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RESEARCH REPORT

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Genetic background modulates the effect of glucocorticoids on proliferation, differentiation and myelin formation of oligodendrocyte lineage cells

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Abstract

Anxiety disorders are prevalent mental disorders. Their predisposition involves a combination of genetic and environmental risk factors, such as psychosocial stress. Myelin plasticity was recently associated with chronic stress in several mouse models. Furthermore, we found that changes in both myelin thickness and node of Ranvier morphology after chronic social defeat stress are influenced by the genetic background of the mouse strain. To understand cellular and molecular effects of stress-associated myelin plasticity, we established an oligodendrocyte (OL) model consisting of OL primary cell cultures isolated from the C57BL/6NCrl (B6; innately non-anxious and mostly stress-resilient strain) and DBA/2NCrl (D2; innately anxious and mostly stress-susceptible strain) mice. Characterization of naïve cells revealed that D2 cultures contained more pre-myelinating and mature OLs compared with B6 cultures. However, B6 cultures contained more proliferating oligodendrocyte progenitor cells (OPCs) than D2 cultures. Acute exposure to corticosterone, the major stress hormone in mice, reduced OPC proliferation and increased OL maturation and myelin production in D2 cultures compared with vehicle treatment, whereas only OL maturation was reduced in B6 cultures. In contrast, prolonged exposure to the synthetic glucocorticoid dexamethasone reduced OPC proliferation in both D2 and B6 cultures, but only D2 cultures displayed a reduction in OPC differentiation and myelin production. Taken together, our results reveal that genetic factors influence OL sensitivity to glucocorticoids, and this effect is dependent on the cellular maturation stage. Our model provides a novel framework for the identification of cellular and molecular mechanisms underlying stress-associated myelin plasticity.

Abbreviations: B6, C57BL/6NCrl inbred mouse strain; BNST, bed nucleus of stria terminalis; CORT, corticosterone; CSDS, chronic social defeat stress; DEX, dexamethasone; DIV, days in vitro; MGC, mixed glial cell culture; mPFC, medial prefrontal cortex; OL, oligodendrocyte; OPC, oligodendrocyte progenitor cell; vHP, ventral hippocampus.

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KEYWORDS
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1 | INTRODUCTION

Anxiety disorders encompass panic disorder, generalized anxiety disorder, social anxiety disorder, agoraphobia and specific phobias. Both genetic and environmental factors, such as psychosocial stress, contribute to the onset of anxiety disorders. Among the European population, these mental disorders hold the highest prevalence rate, affecting approximately 14% of individuals (Wittchen et al., 2011). They greatly affect the quality of life and result in significant healthcare costs (Bandelow & Michaelis, 2015). Critically, the recent COVID-19 pandemic, forcing physical and social distancing, increased rates of social isolation and loneliness and doubled the number of children and adolescents suffering from anxiety symptoms (Racine et al., 2021). Current treatments include psychotherapy and symptom-targeted anxiolytics, but they show limited efficacy with a high risk of relapse (Bandelow et al., 2017). There is a pressing need to develop more effective treatments for anxiety disorders by gaining a deeper understanding of the cellular and molecular mechanisms involved.

To identify biological pathways associated with chronic stress-induced anxiety-like behaviour, we previously performed gene expression profiling in stress and anxiety-related brain regions including the medial prefrontal cortex (mPFC), ventral hippocampus (vHP) and bed nucleus of the stria terminalis (BNST) of the C57BL/6NCrl (B6) and the DBA/2NCrl (D2) mouse strains after chronic social defeat stress (CSDS, [Laine et al., 2018]). We identified a significant over-representation of myelination-related genes among the differentially expressed genes, accompanied by differences in myelin thickness and node of Ranvier morphology. Critically, these changes differed between the B6 and D2 mice, suggesting genetic control of stress-induced myelin plasticity (Koskinen et al., 2023; Laine et al., 2018). Mice from the D2 strain have high levels of innate anxiety-like behaviour (Hovatta et al., 2005) and 95% are susceptible to CSDS (Laine et al., 2018). Conversely, B6 mice are innately non-anxious (Hovatta et al., 2005), and only 31% are susceptible to CSDS (Laine et al., 2018). *Glyoxalase 1* gene has been identified as a critical mediator of innate anxiety levels in these strains, with D2 mice having higher expression levels of this gene due to a copy number variant (Hovatta et al., 2005; Williams et al., 2009). However, little is known about the molecular mechanisms underlying differences in their stress-susceptibility.

