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Laminin α2 controls mouse and human stem cell behaviour during midbrain dopaminergic neuron development

Maqsood Ahmed*[1], Leandro N Marziali[2], Ernest Arenas[3], M. Laura Feltri[2] and Charles ffrench-Constant[1]

1. MRC Centre of Regenerative Medicine, University of Edinburgh, EH16 4UU, UK

2. Departments of Biochemistry and Neurology, School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, NY, United States

3. Laboratory of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

* Corresponding author: Maqsood Ahmed max.ahmed@ed.ac.uk

Abstract

The development of the central nervous system requires the coordination of proliferation and differentiation of neural stem cells. Here, we show that laminin alpha 2 (lm-α2) is a component of the midbrain dopaminergic (mDA) progenitor niche in the ventral midbrain (VM) and identify a concentration-dependent role for lm211 in regulating mDA progenitor proliferation and survival via distinct set of receptors. At high-concentrations, lm211 rich environments maintain mDA progenitors in a proliferative state via integrins α6β1 and α7β1. Whereas low concentrations of lm211 support mDA lineage survival via dystroglycan receptors. We confirmed our findings in vivo where, in the absence of lm-α2, the VM was smaller, with increased apoptosis, and the progenitor pool depleted through premature differentiation resulting in fewer mDA neurons. In examining mDA neuron subtype composition we found a reduction in later-born mDA neurons of the ventral tegmental area, which control a range of cognitive behaviours. Our results identify a novel role for lm in neural development and provide a possible mechanism for autism-like behaviours and brainstem hypoplasia seen in some patients with mutations of the human lm-α2 gene.

Keywords: Extracellular matrix, neural stem cells, laminin, integrin, dystroglycan, dopaminergic neurons, congenital muscular dystrophy

Introduction

During development, the embryonic central nervous system (CNS) consists of proliferating neural stem cells (NSCs) that are exposed to a balance of intrinsic and extrinsic factors that regulate cell fate decisions (1,2). One such extrinsic determinant is the extracellular matrix (ECM), a multifunctional network of proteins interacting with and regulating a range of cell functions. Transcriptional analysis of mouse and human neocortices display an enrichment of ECM genes such as laminins (lms), proteoglycans and integrins in the germinal zones suggesting they may play a role in regulating NSC behaviour (3,4).

Lms are high-molecular weight, heterotrimeric glycoproteins composed of an α, β and γ chain. Currently 5α, 3β and 3γ chains have been identified combining to form at least 16 different combinations in the mouse, creating considerable tissue heterogeneity. Mutations to the human gene (lama2) encoding the lm-α2 protein results in merosin-deficient muscular dystrophy (MDC1A)(5–7). Whilst this congenital muscular dystrophy (CMD) results primarily in skeletal muscle damage, patients often exhibit a broad spectrum of neuroanatomical defects including white matter abnormalities, cerebellar cysts and brainstem (midbrain, pons and medulla) hypoplasia (8,9). However, the specific functions of lm-α2, particularly in the developing CNS, that lead to these brain abnormalities in MDC1A are unknown.

A further set of clinical symptoms seen in these patients includes neurological deficits in executive functions, intellectual disability and attention deficit hyperactive disorder (ADHD) (8–11). This phenotype highlights a possible involvement of the ventral domain of the midbrain, consisting of dopaminergic (mDA) neurons that can be subdivided into two main nuclei: substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). SNc mDA neurons are generated first, project largely to the striatum and contribute to the control of voluntary movement, with the selective death of these neurons being the pathological hallmark of Parkinson’s disease (PD) (12). In contrast, later-born VTA mDA neurons innervate the hippocampus and the prefrontal cortex (PFC) regulating a range of cognitive functions (13). A dopamine imbalance in the VTA mDA neurons has been implicated in the aetiology of ADHD, obsessive-compulsive disorder (OCD), addiction and schizophrenia (14–16).

In this study, we have explored the role of lm-α2 protein in NSC development, focussing on the hypothesis that mutations in lama2 gene disrupt mDA neurogenesis resulting in a dopamine imbalance that may contribute to some of the neuropsychiatric deficits found amongst CMD patients. We first confirm the expression of lm-α2 in the human embryonic ventral midbrain (VM) during mDA neurogenesis. We then utilised a human embryonic stem (hES) cell model of mDA differentiation to define a functional role for lm-α2 in mDA progenitor proliferation and survival. Finally, we confirmed our findings in vivo using a lm-α2 null transgenic mouse model. Our findings show that lm-α2 regulates NSC behaviour by controlling the survival of mDA progenitors and their timely differentiation into neurons. In the absence of lm-α2, mDA progenitors are prematurely depleted resulting in a reduction in the number of late-born mDA neurons in the VTA.

