arena and allowed to explore for 10 min. For the purpose of data collection, the

679 arena was conceptually partitioned into two zones: a virtual center zone of 23x23 cm

680 and a peripheral zone occupying the remaining area. Lower percentages of time

681 spent in the center zone was used to indicate a higher level of anxiety.

682 Elevated plus maze test. The elevated plus maze consisted of a central square (8×8

683 cm) and four arms (29 cm long $\times$ 8 cm wide, two open arms with no railing and two

684 closed arms enclosed by a transverse wall 20 cm in height). A mouse was placed in

685 the center of the central square facing the open arm and allowed to explore the maze

686 apparatus for 10 min. The time spent in any of the open arms was recorded and used

687 as a measure of anxiety.

688 Y maze test. The apparatus consisted of three arms with an angle of 120° between

689 each of the two arms. Each arm was 40 cm long $\times$ 8 cm wide $\times$ 15 cm high. Visual

29

690 cues were placed on the walls of the mazes. The Y-maze test consisted of two trials

691 separated by an inter-trial interval (ITI) to assess spatial recognition memory. The

692 first trial (training) had a 5 min duration and allowed the mouse to explore only two

693 arms of the maze, with the third arm (novel arm) being blocked. After 30min ITI, the

694 second trial was conducted, during which all three arms were accessible for 5 min.

695 Trials were recorded using a ceiling-mounted camera and analyzed by a video

696 analyzer (Ethovision XT, Noldus) to determine the period that each mouse spent in

697 each arm of the maze.

698

699 **Statistics**

700 All statistical analyses were performed using Graphpad prism 8, with methods and

701 values summarised in each figure legend.

702

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709

710 **Competing interests**

711 The authors declare no competing interests.

712

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727

728   Data availability

729   The accession number for all sequencing data reported in this paper is GEO:

730   GSEXXXXXX.

731

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917

918   **Figure legends**

919   **Fig.1 *Grem1*-expressing cells give rise to cortical neurons in the developing mouse**

920   **brain**

921   (A) Schematic showing tamoxifen and BrdU administration to *Grem1creERT;*

922   *Rosa26LSLTdtomato* (*Grem1*-reporter) mice.

923   (B) Representative images of TdTomato+ (red) cells in the telencephalon at 14.5dpc

924   (24h post-induction), 17.5dpc (3d post-induction), and 20.5dpc (6d post-induction)

925   in *Grem1*-reporter mice treated with tamoxifen at 13.5 dpc, DAPI (blue). Scale bar

926   = 200 μm.

927   (C) Representative images of immunohistochemical staining of boxed region from

928   (B) to visualize TdTomato+ cells (red) and *Grem1* mRNA by ISH (green) at 14.5dpc

929   (I, 24h post-induction) and 20.5dpc (III, 6d post-induction) in *Grem1*-reporter mice

930   treated with tamoxifen at 13.5 dpc, DAPI (blue). The boxed areas were further

931   magnified in adjacent panels. Scale bar = 50 μm.

932   (D) Quantification of (C) showing the percentage of TdTomato+ cells that were also

933   *Grem1* RNA+ in 4 HPFs of 2-3 biological replicates, t-test.

934   (E)   Representative   images   of   immunofluorescence   staining   of   (II)   17.5dpc

935   telencephalon from *Grem1*-reporter (red) mice induced with tamoxifen at dpc13.5,

936   BrdU (green), DAPI (blue). Scale bar = 50 μm.

937   (F) Quantification of (E) showing the percentage of TdTomato+ cells that were also

938   BrdU+ 2 HPFs of 3 biological replicates, t-test.

939   (G) Representative images of immunofluorescence staining of 14.5 and 20.5 dpc

940   neocortex from *Grem1*-reporter (red) mice induced with tamoxifen at dpc13.5, NeuN

941   (green), DAPI (blue). Layer V and VI boxed in (i) and (ii), respectively. Scale bar =

37

942   50 µm. MZ, marginal zone ; CP, cortical plate ; SP, subplate

943   (H) Quantification of (G) showing the percentage of TdTomato$^+$ cells that were also

944   NeuN$^+$ in 3 HPF from 3 biological replicates. One way ANOVA with Tukey's multiple

945   test.