Psychological stress can result in remodelling of myelin sheaths (Lehmann et al., 2017; J. Liu et al., 2012; Makinodan et al., 2012). Chronic stress also influences oligodendrogenesis and induces widespread gene expression changes in oligodendrocytes (OLs) and oligodendrocyte progenitor cells (OPCs) (Liu et al., 2018; Teissier et al., 2020). OPCs remain active in the adult central nervous system, with neuronal activity regulating their migration and further differentiation into myelin-producing mature OLs (Gibson et al., 2014). OLs express glucocorticoid receptors that can translocate to the nucleus in the presence of their ligands (Matsusue et al., 2014; Vielkind et al., 1990). Most in vitro work suggests that glucocorticoids promote the survival of OPCs and push them from proliferation to differentiation, inducing expression of myelin-related genes and proteins (Barres et al., 1994; Clarner et al., 2011). However, it is not known whether, and how, the genetic background affects these processes.

Here, we established an in vitro OL model of primary cell cultures consisting of OPCs isolated from B6 and D2 inbred mouse strains (further referred to as B6 and D2 cultures). This model allows studies of myelin-producing cells in response to specific exposures in a controlled environment taking into account genetic background effects. We showed that under naïve conditions, the genetic background of OPCs influences the extent of their proliferation and differentiation into mature OLs. To assess whether the response of OLs to glucocorticoids is influenced by the genetic background, we exposed B6 and D2 cultures acutely to corticosterone (CORT), the major stress hormone in mice or prolongedly to the synthetic glucocorticoid dexamethasone (DEX) and let them differentiate in culture. We found that the effects of glucocorticoids on OPC proliferation, differentiation, maturation and the size of myelin sheet area were influenced by the genetic background of the cells and by the cellular maturation stage at glucocorticoid exposure.

2 | MATERIAL AND METHODS

2.1 | Animals

B6 and D2 breeding pairs were purchased from Charles River Laboratories and bred locally. Mice were provided with water and food ad libitum and housed in groups of 4–5 mice in a temperature (22 ± 2°C) and humidity (50

± 15%) controlled facility on a 12/12 h light/dark cycle (lights on 6 AM to 6 PM). All animal care conformed to the European Communities Council Directive 86/609/EEC, and the experiment was approved by the Laboratory Animal Center of the University of Helsinki. Mice occupied the same rooms and racks throughout breeding. To avoid genetic drift, mice housed in our facility were backcrossed with mice re-ordered from the supplier every 3 generations.

2.2 | Cell culture

Cultures were derived from neonatal B6 and D2 cortices as previously described (O'Meara et al., 2011). First, mixed glial cell cultures (MGCs) were obtained from cortices dissected from P0-P2 mice of undetermined sex. The MGCs were grown for 9 days, after which OPCs were isolated by high-speed mechanical dissociation overnight. OPCs were seeded on coverslips coated with poly-L-Lysin (1 mg/mL, Sigma-Aldrich, P2636) and left to differentiate. Over the following 6 days, OPCs progressively matured into mature OLs that produced myelin membranes. For acute CORT exposure, freshly seeded OPCs were exposed to 20-μM CORT (Supelco, 46148), dissolved with .78% DMSO in MGC medium. After 2 h of exposure, the medium was replaced by fresh differentiation or proliferation medium. For prolonged DEX (Sigma-Aldrich, D2915) exposure, OPCs were exposed to 50-μM DEX and let to differentiate. The medium was replenished at 50% after 3 days of culture. To investigate the effect of prolonged glucocorticoid treatment on fully mature OLs, we exposed mature OLs cultured for 6 days to 50-μM DEX for 2 or 4 days. The chosen CORT and DEX concentrations were based on previous literature (Miguel-Hidalgo et al., 2019). Our study deliberately employed high glucocorticoid concentrations to saturate the glucocorticoid receptors. This strategic choice was made to enhance our ability to detect subtle effects. MGC medium was composed of 10% fetal bovine serum (FBS; Gibco, 10091148), 1% GlutaMAX (Thermo Fisher Scientific, 35050038), 33 μg/mL penicillin and 33 μg/mL streptomycin in DMEM (52100-021, Gibco). Differentiation medium comprised of 5 μg/mL insulin (Sigma-Aldrich, I6634), 2% B27 (Gibco, 0080085SA), .1% CNTF (PeproTech, 450–50), 1% GlutaMAX, .15% holotransferine (Sigma-Aldrich, T0665), .5% FBS, 33 μg/mL penicillin and 33 μg/mL streptomycin, 100X SATO (OL supplement; see O'Meara et al., 2011) in DMEM. Proliferation medium was composed of 10% FBS, 10-nM biotin, 30-nM sodium selenite (Sigma-Aldrich, S5261) 50-μg insulin, 20 ng/mL bFGF (PeproTech, 450-33), 20 ng/mL PDGF-AA (PeproTech, 315-17), 1% GlutaMAX, .15% holotransferine, 1% bovine serum albumin (BSA; Sigma-Aldrich, A4503), 33 μg/mL penicillin and 33 μg/mL streptomycin in DMEM. All cell culture experiments were executed in a standardized manner for both strains. This standardization encompassed every step from the dissection process to the final data analysis. It involved the use of identical dissection equipment, cell culture reagents, incubators and analytical techniques. These stringent protocols were implemented to minimize any potential experimental biases and to ensure that the observed differences can be attributed to genetic differences between the two strains.