Results

Lm-α2 is present in the developing human VM surrounding mDA progenitors

84 Using immunohistochemistry (IHC), chain-specific lm expression in the mesencephalon of
85 human embryos at 6-10 post-conception weeks (pcw) revealed the early embryo (6 pcw) to
86 be rich in lm-α2 (fig 1). The VM was immuno-positive for lm-α2 throughout the entire apico-
87 basal axis surrounding a population of rapidly dividing (Ki67+) mDA progenitors. Analysis of
88 a previously published single-cell RNA sequencing (scRNA-seq) study of the human VM
89 between 6-11 pcw suggests that pericytes are a potential contributing cell source and could
90 potentially secrete this lm into the interstitial space (fig 1) (17). A developmental time-course
91 analysis revealed that protein expression diminished over time and by 10 pcw, lm-α2
92 expression was largely absent from the human VM. Similarly, in the mouse VM, lm-α2
93 protein expression rapidly diminished after E10.5 with little or no detectable lm-α2 at E14.5
94 (fig S1). The loss of interstitial lm-α2 expression correlated with the loss of proliferating mDA
95 progenitors, suggesting a potential role for lm-α2 in supporting self-renewal. Examination of
96 the spatiotemporal expression patterns of the remaining four lm-α chains revealed the basal
97 laminae of the blood vessels within the VM to be rich in lm-α4 (fig S1), consistent with the
98 scRNA-seq data showing expression to be greatest in endothelial cells and pericytes.
99 Expression of the lm-α1 was most pronounced in the basement membrane lining the basal
100 surface of the neural tube whereas lm-α5 could be seen on the ventricular surface and
101 interspersed around the cells of the VM. This again is consistent with the scRNA-seq data
102 showing expression of these laminin genes in radial glial cells (Rgl – whose processes span
103 the two surfaces of the developing mesencephalon) and progenitors (fig S1). Lm-α3 protein
104 and mRNA expression were absent in the midbrain floor plate, underlining the specificity of
105 the different laminin expression patterns.
106
107 **Lm211 regulates the balance between proliferation and differentiation in a**
108 **concentration-dependent manner and controls the survival of human mDA**
109 **progenitors**
110 To assess the functional role of lm-α2, a hES cell model of mDA differentiation was
111 established (fig S2) using a previously published protocol capable of yielding mDA neurons
112 positive for a range of markers such as TH, Nurr1, DDC, Pbx1a, Girk2 and Calb1 (fig S2)
113 (18). Day 14 VM progenitors that are positive for FoxA2+, Lmx1a+ and Otx2+ (fig S2),
114 analogous to mDA progenitors in vivo at 6pcw, were incubated with the recombinant version
115 of the lm-α2 containing trimeric protein, lm211, at a concentration ranging from 0.5-12 $\mu g ml^{-1}$
116 for a period of 14 days. The thymidine analogue, EdU, was administered prior to fixation to
117 label proliferating cells and the cultures were stained for tyrosine hydroxylase (TH), which
118 marks mDA neurons. Increasing concentrations of lm211 increased the number of mDA
119 progenitors that were EdU+, with a concomitant reduction in the number of TH+ mDA
120 neurons (fig 2A-C).
121
122 Focussing on two concentrations favouring proliferation (4 $\mu g ml^{-1}$) and differentiation (1
123 $\mu g ml^{-1}$), the differentiation dynamics were interrogated further. Cultures were fixed at day
124 21, 28 and 35 and were examined for proliferation (EdU), differentiation (Nurr1 and TH) and
125 cell death (active caspase 3, aC3). No differences were detected at day 21 between either
126 conditions; however, the lm211-rich environment (4$\mu g ml^{-1}$) sustained cells in a proliferative
127 state (fig 2D) and consequently delayed differentiation resulting in fewer Nurr1+ post-mitotic
128 neuroblasts (fig 2E) and TH+ neurons (fig 2F) being detected at day 28 and 35 compared to
129 lm211-poor condition (1$\mu g ml^{-1}$). Whilst the number of apoptotic cells increased over the
130 duration of the culture, no significant difference was detected between the two lm211
131 concentrations (fig 2G).

132 Having shown a role for lm211 in promoting VM progenitor proliferation, we next determined
133 whether this property was specific to the lm-α2 isoform by treating the cultures with three
134 other lm isoforms – lm111, lm411 and lm511 – at the previously identified concentrations (1
135 and 4 $\mu g ml^{-1}$). Rates of proliferation of mDA progenitors were the same on all lm isoforms,
136 with no significant difference detected in EdU labelling at either concentration (fig S3A, B,
137 D). Similarly, no effect on the number of TH+ cells was detected at high concentrations of
138 lm-α isoforms (fig S3E). Surprisingly, however, a significant increase in the number of TH+
139 neurons was detected on lm211 at low concentration, compared to the other three isoforms
140 (fig S3C). We therefore next determined whether this increase in TH+ neurons on low
141 concentrations of lm211 was due to enhanced differentiation or survival by examining aC3
142 immunoreactivity (a marker of apoptosis) on the four different lm isoforms. Significantly
143 fewer aC3+ cells were seen on lm211 (fig S3F) suggesting the increase in the number of
144 mDA neurons is due to improved cell survival. Taken together, these results suggest lm211
145 has distinct concentration-dependent effects, with a lm-rich environment supporting
146 proliferation and expansion of the progenitor pool irrespective of isoform, whilst low
147 concentrations of lm-α2 containing isoform promote survival of mDA lineage cells.
148
149 **Interaction of Lm211 and human mDA progenitors is mediated by distinct receptor**
150 **engagement**
151 To identify the cell surface receptors responsible for the different effects of lm-α2 on mDA
152 progenitors, we used a functional blocking antibody approach. As the lm-rich environment
153 maintains cells in a proliferative state irrespective of isoform, we speculated that the integrin
154 family of receptors were involved. Three principle lm-binding integrins have been identified:
155 α3β1, α6β1 and α7β1. All were expressed in the hVM; scRNA-seq analysis showed that the
156 radial glia (hRgl) and midline progenitors (hProgM) express significant levels of α6 integrin
157 and detectable levels of α7 and β1 integrins (fig S4). We further confirmed the presence of
158 these three integrin subunits via immunostaining hES cell derived mDA progenitors (fig S4).
159 Blocking the β1 integrin subunit resulted in a complete loss of adhesion and cell detachment
160 (data not shown) whilst blocking the individual α-subunits had a negligible effect on mDA
161 progenitor proliferation (fig 3B). As the integrin α-subunits may functionally compensate for
162 each other, we next blocked pairwise combinations of α-subunits. Blocking α3 integrin had
163 no discernible effect, but the combined blockade of both integrin α6 and α7, in the presence
164 of 4$\mu g ml^{-1}$ lm211, resulted in the loss of the lm211-driven increase in EdU+ mDA progenitors
165 and an increase in the number of TH+ mDA neurons (fig 3B and 3C). We further confirmed
166 the role of integrin α6 and α7 in mediating the proliferative response by blocking both
167 receptors in the presence of lm111, lm411 and lm511 at 4$\mu g ml^{-1}$ and successfully reversing
168 the increase in proliferating mDA progenitors (fig S5).
169
170 As the previously seen increased cell survival of hES derived mDA lineage cells (fig S3C
171 and G) was specific to low concentrations of lm-α2, we speculated that dystroglycan – a
172 high-affinity lm-α2 receptor enriched in the floor plate, point of origin of mDA neurons, may
173 be mediating the effect (19–21). We confirmed the expression of dystroglycan in the hVM
174 via immunostaining and also in the scRNA-seq dataset, in which it is expressed at significant
175 levels in hRgl (fig S4). Blocking dystroglycan with a targeted antibody (IIH6C4) in vitro
176 reversed the laminin-isoform specific increase in TH+ neurons seen on low-concentrations of
177 lm211 to levels comparable to those seen on lm111 (fig 3D-F). When we administered the
178 antibody without any exogenous lm211, we did not observe any significant differences in
179 TH+ cells confirming the lm-specific nature of the interaction (fig S5).