946   *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

947

948   **Fig.2 Grem1 expressing cells have neuron associated gene signatures**

949   (A) TdTomato$^+$ and TdTomato$^-$ cells were isolated from 14.5 dpc brains of *Grem1-*

950   *CreERT; R26-TdTomato* mice induced with tamoxifen at 13.5dpc. Representative

951   FACS plot is shown, n=5 mice sorted for bulk RNAseq analysis.

952   (B) Volcano plot to show differentially expressed genes (DEG) between

953   TdTomato$^+$.and TdTomato$^-$ cells from (A). *Grem1* was significantly upregulated,

954   whereas BMP target genes, *Id1,3,4* were significantly downregulated in TdTomato$^+$

955   cells compared to TdTomato$^-$ cells . Absolute value of log2 fold change $\geq$ 2.0,

956   FDR<0.05

957   (C) Network visualization of correlated DEG modules in TdTomato$^+$ cells. Each dot

958   represents a gene, dot perimeter colour indicates module membership (see Fig S3),

959   dot interior colour denotes upregulation (red) or downregulation (blue) of gene

960   transcript in TdTomato$^+$ cells compared to TdTomato$^-$, dot size indicates the

961   magnitude of gene expression correlation to neighboring genes, lines connecting dots

962   represent topological distance. The module containing highly correlated genes that

963   were predominantly upregulated in TdTomato$^+$ cells is boxed in magenta.

964   (D) Significantly enriched gene sets in magenta boxed module in (C). FDR<0.001

965   (E) Genes associated with Id1, a hub gene in the magenta boxed module in (C). Dot

966   perimeter colour indicates module membership (see Fig S3), dot interior colour

967   denotes upregulation (red) or downregulation (blue) of gene transcript in TdTomato[+]

968   cells compared to TdTomato[-].

969

970   **Fig.3 *Grem1* is expressed in the glutamatergic excitatory neuron lineage cells**

971   (A) Heatmap depicting unsupervised clustering of TdTomato[+] and TdTomato^{-} cells

972   isolated from 14.5dpc *Grem1*-reporter (red) mice induced with tamoxifen at dpc13.5

973   based on expression of representative differentiation marker transcripts for neural

974   stem cell (NSC), radial glia, intermediate progenitor, immature neuron, and mature

975   neuron.

976   (B) Gene set enrichment analysis (GSEA) for Glutamate Secretion genes between

977   TdTomato+ and TdTomato- samples from (A). Normalised enrichment score (NES)

978   = 2.16, p = 0.0049.

979   (C) Representative images of immunofluorescence staining of 14.5 and 20.5 dpc

980   telencephalon from *Grem1*-reporter (red) mice induced with tamoxifen at dpc13.5,

981   TBR1 (green), DAPI (blue). Scale bar = 100 μm.

982   (D) Quantification of (C) showing the percentage of TdTomato^{+} cells that were also

983   TBR1^{+} in 3 representative fields from 3 biological replicates.

984   (E) tSNE plot of human scRNAseq dataset. *GREM1* expressing cells outlined in black.

985   Dot size represents Grem1 expression value as indicated.

986

987   **Fig.4 Gremlin1, a BMP antagonist, promotes proliferation and neuronal**

988   **differentiation in neural stem / progenitor cells *ex vivo***

989   (A) Detection of Grem1 protein levels in control *Grem1^{flox/flox}, Grem1^{-/-}* and *Grem1^{O/E}*

990   NSPC by western blot.

991   (B) BRE-reporter activity relative to internal control reporter. Data obtained from 4

992   independent experiments each performed in triplicate.

993   (C) BMP target gene, *Id1*, mRNA expression normalized to *Gapdh* in *Grem1flox/flox*,

994   *Grem1-/-* and *Grem1O/E* NSPC treated with vehicle or rBMP2 for 24h. Columns, mean;

995   bars, SD. One way ANOVA with Tukey's multiple test. Results from 5 independent

996   experiments performed in triplicate.