2.3 | Immunocytochemistry

OPC-enriched cultures were fixed in 3% PFA for 15 min at 1, 3 and 6 days after the initial timing of glucocorticoid exposure. For the prolonged DEX exposure on mature OLs, cells were cultured for 6 days before DEX exposure and fixed 2 or 4 days later. After fixation, coverslips were washed for 3 × 5 min in PBS followed by incubation in PBS + .5% Triton X-100 (PBST) for 10 min. After a 10-min wash in PBS, coverslips were blocked with 2% normal goat serum + PBST (NGS; 005-000-121, Jackson ImmunoResearch). Coverslips were then incubated 24 h with mouse anti-MAG (Millipore, MAB1567, 1:100 dilution), rat anti-MBP (Serotec, MCA409S, 1:100 dilution), rabbit anti-OLIG2 (Millipore, AB9610, 1:500 dilution), rabbit anti-cleaved caspase 3 (Cell Signaling, 9661S, 1:1000 dilution) or rat anti-Ki67 (ThermoFisher Scientific, #14-5698-80, 1:1000 dilution) in blocking solution at 4°C overnight. Cells were washed for 3 × 5 min with PBS and incubated for 2 h with secondary antibodies (Goat anti-Rabbit Alexa 568 IgG, A-11011, Invitrogen; Goat anti-Mouse Alexa 488 IgG, A-11029, Invitrogen; Goat anti-Rat IgG Alexa 647, A-21247, Invitrogen, all 1:200 dilution). After 3 × 5 min wash with PBS, coverslips were mounted on glass using VECTASHIELD Antifade Mounting Medium with DAPI (VECTOR Laboratories). For phalloidin staining, cells were instead incubated with 1% BSA for 20 min after Triton X-100 incubation and added with 1 drop/500 μL of ActinRed 555 ReadyProbes Reagent (Thermo Scientific) for 40 min. Coverslips were washed for 3 × 5 min in PBS before mounting and sealed with nail polish.

2.4 | Image acquisition and analysis

Images were acquired with a Zeiss fluorescent microscope (Axio Imager.Z2 upright epifluorescence wide-field microscope coupled with a Zeiss AxioCam 105 colour camera) running with the Zeiss Zen 2 software. About

10–30 images were acquired per coverslip with a 20× objective (Plan Apochromat) to count cells or a 63× oil objective (Plan Apochromat) for morphological analysis of phalloidin-stained OLs. Image analysis was conducted with a semi-automated macro in ImageJ (Version 1.54c). All analysed cells were systematically controlled as DAPI positive before quantification. We calculated the number of cells positive for the different markers per field of view. Because of their overlap with other cell nuclei, MAG^+ and MBP^+ cells were counted manually. $Ki67^+/OLIG2^+$ double-positive cells were identified by measuring the positivity of the $Ki67^+$ channel within $OLIG2^+/DAPI^+$ positive cells. The Sholl Analysis plugin (SNT-4.1.2.) was used to perform Sholl analysis with 10-µm incrementing circles drawn from the cell soma.

2.5 | Statistical analysis

We analyzed the data using generalized linear mixed-effects model (GLMM) in R (v4.2.1) with the lme4 package (v1.1-33). This approach was chosen due to the hierarchical nature of the data (i.e., data deriving from multiple independent cell culture batches and microscope images). GLMM considers dependencies within independent images and across cell batch replicates (consisting of cells from litters of 3–8 pups), making it suitable for capturing complex experimental structures. The outcome was analysed in terms of the percentage of cells per image that expressed the given marker. We investigated whether this percentage differed either between strains (naïve conditions) or between treatments (drug-exposure experiments). The final number of images and cells analysed for each culture experiment is indicated in the corresponding figure legend. Each experiment consisted of 2–3 independent cell batch replicates. The inbred mouse strain or the treatment was used as a fixed effect and the cell batch replicate as a random effect to control for within-cell batch replicate dependencies.

Data obtained from the Sholl analysis were analysed using Student *T* tests at every circle radius followed by Bonferroni correction.

3 | RESULTS

3.1 | Genetic background influences OPC proliferation, differentiation and OL maturation programmes

To determine how the genetic background affects the basic properties of OLs and their response to glucocorticoid exposure, without the various confounders of the in vivo psychological stress experience, we isolated primary OLs from B6 and D2 inbred mouse strains. We obtained OL-enriched primary cultures (O'Meara et al., 2011) where OL lineage cells represented 56.4 ± 12.6% of all cells (data not shown). We determined whether the genetic background of the cells affects their intrinsic ability to proliferate, differentiate into pre-myelinating and mature into myelin-producing OLs, which are key processes in adaptive myelination (Xin & Chan, 2020).