180

181 **A loss of progenitors and premature differentiation results in reduced number of**
182 **neurons in the ventral midbrain of Lm-α2 null mice**

183 Together, results using human cells and tissue suggest the hypothesis that the fall in lm-α2
184 expression levels in the human VM between 6 and 10pcw controls VM NSC cell cycle exit
185 and mDA neuron differentiation, with the lower concentrations of lm-α2 then supporting cell
186 survival. To confirm that lm-a2 is such an instructive component of the niche in vivo, we
187 examined the VM of lm-α2 null mouse embryos during mDA neurogenesis at embryonic (E)
188 day 10.5-14.5, corresponding to 6-10pcw in humans. We first confirmed that the lm-α2 null
189 embryos do not express lm-α2 (fig S6).
190

191 Our hypothesis predicts that in the absence of lm-α2 there would be fewer mDA progenitors
192 and more neurons due to the loss of the proliferative effect of this laminin, resulting in
193 premature differentiation. This was confirmed; the *lama2*$^{-/-}$ embryos were smaller than the
194 WT littermate controls and the ventral midbrain (VM) domain, demarcated by FoxA2
195 expression, consisted of significantly fewer cells (fig S7A-D). Premature differentiation at
196 E10.5 could be detected in the lama2$^{-/-}$ embryos as evidenced by increased number of
197 Nurr1+ and TH+ cells at this age, with a concomitant reduction in proliferating VM
198 progenitors (double Ki67+ and FoxA2+ cells) (fig 4A-D). Nascent mDA neurons were seen
199 not only in the marginal zone along the basal surface of the VM but also in ectopic positions
200 at the ventricular surface of the lama2$^{-/-}$ VM (fig S8) – a region where the high concentration
201 of lm-α2 would normally prevent differentiation.
202

203 To exclude the possibility that the observed reduction in size of the VM resulted from
204 patterning defects rather than changes in proliferation, we examined the position of cells
205 expressing FoxA2, Lmx1a or Corin and calculated the area of their domains, normalised to
206 the area of the ventricle. No significant difference in position or domain sizes was seen at
207 E10.5 or E12.5 (fig S7), suggesting patterning is unaffected in the mutant embryos. We also
208 examined the expression patterns of Nkx6-1 and *Wnt1* in wild-type and mutant embryos at
209 E12.5 and found no differences (fig S7H,I). We did however note a slight delay in the lateral
210 expansion and medial inhibition of Shh expression, with low levels of Shh in the mutant
211 basal plate compared with the controls (fig S7I). This finding is in agreement with both the
212 known function of Shh to control the expansion of the VM (22,23) and with our findings of
213 decreased number of FoxA2^{+} cells (figS7K), impaired growth and reduced proliferation in the
214 VM.
215

216 Our original hypothesis also predicts increased levels of apoptosis due to the loss of the
217 survival-promoting effect of low concentrations of lm-α2. Again, this was confirmed; by
218 E12.5 numerous active caspase 3+ (aC3) cells were found along the border of the
219 ventricular zone and intermediate zone that separates progenitors from postmitotic
220 neuroblasts, suggesting that apoptosis takes place during cell-cycle exit (fig 4E).
221 Quantification revealed a near two-fold increase in aC3+ cells and fewer FoxA2+ progenitors
222 (double Foxa2+ and KI67+ cells) in the VM (fig 4F, J).
223

224 Ectopic TH+ neurons were once again detected at the ventricular surface of the mutant VM
225 at E12.5 (fig S8). However, in contrast to the situation at E10.5, by E12.5 the intermediate
226 and marginal zone of the *lama2*$^{-/-}$ embryos contained fewer Nurr1+ and TH+ cells (fig 4H

227 and I). To determine the cause of this reduction in neuron number, we next examined the
228 expression of the proneural gene Ngn2 in Lmx1a+ mDA progenitors (24). Whilst these
229 experiments confirmed the reduction of midbrain floor plate mDA progenitors, identified as
230 Lmx1a+, we found no significant difference in the subset of progenitors undergoing
231 neurogenesis, identified as Ngn2+, in the lama2$^{-/-}$ embryos (fig 4L). Consequently, we
232 conclude that the proportion of progenitors undergoing neurogenesis is greater in the mutant
233 embryos, leading to accelerated differentiation and depletion of the progenitor pool. To test
234 this directly, we injected pregnant mice subcutaneously with EdU at E11.5 and dissected the
235 embryos 24 hours later at E12.5. We then quantified the number of dividing cells (EdU+)
236 that had exited cell cycle and were positive for the post-mitotic marker Nurr1. Confirming
237 our previous results, we found a significant increase in neurogenesis with a 26% increase in
238 the number of cells positive for both EdU and Nurr1 in the mutant embryos compared with
239 wild-type littermate controls. Meanwhile, there was a significant decrease in the number of
240 Lmx1a+ EdU+ cells that are Nurr1- (i.e. mDA progenitors that are still in cell cycle).
241 Consistent with this, by E14.5, there were significantly fewer TH+ neurons in the mutant
242 embryos than in the WT littermate controls (fig S9).
243
244 To determine whether these effects are restricted to the mDA lineage, we next examined
245 non-mDA neurons in the VM. Brn3a is a marker of neurons within the red nucleus,
246 immediately lateral to the mDA neuron domain. The number of Brn3a+ neurons in *lama2*$^{-/-}$
247 mutants were significantly reduced compared to wild-type littermate controls at E14.5 (fig
248 S10), indicating that basal plate neurogenesis is also impaired.
249
250 **Lm-α2 null mice have fewer Calbindin+ mDA VTA neurons**
251 Birth-dating experiments have demonstrated that mDA neurons are born sequentially with
252 more lateral mDA neurons that project to the striatum born first and the medial mDA neurons
253 projecting to the PFC, born later in development (25,26). To examine how the altered
254 differentiation dynamics in the mutant embryos affects the distribution and subtype
255 composition of mDA neurons in the postnatal brain we examined the expression of Girk2
256 and Calbindin (Calb1), markers expressed predominantly in the lateral SNc mDA neurons
257 and the medial VTA mDA neurons respectively at postnatal (P) day 15. Both subpopulations
258 of TH+ mDA neurons can be identified in the VM of both WT and mutant brains, with the
259 Calbindin+/TH+ cells clustered around the ventral midline whereas the Girk2+/TH+ cells are
260 distributed more laterally (fig 5). A small sub-section of mDA neurons expressing both Girk2
261 and Calbindin were found largely restricted to the dorsolateral VTA in the transition zone
262 between the SNc and VTA. The lama2$^{-/-}$ brains were found to be smaller at P15 (fig S11)
263 and to have fewer TH+ mDA neurons in the midbrain compared to WT littermate controls (fig
264 5A). The loss of neurons was particularly apparent in the mediocaudal VM (fig S12),
265 suggesting that the later-born medial mDA neurons were preferentially depleted in the
266 mutant mice. Quantifying Girk2 and Calbindin expression confirmed this, revealing a
267 reduction in the number of TH+ Calbindin+ (later-born) mDA neurons with no significant
268 decrease in the number of Girk2+ TH+ (early-born) mDA neurons (fig 5B-C). As expected,
269 quantification of the ratio of TH+ mDA neurons that are either Girk2 or Calbindin+ in the
270 mutant brains revealed proportionately more Girk2+ and fewer Calbindin+ mDA neurons (fig
271 5D). These results suggest that premature depletion of the mDA progenitor pool, due to a
272 smaller progenitor domain combined with accelerated differentiation and increased
273 apoptosis, leads to a loss of late-born Calbindin positive mDA neurons creating an