997   (D) Representative images of *Grem1flox/flox*, *Grem1-/-* and *Grem1O/E* neurosphere

998   cultures. Scale bar = 100 μm.

999   (E) Quantification of neurosphere number and size from (D) in 4 representative fields

1000   from 4 independent experiments. One way ANOVA with Tukey's multiple test.

1001   (F) Cell viability normalized to 0h timepoint for each culture. Results from 3

1002   independent experiments each performed in triplicate and analyzed using linear

1003   regression.

1004   (G) Representative images of immunofluorescence staining to identify Tuj1+ neurons,

1005   GFAP+ astrocytes and O4+ oligodendrocytes in differentiated cultures from wild type

1006   NSPCs treated with vehicle or rGrem1.

1007   (H) Quantification of (G), showing the percentage of marker+ cells in 3 representative

1008   fields from 3 independent experiments. t-test. Scale bar = 100 μm.

1009   *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

1010

1011   **Fig.5 Cortical development, motor balance, and fear is impaired in Grem1**

1012   **conditional knock out mice**

1013   *Grem1* is required for normal cortical development

40

1014   (A)   Representative *Grem1* expression in the dorsal telencephalon of *Grem1^cKO^* mice

1015   and *Grem1^flox/flox^* littermate controls by ISH at 14.5 dpc. Monochrome images were

1016   prepared for quantitation of the *Grem1* ISH signal with ImageJ. n = 3

1017   (B)   *Grem1* mRNA levels normalized to *Gapdh* were determined using real time

1018   qRT-PCR in mouse cortical brain samples collected at postnatal day 0 from *Grem1^cKO^*

1019   mice and littermate controls n=4 biological replicates. Columns, mean; bars, SD. t-

1020   test. ****p<0.0001

1021   (C) Representative histological images of cortical layers of *Grem1^cKO^* mice and

1022   *Grem1^flox/flox^* littermate controls using Nissl at 10 weeks of age. Scale bar = 100 μm.

1023   (D) Quantification of cortical layer thickness from (C) compared in 8 pairs of male

1024   *Grem1^cKO^* mice and littermate controls at 10 weeks of age. Female, n=7 control and

1025   n=7 *Grem1^cKO^* . t-test, *p<0.05.

1026   (E) Representative images of immunofluorescence staining of cortex of *Grem1^cKO^*

1027   mice   and littermate controls at 10 weeks of age to visualise layer Ⅱ‐Ⅳ with

1028   marker CDP (green), layer Ⅴ and Ⅵ with marker Ctip2 (red), and DAPI (blue). n =

1029   3 Scale bar = 100 μm.

1030   (F) Representative images of immunohistochemical staining of cortex of *Grem1^cKO^*

1031   mice   and littermate controls at 10 weeks of age to visualise layer Ⅵ marker Foxp2

1032   IHC (shown in red arrowheads). n = 3 Scale bar = 100 μm.

1033   (G) Behavioral testing was performed to compare *Grem1^cKO^* and *Grem1^flox/flox^* mice

1034   using age and sex matched littermates at 7-10 weeks of age. Columns, mean; bars,

1035   SEM.

1036   (i) Rotarod test latency to fall. Male, n=14 control and n=11 *Grem1^cKO^* , Female, n=12

41

1037   control and n=13 *Grem1^cKO* . t-test, *p<0.05.

1038   （ⅱ）Open field test, cumulative duration spent in the center area. Male, n=13 control

1039   and n=14 *Grem1^cKO* , Female, n=13 control and n=13 *Grem1^cKO* . t-test, *p<0.05.

1040   （ⅲ, ⅳ）Elevated plus maze test.（ⅲ）Cumulative duration spent in open arms.（ⅳ）

1041   The number of entries to open arms. Male, n=12 control and n=11 *Grem1^cKO* , Female,

1042   n=14 control and n=14 *Grem1^cKO* . t-test, *p<0.05.