First, we let OPCs from B6 and D2 mice proliferate in the proliferation medium without intervention for up to 6 days (naïve cells, Figure 1a). We quantified the number of OPCs expressing the proliferation marker Ki67 ($Ki67^+/OLIG2^+$ double-positive cells). After 1 day of culture, we observed 32.1% less proliferating OPCs in cultures obtained from D2 compared with B6 mice ($p = 0.003$, Figure 1b,d). However, when the same evaluation was conducted after 3 or 6 days, no such difference was observed. This indicates that the genetic background of OPCs may influence their initial response to proliferation stimuli but does not affect their overall capacity for proliferation over time.

Next, we cultured B6 and D2 cells in the differentiation medium. Under these conditions, oligodendroglia began to form myelin sheets and express myelin marker proteins at day in vitro (DIV) 3 and were fully mature at DIV6 (Figure 1c,e–g). We counted the number of pre-myelinating OLs expressing myelin-associated glycoprotein ($MAG^+/OLIG2^+$ double-positive cells). D2 cultures had 95.3% and 24.6% more pre-myelinating OLs after 3 and 6 days of culture, respectively ($p = 0.002$ and $p = 0.02$, Figure 1e), compared with B6 cultures. The morphology of OLs may also serve as an indicator of their maturation stage, with increasingly complex structures signifying more advanced stages of maturation (Pfeiffer et al., 1993). To study a possible effect of the genetic background in modulating OL morphology, we used Sholl analysis (Sholl, 1953) (Figure S1). We compared the complexity of the cell arborization from phalloidin-stained OPCs obtained from B6 and D2 cultures. Concurring with our results obtained with maturation markers, we found that OLs from B6 cultures had a simpler arborization than OLs from D2 cultures after 3 days of culture, as measured by a 32.5% lower ramification index ($p = 0.009$, Figure S1b,e).

We also quantified the number of mature OLs forming myelin sheets and expressing myelin basic protein ($MBP^+/OLIG2^+$ double-positive cells) in B6 and D2 cultures. Also here, D2 cultures had 114% more mature OLs after 3 days and 34.1% more OLs after 6 days when compared to B6 cultures ($p = 1.58e\text{-}06$ and $p = 0.041$, respectively, Figure 1f). These results were not due to a higher rate of apoptosis in the B6 cultures,