274 imbalance between these two populations of dopaminergic neurons in the VM of *lama2*$^{-/-}$
275 brains.
276
277 ## Discussion
278 In this study, we describe the expression and distribution of laminin isoforms in the mouse
279 and human VM and then identify a dual functional role for one of these, lm-α2, in controlling
280 cell cycle exit and survival in the mDA neuron lineage. We propose a model whereby the
281 lm-α2 rich environment, found early in development, sustains VM progenitor proliferation via
282 an integrin-dependent pathway. As lm-α2 expression diminishes, cells are able to exit from
283 the cell cycle and differentiate with low levels of lm-α2 then promoting the survival of
284 progenitors through dystroglycan receptors. In mutant lama2$^{-/-}$ mice, the loss of lm-α2
285 protein results in a smaller mDA progenitor pool that is subsequently depleted by a
286 combination of increased cell death and premature differentiation, leading to a defect in late
287 generated mDA neurons of the VTA subtype.
288
289 Previous studies examining lm expression in the brain describe the murine neocortex as
290 being rich in lm-α2 and lm-α4, with expression greatest at the ventricular surface (3,27).
291 Moreover, the subventricular zone, one of two adult stem cell niches in the CNS, was found
292 to be a lm-rich environment compared to neighbouring non-neurogenic regions suggesting
293 lms may play a role in regulating NSC self-renewal (28). Consistent with this, we find the
294 presence of high levels of interstitial lm-α2 in the early human embryo during a period of
295 rapid growth, after which levels decline. Three other laminin isoforms, lm-α1, lm-α4 and lm-
296 α5 were largely restricted to the basal lamina. Together, our immunostaining and analysis of
297 a previously-published scRNA-seq dataset reveals lm expression to be highly specific and
298 dynamically regulated over the course of development.
299
300 Several independent studies have implicated the lm-integrin interaction in controlling NSC
301 proliferation in both the embryonic cortex and in the adult stem cell niches (29–32).
302 Additionally, in pathological conditions characterised by rapid proliferation such as
303 glioblastoma multiforme (GBM), the cellular niche has been described to be rich in lm-α2
304 whilst the glioblastoma stem-like cells that drive tumour growth are enriched with integrin α6
305 and α7 (33–35). Disruption of the lm-integrin interactions in GBM significantly impaired
306 tumour growth (33–35). Given our results showing the expression of lm-α2 in mDA
307 progenitors maintains cells in a proliferative state, it would be interesting to test the
308 hypothesis that a common mechanism underpins the lm-integrin driven NSC proliferation in
309 embryonic, adult and pathological conditions. Identifying downstream mechanisms as well
310 as positive and negative regulators of lm expression could be important not only to control
311 proliferation in cancer, but also in a regenerative context, offering the potential to counteract
312 the age-dependent decline in NSC self-renewal.
313
314 Over the course of development, laminin expression diminishes and is restricted to the
315 ventricular zone, limiting the cells it can interact with to those rapidly dividing progenitors
316 juxtaposed to the surface of the ventricle. The interaction with lm-α2 in this region prevents
317 exit from the cell cycle and differentiation. As predicted from this hypothesis, in the complete
318 absence of lm-α2 precocious mDA neurons can be seen at both the basal surface and
319 ectopically at the ventricular surface. An increase in the number of apoptotic cells in the
320 interface between the ventricular and intermediate zone can also been seen, reflecting the
321 requirement for lower concentrations of lm-α2 to maintain the survival of the newly-