1043

1044   **Fig.S1 *Grem1* starts to express after 12.5 dpc and is dramatically decreased after**

1045   **birth**

1046   (A) Representative coronal images of immunofluorescence staining of 12.5 dpc

1047   telencephalon from *Grem1*-reporter (red) mice induced with tamoxifen at 11.5 dpc.

1048   Scale bar = 100 μm. LV, lateral ventricle ; NCx, neocortex

1049   (B) *Grem1* mRNA levels normalized to *Gapdh* were determined using real time qRT-

1050   PCR in mouse cortical brain samples collected at p0, p10 and 4 weeks post-birth

1051   (n=4-6 mice/group, PCR conducted in triplicate) Columns, mean; bars, SD. One way

1052   ANOVA with Tukey's multiple test. ****p<0.0001

1053

1054   **Fig.S2 Clustering of differentially expressed genes**

1055   A heat map was generated to visualise the unsupervised hierarchical clustering of

1056   correlation scores for gene expression of DEG in TdTomato+ cells. Yellow colour

1057   indicates closely related and red not related. The color bar between the hierarchical

1058   clustering tree and plotting fields denotes the module membership of each gene.

1059

1060 **Fig.S3 *Grem1*-expressing cells are radial glial stem cell markers, Sox2 and Pax6**

1061 **negative**

1062 Representative images of immunofluorescence staining of 14.5 dpc neocortex from

1063 *Grem1*-reporter (red) mice induced with tamoxifen at dpc13.5, (A) Sox2 (green),

1064 DAPI (blue). (B) Pax6 (green), DAPI (blue). Scale bar = 100 μm.

1065

1066 **Fig.S4 Extended transcriptome analysis of bulk mRNAseq data from *Grem1*-**

1067 **expressing TdTomato⁺ and TdTomato⁻ telencephalon cells at 14.5dpc**

1068 (A) Gene set enrichment analysis (GSEA) for genes involved in the regulation of (i)

1069 GABAergic synaptic transmissions and (ii) dopamine secretion that were

1070 differentially regulated between *Grem1*-expressing TdTomato⁺ and TdTomato⁻

1071 cells. Normalised enrichment score (NES) = -0.93, p = 0.75 and NES = -0.69, p =

1072 0.87.

1073 (B) Unsupervised clustering of *Grem1*-expressing TdTomato⁺ and TdTomato samples

1074 based on expression of representative markers for glutamatergic, GABAergic, and

1075 Dopaminergic neurons, in bulk mRNAseq data. Marker transcript expression is

1076 displayed as a heat map with high expression in red to low expression in blue.

1077 (C) Multidimensional scaling plot comparison of transcriptome of TdTomato⁺ and

1078 TdTomato⁻ cells isolated from 14.5dpc *Grem1*-reporter mice induced with tamoxifen

1079 at dpc13.5 and *GREM1*⁺ and *GREM1*⁻ cells from scRNAseq of human mid-gestational

1080 cortex.

1081 (D) GREM1 expression is associated with the excitatory neuronal lineage in the

1082 developing human cortex. Unsupervised clustering of human developmental brain

1083 cell populations from scRNAseq GSE103723 based on expression of lineage markers

43

1084   and transcripts encoding BMP antagonists (*FST, NOG, CHRD, BMPER, GREM1,*

1085   *SOSTDC1*), and BMP target genes *(ID1-4)*. Cell type is indicated by colour legend as

1086   per tSNE plot shown in Figure 4E. Chi-square testing was used to evaluate the level

1087   of each transcript in each cell population and the resulting standardized residual

1088   values are depicted in the heat map.

1089

1090   **Fig.S5 Grem1 suppresses intrinsic BMP signaling in developing human excitatory**

1091   **neurons, likely via antagonism of paracrine BMP ligands.**

1092   (A) Heat map depicting expression of BMP ligands in human developmental brain

1093   cell populations from scRNAseq GSE103723. Cell types as per tSNE plot shown in

1094   Figure 4E. Chi-square testing was used to evaluate the level of each transcript in

1095   each cell population and the resulting standardized residual values are depicted in

1096   the heat map.