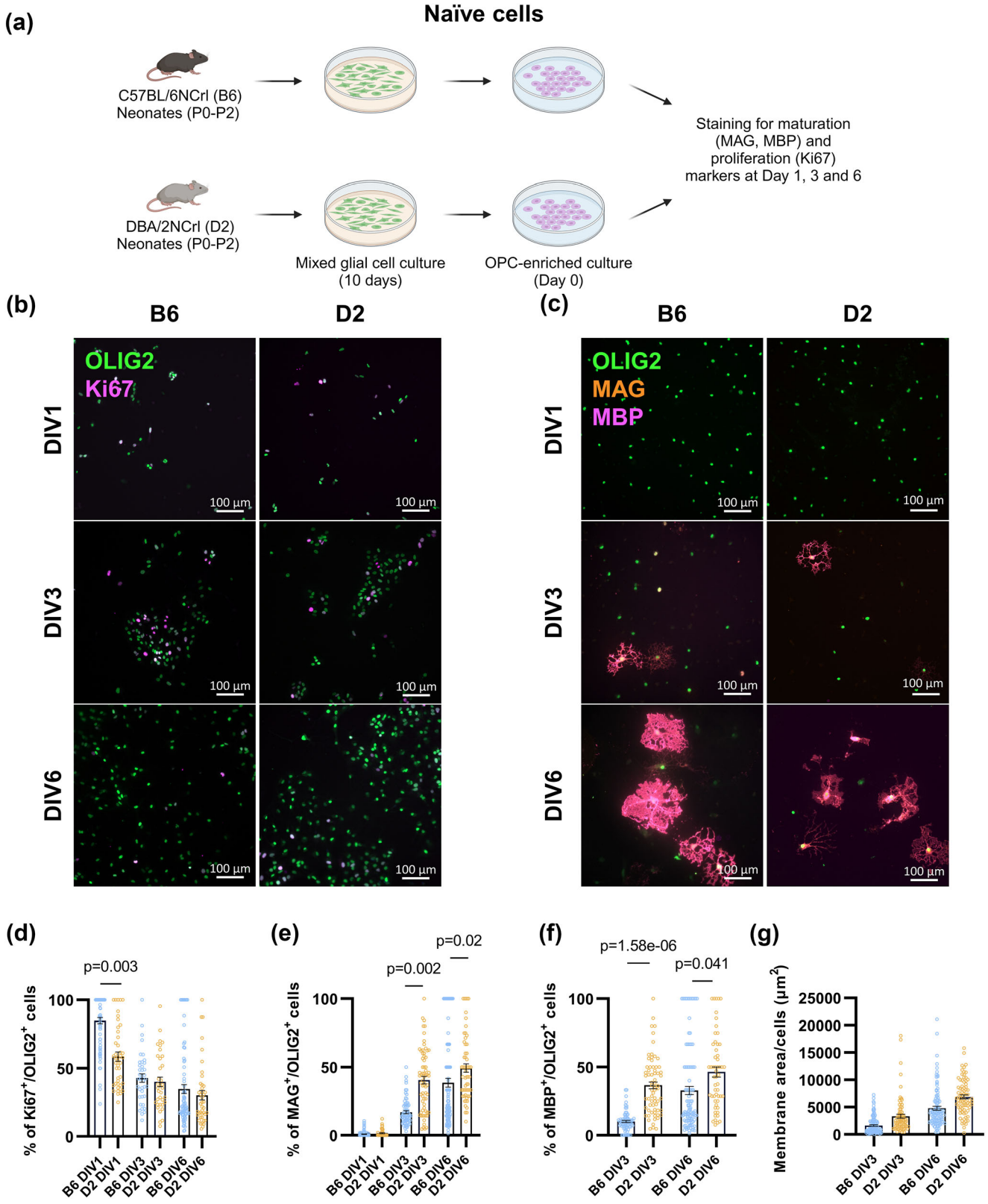

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FIGURE 1 Oligodendrocyte progenitor cell (OPC) proliferation, differentiation and oligodendrocyte (OL) maturation programmes are influenced by the genetic background. (a) Cultures obtained from D2 and B6 mice were let to proliferate or differentiate without intervention (naïve condition). (b) Representative fluorescence images of OLs isolated from B6 and D2 mice ($OLIG2^+$ cells) engaged in proliferation ($Ki67^+/OLIG2^+$ cells). (c) Representative fluorescence images of OLs obtained from B6 and D2 mice and stained for maturation markers (OLIG2 for the whole OL lineage, myelin-associated glycoprotein [MAG] for mid and myelin basic protein [MBP] for late-stage differentiation) after 1, 3 or 6 days of culture. (d) Proportion of proliferating OLs ($Ki67^+/OLIG2^+$ cells) in B6 (DIV1: $N = 3$ cell batches, 104 images, 1360 cells; DIV3: $N = 2$ cell batches, 59 images, 3280 cells; DIV6: $N = 3$ cell batches, 99 images, 8200 cells) and D2 cultures (DIV1: $N = 2$ cell batches, 44 images, 680 cells; DIV3: $N = 2$ cell batches, 38 images, 775 cells; DIV6: $N = 2$ cell batches, 42 images, 2717 cells). (e) Proportion of pre-myelinating OLs ($MAG^+/OLIG2^+$ cells) in B6 (DIV1: $N = 3$ cell batches, 123 images, 3848 cells; DIV3: $N = 3$ cell batches, 88 images, 2492 cells; DIV6: $N = 3$ cell batches, 102 images, 2240 cells) and D2 (DIV1: $N = 2$ cell batches, 44 images, 1417 cells; DIV3: $N = 3$ cell batches, 88 images, 1365 cells; DIV6: $N = 3$ cell batches, 87 images, 724 cells) cultures. (f) Proportion of mature OLs ($MBP^+/OLIG2^+$ cells) in B6 (DIV3: $N = 2$ cell batches, 58 images, 2058 cells; DIV6: $N = 2$ cell batches, 89 images, 2102 cells) and D2 (DIV3: $N = 2$ cell batches, 40 images, 396 cells; DIV6: $N = 2$ cell batches, 60 images, 443 cells) cultures. (g) Area of myelin membrane formed by mature OLs in B6 (DIV3: $N = 3$ cell batches, 99 images, 675 cells; DIV6: $N = 3$ cell batches, 102 images, 401 cells) and D2 cultures (DIV3: $N = 3$ cell batches, 105 images, 491 cells; DIV6: $N = 3$ cell batches, 93 images, 305 cells). Bar graphs show mean ± SEM. Each dot represents an average of all cells in one microscope image.

because the proportion of cells expressing the cell death marker cleaved caspase 3 ($CC3^+$ cells) did not differ between B6 and D2 cultures at any observed time point (Figure S2a,b). After 3 days of culture, OLs from both B6 and D2 cultures started to express myelin proteins and form a membranous myelin sheet reminiscent of myelin. To investigate a possible difference in the size of the area of myelin sheets formed by the cells, we measured the MBP^+ area of OLs (Figure 1c,g). It did not differ between B6 and D2 cells after 3 or 6 days of culture (Figure 1g). Overall, these results suggest that the genetic background influences the proliferation, differentiation, maturation programmes and morphology of OLs but not the size of myelin sheet area they form.