322 postmitotic cells in this region. A previous study identified that lm-α5 driven YAP activation
323 promotes the survival of mDA neurons in the marginal zone (36), at a later differentiation
324 stage compared to the one described in this study. Interestingly, lm-α2 has also been
325 reported to activate YAP and further upregulate the expression of lm receptors such as
326 integrins and dystroglycan in Schwann cells (37), suggesting a possible interaction between
327 these two lm-activated pathways and a general role of lms in controlling cell survival at
328 different developmental stages along the same lineage. Indeed, it is perfectly feasible that
329 the lm511 present in the VM may be compensating for the loss of lm211, masking the extent
330 of the phenotype.
331
332 We have previously shown that the principal lm receptor, integrin β1, is capable of regulating
333 both NSC self-renewal and differentiation in the chick midbrain via distinct mechanisms (38).
334 Meanwhile, the relative abundance of the lm protein has been shown to control the balance
335 between quiescence and activation in epidermal stem cells (39). Our present results extend
336 these prior studies by showing a concentration-dependent effect of lm-α2 controls the
337 proliferation and survival of human progenitors, at least in part, by sequential receptor
338 engagement with integrins and dystroglycan. It is interesting to note that a similar interplay
339 between the two receptors has been identified in lm-mediated pancreatic β-cell development
340 (40). Moreover, αβ integrins and dystroglycan have been shown to act sequentially during
341 Schwann cell development in the peripheral nervous system (PNS), activating different
342 signalling pathways during axonal sorting (41). In agreement with these previously
343 published studies, the data presented here suggests that the two lm receptors are
344 responsible for distinct steps during mDA development. How lm-211 is able to regulate
345 these specific processes in a concentration-dependent manner remains an open question.
346 One possible explanation may lie in receptor affinities. Previously published solid-phase
347 binding assays of lm211 with dystroglycan and a number of α-integrins demonstrated that
348 the dystroglycan interaction is an order of magnitude greater than with integrins (42,43), and
349 so is likely to be favoured at the lower lm concentrations later in the developmental process.
350
351 Mutations affecting lm-α2, dystroglycan and more rarely integrin α7 result in CMD, a
352 clinically and genetically heterogeneous group of disorders affecting primarily muscle and
353 brain. Neurological deficits that includes structural malformations and cognitive impairment
354 are most abundant in patients carrying mutations affecting dystroglycan (44,45). However, a
355 number of patients carrying a lama2 mutation also report neurological abnormalities
356 including brainstem hypoplasia (8,9). In the lm-α2 null embryos described in this study, we
357 found a VM significantly reduced in size likely due to the impairment of proliferation,
358 premature differentiation and increased cell death resulting in a depletion of the progenitor
359 pool; a phenotype reminiscent of integrin β1 mutant mice in which cerebellar granule cell
360 precursors cease proliferating and differentiate prematurely (46). Interestingly, impaired Shh
361 signalling contributed to the premature differentiation in integrin β1 mutant mice following
362 disruption of the integrin-laminin-Shh complex (46). Moreover, Shh-proteoglycan interactions
363 were previously shown to be necessary for regulating proliferation but dispensable for tissue
364 patterning (47). As we find a defect in proliferation but with correct patterning and a subtle
365 delay in *Shh* expression in lm-α2 null embryos, a contribution of Shh signalling to the
366 proliferation phenotype cannot be excluded.
367

368 A well-established consequence of premature differentiation and depletion of the progenitor
369 pool is a loss of later-born neurons (48–50). In accordance with these studies, we found a
370 disruption of mDA neuron subtype composition, with a reduction in the later-born, Calbindin
371 positive mDA neurons of the VTA. The mDA neurons of the VTA project to the caudal
372 brainstem, hippocampus and PFC and play an important role in a number of processes
373 including cognition, motivation and attention related behaviour. Indeed a subset of these
374 late-born mDA neurons project almost exclusively to the GABAergic interneurons of the PFC
375 and regulate perseveration-like behaviour (51). This loss of late-born neurons may
376 contribute to the cognitive defects described in the few published reports of behavioural and
377 psychiatric problems in MDC1A patients. Of potentially more importance, however, is the
378 possibility that abnormalities in the laminin-dystroglycan-dystrophin pathway caused by
379 dystrophin mutations in Duchenne muscular dystrophy (DMD) patients may, by altering mDA
380 neuron production, contribute to the high prevalence for ADHD and autism spectrum
381 disorder (ASD) described in a recent study on 130 Duchenne muscular dystrophy (DMD)
382 patients (10). In keeping with this, a separate study reported a reduction in $GABA_A$ receptor
383 clustering in the PFC – a target of the late-born mDA neurons – of 14 DMD patients (52).
384 Given the roles we have ascribed to lm-α2, a more thorough behavioural and psychiatric
385 analysis of MDC1A patients would be of great value and provide some support to the notion
386 that a dopamine imbalance underpins the behavioural deficits observed in muscular
387 dystrophies.
388
389 **Methods**
390 **Human and mouse tissue processing**
391 OCT Tissue-Tek embedded human foetal midbrain tissue (5-10 pcw) was obtained from the
392 MRC-Wellcome Trust Human Developmental Biology Resource (HDBR). Sequential
393 coronal sections covering the length of the mesencephalon were collected on superfrost
394 glass slides (ThermoFisher) using a cryostat (ThermoFisher).
395
396 All mouse experiments were conducted following the procedures approved by Roswell Park
397 Institute Animal Care and Use Committee (UB1188M, UB1194M, and UB1196R). The
398 protocols follow the guidelines of the "Guide For The Use of Laboratory Animals," National
399 Research Council, National Academy Press, Washington D.C., 1996. All animals used in
400 this work were congenic into the C57/BL6N background and genotyping of *Lama2* mutant
401 mice was done by PCR of tail genomic DNA. Briefly, PCRs were done 45s at 95°C, 45s at
402 50°C, and 60s at 72°C for 30 cycles. The primers used were as follows: 5'-
403 CCCGTGATATTGCTGAAG-3', 5'-CCTCTCCATTTTCTAAAG-3' and 5'-
404 CAGGTGTTCCAGATTGCC-3'. The lama2 mutant used in this study was the dy3k mutant
405 comprising a complete knockout of the laminin alpha 2 protein (53).
406
407 To obtain embryos, matings were set late in the evening and plugs checked the next
408 morning before 9 AM with those that were positive designated as E0.5. At E10.5, E12.5 and
409 E14.5, pregnant mice were sacrificed. E10.5 whole mount embryos and the dissected heads
410 of E12.5 and E14.5 embryos were fixed in 4% PFA overnight at 4°C.
411
412 From the EdU labelling studies, pregnant mice were subcutaneously injected with 50mg/kg
413 of 5-Ethynyl-2′-deoxyuridine (EdU, Sigma 900584) at E11.5. Embryos were dissected 24
414 hours later at E12.5.
415