1097   (B) GREM1 expression inversely correlates with enrichment of BMP2,7 and BMP

1098   target genes ID1, 3, 4 expression in human excitatory neurons in mid-gestation.

1099   Expression of transcripts encoding BMP ligands antagonised by GREM1 and ID

1100   target genes in *GREM1+* and *GREM1-* cells is displayed from human scRNAseq

1101   GSE103723. One way ANOVA with Tukey's multiple test. ns $p>0.05$, *$p<0.05$,

1102   **$p<0.01$,****$p<0.0001$

1103

1104   **Fig.S6 Grem1 acts to antagonize BMP signaling in NSPCs resulting in altered**

1105   **downstream transcript levels**

1106   Transcript levels of BMP target genes, *Id2, Id3* and *Id4,* normalized to *Gapdh* in

1107   *Grem1flox/flox, Grem1-/-* and *Grem1O/E* NSPC treated with vehicle or rBMP2 for 24h.

44

1108   Results from 5 independent experiments performed in triplicate. Columns, mean;

1109   bars, SD. One way ANOVA with Tukey's multiple test. *p<0.05, ***p<0.001

1110

1111   **Fig.S7 Extended morphological, immunostaining and transcript analysis of**

1112   ***Grem1^{cKO}*** **mice and littermate controls**

1113   (A) Representative coronal section of TdTomato+ (red) cells in the dorsal

1114   telencephalon at 14.5dpc in *Emx1-cre ; Rosa26LSLTdtomato* mouse brain. This cre-

1115   driver is broadly active across the telencephalon region in which *Grem1* is highly

1116   expressed. Scale bar = 100 μm. NCx, neocortex ; H, hippocampus ; Str, striatum ; T,

1117   thalamus

1118   (B) Quantification of cortical layer thickness compared between age and sex matched

1119   littermates at 10 weeks of age. Female, n=7 control and n=7 *Grem1^{cKO}*,. t-test,

1120   *p<0.05.

1121   (C) Representative histological images of neocortical layer 5 and 6 from *Grem1^{cKO}*

1122   mice and *Grem1^{flox/flox}* littermate controls at 10 weeks of age using Nissl. Scale bar =

1123   20um.

1124   (D) Quantification of (C) showing the cell number per area of layer V and Ⅵ using

1125   Nissl in 2 HPF of 3 biological replicates. Columns, mean; bars, SD. t-test. *p<0.05.

1126   Scale bar = 20um.

1127   (E) Representative histological images of hippocampus from *Grem1^{cKO}* mice and

1128   *Grem1^{flox/flox}* littermate controls at 10 weeks of age using Nissl. 8 pairs of males and

1129   7 pairs of females were analyzed. Scale bar = 200 μm.

1130   (F) Transcripts from the *Id1* associated gene cluster (Fig3E), *Ryr3* and *Lrrtm3* were

1131   evaluated in *Grem1^{cKO}* and *Grem1^{flox/flox}* littermate control cortex at p10 using real

1132   time qRT-PCR. *Ryr3* and *Lrrtm3* levels were normalized to *Gapdh*. n=4-6 biological

1133   replicates. Columns, mean; bars, SD. t-test. *p<0.05, ****p<0.0001

1134

1135   **Fig.S8 Memory is not impaired in *Grem1^{cKO}* mice compared to *Grem1$^{flox/flox}$***

1136   **littermate controls**

1137   (A,B) Y maze test. Behavior was compared between age and sex matched *Grem1^{cKO}*

1138   mice and *Grem1$^{flox/flox}$* littermate controls at 7-10 weeks of age. Male, n=12 control

1139   and n=10 *Grem1^{cKO}*, Female, n=14 control and n=13 *Grem1^{cKO}*. t-test. (A)

1140   Cumulative duration spent in new arm of Y maze. (B) The number of entries to new

1141   arm of the Y maze. Columns, mean; bars, SEM.

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Ichinose_Fig2

Ichinose_Fig4