3.2 | Acute corticosterone exposure of OPCs

Acute stress, in the form of fear conditioning, increases OPC proliferation (Pan et al., 2020). To examine the effect of acute CORT exposure on freshly isolated OPCs, we treated them with 20-μM CORT for 2 h after which the culture medium was fully replenished (Figure 2a). We first quantified the number of proliferating cells after 1, 3 and 6 days. We observed 25% more proliferating OPCs in D2 cultures compared with vehicle treatment ($p = 1.31e\text{-}07$) 24 h after the initial timing of the acute CORT exposure but not at later time points (Figure 2b,c). Acute CORT treatment had the opposite effect in B6 cultures, in which OPC proliferation was inhibited by 3% ($p = 0.0024$) after 1 day of culture and by 24.6% after 3 days of culture compared with vehicle treatment ($p < 2e\text{-}16$). None of these effects were observed at DIV6.

We next investigated the effect of acute glucocorticoid treatment on OL differentiation (Figure 2d,e). In B6 cultures, we found that acute CORT exposure resulted in an 18.3% ($p = 1.2e\text{-}07$) reduction in the number of $MAG^+/OLIG2^+$ double-positive cells 3 days post-exposure and in a 26.7% reduction ($p = 0.021$) 6 days post-exposure, compared with the vehicle treatment group. In contrast, acute CORT exposure led to a 9.8% ($p = 0.003$) higher number of pre-myelinating OLs at 3 days post-exposure compared with vehicle treatment, but there were no differences at the 1- or 6-day time points. In contrast to the cultures in naïve conditions, Sholl analysis did not concur with these observations, as acute CORT exposure did not affect OL morphology in B6 or D2 cultures at any time point compared with vehicle treatment (Figure S1c,f). However, the vehicle-treated cells did not show comparable complexity as in the naïve conditions, where OLs from D2 had a more complex arborization compared to OLs from B6 after 3 days of cultures. This may indicate that the DMSO used to dissolve CORT simplified OL morphologies.

The genetic background affected OL maturation at different time points in response to acute CORT exposure. In B6 cultures, acute CORT did not affect the number of $MBP^+/OLIG2^+$ double-positive cells 3 days post-exposure compared with vehicle treatment, but we observed 29.8% ($p = 0.009$) fewer mature OLs at 6 days post CORT exposure (Figure 2d,f). This effect was in contrast with D2 cultures, where we observed 32% more mature OLs ($p = 7.5e\text{-}06$) 3 days post-exposure compared with vehicle treatment, whereas no differences were observed at DIV6.

We asked whether the differences in OL numbers in response to acute CORT exposure could originate from differences in cell viability. The number of apoptotic $CC3^+$ cells varied depending on the time point post-CORT exposure and the genetic background of the cells. For example, cell survival was promoted by 29.2% at