416 Embryos were washed in PBS (3×5mins) and incubated in 15% sucrose (Sigma) in PBS at
417 4°C overnight. The embryos were then placed in embedding solution that consisted of 15%
418 sucrose and 7% gelatin (Sigma) in PBS at 37°C for a period of 2 hours. Finally, the embryos
419 were placed in moulds and orientated for coronal sections and snap frozen in liquid N_2 and
420 stored at -80°C. 10μm serial sections spanning the length of the midbrain were taken from
421 each sample. Two embryos from 2-3 different litters were analysed for each time-point
422 (N=4-6). Individual figure captions make clear the N used for each experiment.
423
424 In the case of P15 brains, animals were anesthetized with 20 mg/ml Avertine (2,2,2-
425 tribromoethanol, Sigma, T48402) and sequentially perfused through the left ventricle with ice
426 cold 1X PBS and 4% PFA. Next, the brains were dissected and post fixed in 4% PFA
427 overnight at 4˚C and transferred to 30% sucrose for 48 hours at 4°C and snap frozen in
428 liquid N_2. 20μm sections were collected on glass slides. Two brains from two different litters
429 were examined for both mutant and control animals (N=4).
430
431 **Cell cultures**
432 Undifferentiated RC17 ES cells (passages 23-58, Roslin Cells, hPSC reg #RCe021-A) were
433 maintained in E8 media (A1517001) on Geltrex (1%, 12760021) coated plates and
434 passaged weekly with EDTA (0.5 mM). To start differentiation (day 0), hESC colonies were
435 detached using EDTA (0.5 mM) and placed in non-treated 60mm culture dishes in
436 differentiation media consisting of DMEM:F12/Neurobasal (1:1), N2 supplement (1:100), B27
437 supplement (1:50), SB431542 (10uM, Tocris Biosciences), rhnoggin (100 ng/ml, R&D),
438 SHH-C24II (200 ng/ml, R&D) and CHIR99021 (0.9 μM, Tocris Biochem). Media was
439 changed once on day 2. The resultant embryoid bodies were collected on day 4 and placed
440 on polyornithine (PO), fibronectin (Fn) and laminin (lm) coated plates in reduced N2 (1:200)
441 and B27 (1:100) condition. Growth and patterning factors were removed on day 9 with the
442 cultures kept in DMEM:F12/Neurobasal (1:1), N2 supplement (1:200), B27 supplement
443 (1:100). On day 11, the cell clusters were dissociated to single cells with accutase and
444 replated onto dry PO/Fn coated plates for differentiation in Neurobasal, B27 (1:50), BDNF
445 (20 ng/ml), GDNF (10 ng/ml), AA (200 μM), db-cAMP (0.5 mM, Sigma). All culture reagents
446 are from Invitrogen unless otherwise stated here or previously.

447 Experiments were conducted from day 14, where cultures were treated with soluble lm
448 isoform (BioLamina) or vehicle, with each media change, for stated period of time. Media
449 was changed every third day until fixation in 3.7% PFA for 30 minutes. Where stated, an
450 EdU pulse was administered for 24 hours prior to fixation to identify dividing cells.

451 Receptor blocking antibodies or isotype controls were added to the culture media at 5 μg/ml
452 (10 μg/ml for dystroglycan) and refreshed every 3 days with each media change. Antibodies
453 used were: integrin α3 IA3 (R&D MAB1345-SP), integrin α6 GoH3 (R&D MAB13501),
454 integrin α7 6A11 (LifeSpan Biosciences, LS-C179572), Dystroglycan IIH6C4 (Millipore, 05-
455 593), mouse-IgM (ThermoFisher, 02-6800), rat-IgG2a (ThermoFisher, 02-9688), mouse-
456 IgG1 (ThermoFisher, 02-6100).
457
458 **Immunofluorescence, microscopy & image quantification**
459 List of antibodies used, suppliers and dilutions are provided in table S1.
460

461 Human and mouse sections were boiled in antigen unmasking solution (Vector Labs) and
462 pre-incubated at room temperature for 1 hour in blocking solution containing 10% normal
463 donkey serum (Millipore), 1% bovine serum albumin (Sigma), 0.2% Triton X-100 (Sigma).
464 Sections were incubated with primary antibodies diluted in blocking solution at 4°C overnight
465 followed by washing with blocking solution (3×15mins). Sections were then incubated with
466 fluorophore conjugated secondary antibodies (Invitrogen) diluted 1:1000 in blocking solution
467 for 1 hour at room temperature, followed by washing (3×15mins), hoechst nuclear counter
468 stain (10mins at room temperature) and mounted (Fluoromount, SouthernBiotech).
469
470 The Operetta high-content microscope (PerkinElmer) was used for automated image-
471 acquisition of cell cultures that were then quantified using automated quantification pipelines
472 developed using Columbus Image Analysis Software (PerkinElmer).
473
474 Meanwhile, tissue sections (both embryonic and P15) were imaged on an SP8 confocal
475 microscope (Leica) and quantified manually using Fiji CellCounter plug-in. For quantification
476 of embryonic tissue, adjacent sections were used for labelling (FoxA2, Ki67, Nurr1 and TH)
477 and 5 sections spanning the anterior-posterior axis were counted per stain and sample. For
478 P15 brain, TH+ mDA neurons were quantified in sections at three rostrocaudal levels
479 (Bregma: -2.92mm, -3.40mm and -3.88mm). TH+, TH+ Girk2+ and TH+ Calbindin+ cells
480 were counted bilaterally for each level. Tissue sections were quantified whilst blinded to
481 genotype.
482

483 **RNAscope**

484 RNAscope mRNA detection was performed according to the manual, using the
485 RNAScope 2.5 HD reagent Kit-RED (ACD, 322350). PFA fixed sections (10μm) were
486 dehydrated in an ethanol gradient (50%, 70% and 100%) and incubated with H_2O_2 for 10
487 min. Sections were washed in deionized water and then boiled for 5 minutes in antigen
488 retrieval solution then transferred to a dish containing distilled water. After washing,
489 slides were rinsed in fresh 100% ethanol and air dried. RNAscope Protease plus was
490 applied to the slides and incubated for 30 mins at 40°C. Slides were then washed in
491 distilled water with slight agitation. The appropriate RNAscope probe (Shh: cat no
492 314361, Wnt1: cat no 401091) was then applied to the slide and incubated for 2 hours at
493 40°C. Sections were then washed in wash buffer and the colour reaction was performed
494 according to the user manual. Sections were counterstained for 1 min with haematoxylin
495 (Scientific Laboratories, GHS132-1L), and the blue reaction was performed using 0.02%
496 ammonia water. Sections were dried at 60 °C and mounted.
497

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657

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668

669 **Author contributions**

670 Conceived and designed experiments: MA, EA and CffC. Performed experiments: MA.
671 Analysed data: MA. Contributed reagents/materials: LM and MLF. Wrote the paper: MA,
672 EA and CffC.
673

674 **Fig 1: Lm-α2 expression in the human ventral midbrain at 6 and 10 pcw.**
675 Lm-α2 expression (antibody: C13065) is enriched in the human VM at 6pcw, when the hVM is
676 composed of Ki67+ FoxA2+ progenitors. Expression diminishes by 10pcw when the hVM contains
677 few Ki67+ cells and post-mitotic Nurr1+ mDA neurons are established. scRNA-seq of the hVM
678 suggests pericytes (hPeric) are the principle source of Lm-a2 (Right axis shows absolute molecule
679 counts). Scale bar 50 μm.
680
681 **Fig 2: Lm211 regulates proliferation and differentiation in a concentration dependent manner.**
682 (A) Immunostaining of day 28 hES derived mDA cultures treated with lm211 over a concentration
683 range of 0.5-12 μgml^{-1}. Quantification of EdU (B) and TH (C) staining showing a significant increase in
684 proliferating (EdU+) cells at lm211 concentrations of over 4μgml^{-1} which is associated with a reduction
685 in TH+ mDA neurons (One-way ANOVA with Tukeys post-test, $p<0.0001$, n=3). (D-G) Time series
686 examining proliferation (D) differentiation (E, F) and survival (G) at days 21, 28 and 35 of culture at
687 two different lm211 concentrations. mDA progenitors remain EdU+ at day 28 and 35 in the lm211-rich
688 (4μgml^{-1}) environment, resulting in a significant reduction in the number of post-mitotic mDA
689 neuroblasts (Nurr1) and neurons (TH). No significant differences in aC3 staining between the two
690 lm211 concentrations (unpaired, two-tailed t-test, * $p<0.0001$, n=3). (H) Representative images of day
691 35 cultures following lm211 treatment. Scale bar 50 μm in all images.
692
693 **Fig 3: Concentration-dependent effects of lm211 are mediated by distinct receptor**
694 **engagement**
695 (A) Immunostaining of day 28 cultures treated with lm211 (4 μgml^{-1}) in combination with integrin
696 blocking antibodies and a control condition of lm211 (1 μgml^{-1}). Quantification of proliferation (B) and
697 differentiation (C) following blocking of integrin a-subunit demonstrating that blocking both a6 and a7
698 integrins reverses the effect of the lm211-rich environment. (D) Images of day 28 cultures treated
699 with lm isoforms (1 μgml^{-1}) and dystrolgycan blocking antibody or isotype control. Quantification of
700 proliferation (E) and differentiation (F) demonstrating blockade of dystroglycan reverses the gain in
701 mDA neurons seen on lm211 compared to lm111 without effecting proliferation. *$p<0.0001$, unpaired
702 two-tailed t-test, n=3. Scale bar 50 μm in all images.
703
704 **Fig 4: Lm-α2 null VM is reduced in size with fewer cells and exhibits premature differentiation**
705 **and depletion of progenitor pool**
706 Representative images and quantification of wild-type and mutant VMs at (A-D) E10.5 and (E-M)
707 E12.5. At E10.5, mutant embryos display a smaller ventral domain indicated by FoxA2 staining.
708 Fewer FoxA2+ cells are Ki67+ in the $lama2^{-/-}$ embryo (B) with increased numbers of post-mitotic mDA
709 neuroblasts (Nurr1+) and mDA neurons (TH+) quantified in (C) and (D) respectively. By E12.5, there
710 remains fewer proliferating FoxA2+ cells (F) and Lmx1a+ cells (G) in the VM of mutant embryos. The
711 intermediate and marginal zones of the mutant VM contain fewer Nurr1+ (H) and TH+ (I) cells whilst
712 there is an increase in apoptotic (aC3+) cells at the ventricular zone and intermediate zone border in
713 the absence of lm-α2. Whilst there is a reduction in the number of Lmx1a+ mDA progenitors (L), the
714 number of Lmx1a+ Ngn2+ cells remains the same resulting in a greater proportion of progenitors
715 undergoing neurogenesis (12.51 ± 0.89% (WT) vs 20.73 ± 3.49 % ($lama2^{-/-}$)) in the mutant embryos.
716 EdU labelling confirmed this with a significant increase in the number of EdU+ Nurr1+ cells in the
717 mutant embryos (512 ± 41 (WT) vs 663 ± 88 % ($lama2^{-/-}$)) whereas there was a significant decrease in
718 the number of Lmx1a+ EdU+ cells that are Nurr1- (201 ± 35 (WT) vs 116 ± 26 ($lama2^{-/-}$)). N=4-6, two
719 tailed unpaired t-test, *$p<0.01$, scale bar 50μm.

720 **Fig 5: The midbrain of lm-α2 null brains contains fewer late-born Calbindin+ mDA neurons of**
721 **the VTA**
722 Postnatal brains (P15) of wild-type and mutant mice showing fewer TH+ mDA neurons (A) in the
723 $lama2^{-/-}$ brains. In quantifying mDA subtype, there is a modest reduction in the number of Girk2+ mDA
724 neurons (B) but a dramatic loss in Calbindin+ mDA neurons (C) located medially in the VM. In
725 normalising for the number of mDA neurons (D), there is a significant increase (p=0.0033) in the
726 proportion of Girk2+ TH+ mDA neurons in the mutant mice concomitant with a reduction (p=0.0105) in
727 Calbindin+ TH+ mDA neurons. N=3 (WT), 4(KO), unpaired two-tailed t-test. Scale bar 100μm unless
728 stated.