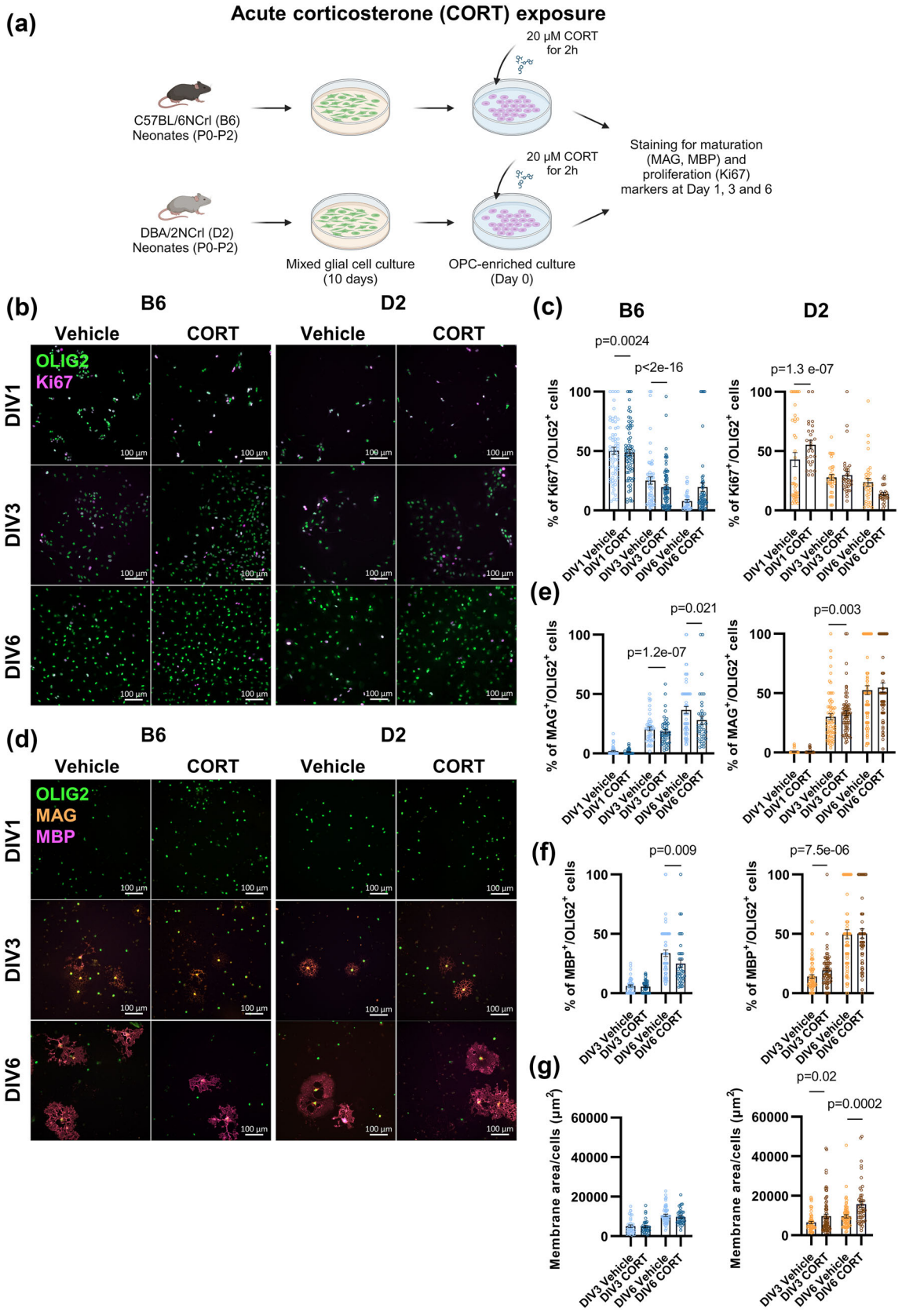

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FIGURE 2 Acute corticosterone exposure affects oligodendrocyte progenitor cell (OPC) proliferation, oligodendrocyte (OL) maturation and myelin production. (a) OPC cultures obtained from D2 and B6 mice were exposed for 2 h to 20-μM CORT. (b) Representative fluorescence images of OLs isolated from B6 and D2 mice ($OLIG2^+$ cells) engaged in proliferation ($Ki67^+/OLIG2^+$ cells) 1, 3 or 6 days after CORT exposure. (c) Proportion of proliferating OLs ($Ki67^+/OLIG2^+$ cells) in B6 (DIV1 vehicle: $N = 3$ cell batches, 73 images, 2212 cells; DIV1 CORT: $N = 3$ cell batches, 71 images, 1985 cells; DIV3 vehicle: $N = 3$ cell batches, 54 images, 3837 cells; DIV3 CORT: $N = 3$ cell batches, 77 images, 5627 cells; DIV6 vehicle: $N = 2$ cell batches, 40 images, 4309 cells; DIV6 CORT: $N = 3$ cell batches, 58 images, 4577 cells) and D2 (DIV1 vehicle: $N = 2$ cell batches, 40 images, 2039 cells; DIV1 CORT: $N = 2$ cell batches, 37 images, 356 cells; DIV3 vehicle: $N = 2$ cell batches, 33 images, 1289 cells; DIV3 CORT: $N = 3$ cell batches, 34 images, 1958 cells; DIV6 vehicle: $N = 2$ cell batches, 34 images, 1893 cells; DIV6 CORT: $N = 2$ cell batches, 26 images, 1116 cells) cultures treated with CORT or vehicle. (d) Representative fluorescence images of OLs obtained from B6 and D2 mice and stained for maturation markers 1, 3 or 6 days after CORT or vehicle treatment. (e) Proportion of pre-myelinating OLs ($MAG^+/OLIG2^+$ cells) in B6 (DIV1 vehicle: $N = 2$ cell batches, 54 images, 1940 cells; DIV1 CORT: $N = 2$ cell batches, 57 images, 2044 cells; DIV3 vehicle: $N = 2$ cell batches, 50 images, 1346 cells; DIV3 CORT: $N = 2$ cell batches, 47 images, 1118 cells; DIV6 vehicle: $N = 2$ cell batches, 57 images, 428 cells; DIV6 CORT: $N = 2$ cell batches, 40 images, 393 cells) and D2 (DIV1 vehicle: $N = 2$ cell batches, 42 images, 772 cells; DIV1 CORT: $N = 2$ cell batches, 42 images, 931 cells; DIV3 vehicle: $N = 3$ cell batches, 65 images, 1144 cells; DIV3 CORT: $N = 3$ cell batches, 79 images, 1007 cells; DIV6 vehicle: $N = 3$ cell batches, 66 images, 462 cells; DIV6 CORT: $N = 2$ cell batches, 49 images, 322 cells) cultures treated with CORT or vehicle. (f) Proportion of mature OLs ($MBP^+/OLIG2^+$ cells) in B6 (DIV3 vehicle: $N = 2$ cell batches, 50 images, 1346 cells; DIV3 CORT: $N = 2$ cell batches, 47 images, 1118 cells; DIV6 vehicle: $N = 2$ cell batches, 57 images, 428 cells; DIV6 CORT: $N = 2$ cell batches, 40 images, 393 cells) and D2 (DIV3 vehicle: $N = 3$ cell batches, 65 images, 1144 cells; DIV3 CORT: $N = 3$ cell batches, 79 images, 1007 cells; DIV6 vehicle: $N = 3$ cell batches, 66 images, 462 cells; DIV6 CORT: $N = 2$ cell batches, 49 images, 322 cells) cultures treated with CORT or vehicle. (g) Area of myelin membrane formed by mature OLs in B6 (DIV3 vehicle: $N = 2$ cell batches, 50 images, 1346 cells; DIV3 CORT: $N = 2$ cell batches, 47 images, 1118 cells; DIV6 vehicle: $N = 2$ cell batches, 57 images, 428 cells; DIV6 CORT: $N = 2$ cell batches, 40 images, 393 cells) and D2 (DIV3 vehicle: $N = 3$ cell batches, 60 images, 127 cells; DIV3 CORT: $N = 3$ cell batches, 77 images, 192 cells; DIV6 vehicle: $N = 3$ cell batches, 90 images, 322 cells; DIV6 CORT: $N = 3$ cell batches, 73 images, 275 cells) cultures treated with CORT or vehicle. Bar graphs show mean ± SEM. Each dot represents an average of all cells in one microscope image.