729 **Fig S1: Lm-α chain immunohistochemistry in human VM**
730 (A) Lm-α1 expression is restricted to the basement membrane surrounding the basal surface neural
731 tube. Lm-a3 is not expressed in the human VM. Lm-a4 is restricted to the basal laminae of blood

vessels at both 6 and 10 pcw. Meanwhile lm-a5 is expressed on both the ventricular and basal surfaces of the VM at 6 pcw as well as some interstitial expression. (B) scRNA-seq data of individual lm-a chains showing cell types for gene expression in human development. Right axis shows absolute molecule counts. (C) Lm-α2 expression in the mouse VM at E10.5-E14.5 displays a similar expression pattern as that seen in the human embryo. Expression can be seen to diminish over time with negligible positive expression at E14.5.

Fig S2: Differentiation protocol and patterning of hES cells into mDA progenitors
(A) schematic of hES differentiation protocol with lm treatment at day 14 till fixation. (B) Immunostainings of day 11 cultures showing cultures to be triple positive for the mDA progenitor markers FoxA2, Lmx1a and Otx2. Cultures are negative for the pluripotency marker Oct4, forebrain marker Pax6 and the lateral domain marker Nkx6.1. (C) TH+ Neurons at day 35 showing positive immunoreactivity for a panel of markers (Nurr1, Pbx1a, DDC, Calb1, Girk2) illustrative of bona fide mDA neurons. Scale bar 50µm.

Fig S3: Lm isoform specificity in regulating mDA progenitor proliferation and survival
(A) Representative images of mDA cultures at day 28 exposed to lm211 at 1 and 4 μgml^{-1}, staining for proliferation (EdU) and neurons (TH). At low concentrations (1 μgml^{-1}), no significant difference in proliferation (B) is detected between any of the lm isoforms whilst an increase in TH+ mDA neurons (C) is observed on lm211. At high concentrations (4 μgml^{-1}), no differences are detected in the number of mDA progenitors that are EdU+ (D) or TH+ (E). *$p<0.001$, ANOVA Tukeys post test, N=3

Fig S4: Expression of lm receptors
Integrins α6, α7, β1 and Dystroglycan are expressed in the mouse VM and on hES derived mDA progenitors at day 14 of culture. scRNA-seq of the hVM identifies radial glial (hRgl1-3) and VM progenitors (hProg) positive for lm receptor expression. Right axis shows absolute molecule counts. Scale bar 50µm.

Fig S5: Specificity of laminin-receptor interactions
(A) Integrin α6 and α7 blocked with antibodies in the presence of 4 μgml^{-1} of lm111, lm411 and lm511. When cultures are exposed to the integrin blocking antibodies, the lm-driven increase in proliferation is abrogated suggesting that the integrin receptors are mediating the proliferative effects of lm. (B) Blocking the α-dystroglycan receptor with no exogenous lm211 does not effect the number of TH+ neurons generated. N=3, two-tailed unpaired t-test, *$p<0.001$.

Fig S6: Lm-α2 expression in the wild-type and lama2$^{-/-}$ mouse VM
No Lm-α2 expression can be detected in the lama2$^{-/-}$ embryos confirming the knock-out.

Fig S7: Lama2$^{-/-}$ exhibit defects in growth but normal patterning
(A) Cross-sections of wild-type and lama2$^{-/-}$ mesencephalon at E10.5 (scale bar 100 µm) with the mutant mesencephalon significantly smaller in area (B). The dopaminergic domain consisting of FoxA2, Lmx1a and Corin (scale bar 50 µm). Fewer FoxA2 cells are present in the lama2$^{-/-}$ midbrain (D). The area of each domain was calculated and normalised to the area of the ventricle. No significant differences were detected in the size of the normalised FoxA2 (E), Lmx1a (F) and Corin (G). Nkx6-1+ cells can be seen laterally and are induced in both the mutant and wild-type embryo at E10.5 (scale bar 50 µm) (H). Potential ectopic expression and a delay in the lateral expansion and medial inhibition of Shh expression in the mutant embryos at E12.5 can be seen. Wnt1 expression is comparable to wild-type and previously published reports (scale bar 100 µm) (I). Dopaminergic domain remains smaller at E12.5 (scale bar 50 µm) (J) in the mutant embryo compared to wild-type, consisting of fewer FoxA2 cells (K) but when normalised for ventricle size, there are no significant differences in FoxA2 (L), Lmx1a (M) or Corin (N) domain size. N=4-6, two-tailed unpaired t-test.

Fig S8: Ectopic mDA neurons in the VM of lama2$^{-/-}$ embryos
TH+ mDA neurons can be seen lining the ventricular surface of lm-α2 null embryos at E10.5 (orange arrows). Ectopic mDA neurons (orange arrowhead) at the ventricular surface (dashed line) continue to be observed at E12.5. Scale bar 50 µm.

Fig S9: Reduced mDA neurons in the VM at E14.5

Significantly fewer TH+ mDA neurons in the VM of mutant embryos compared to wild-type littermate controls. N=6, two-tailed unpaired t-test, scale bar 50 μm.

Fig S10: Development of non-mDA VM neurons compromised in lama2$^{-/-}$ mutants.
Brn3a+ red nucleus neurons, derived from FoxA2+ progenitors, are significantly reduced in the mutant embryos compared to wild-type littermate controls at E14.5. N=6, two-tailed unpaired t-test, scale bar 50μm.

Fig S11: Smaller mutant brains compared to wild-types at P15
Lama2$^{-/-}$ brains are significantly smaller than WT littermate controls, quantified via mass. N=4, two-tailed unpaired t-test.

Fig S12: VM of wild-type and mutant P15 brains showing reduced number of neurons
Large panels are images of the whole VM from wild-type and lama2$^{-/-}$ P15 brains along the rostral-caudal axis showing reduced TH+ immunoreactivity (white) in the mutant brains (scale bar 100 μm). Images to the side are expanded view of the red boxes displaying TH (white) and Calb1 (red) (scale bar 50 μm). Fewer TH+ Calb1+ double positive cells can be seen in the mutant brains, particularly in the more caudal sections.