DIV1 ($p = 0.001$) and by 18.9% at DIV6 ($p = 9.2e\text{-}05$) in B6 cultures and by 28.2% at DIV6 in D2 cultures in response to CORT ($p = 0.012$, Figure S2c). Therefore, the reduced proportion of pre-myelinating and mature OLs in response to acute CORT cannot solely be attributed to differences in cell viability.

Lastly, we examined whether acute CORT treatment influences the size of the myelin sheet area produced by OLs. D2 cultures had a 39.5% larger size of MBP^+ area surrounding OLs at DIV3 ($p = 0.02$) and a 48.3% larger area at DIV6 ($p = 0.0002$) when compared with vehicle treatment (Figure 2d,g). This effect was not observed in B6 cultures. Taken together, these results suggest that acute CORT exposure inhibits OPC proliferation, differentiation and maturation of OLs isolated from B6 but promotes these programmes in OLs isolated from D2 mice.

3.3 | Prolonged dexamethasone exposure of OPCs

Chronic stress in vivo leads to a higher number of OPCs, promotes OL maturation and induces brain region-specific changes in myelin thickness in rodents (Laine et al., 2018; Long et al., 2021; Poggi et al., 2022). DEX is a synthetic glucocorticoid with a longer half-life and duration of action than CORT, making it more suitable for prolonged exposure (Hindmarsh & Geertsma, 2017; Yasir et al., 2022). We next investigated the effects of prolonged glucocorticoid exposure on OPC proliferation, differentiation and maturation by exposing freshly seeded OPCs to 50-μM DEX for 1, 3 or 6 days (Figure 3a).

Prolonged DEX exposure inhibited OPC proliferation in both B6 and D2 cultures compared with vehicle treatment at all observed time points (Figure 3b,c). In B6 cultures, this effect was most noticeable after 1 day of DEX treatment, with 61.2% less $Ki67^+/OLIG2^+$ double-positive cells compared with vehicle treatment ($p < 2e\text{-}16$). In D2 cultures, this effect was most pronounced after 6 days of DEX treatment, with 43.4% less proliferating cells compared with vehicle-treated cells ($p = 0.001$).

We next investigated the effect of prolonged DEX treatment on OL differentiation and maturation. We found 50.7% less $MAG^+/OLIG2^+$ double-positive cells in D2 cultures at DIV3 compared with vehicle treatment ($p = 6.1e\text{-}05$, Figure 3d,e) but no effect in B6 cultures. Results from the morphological analysis were in line with this result, with a 36.4% lower ramification index for OLs isolated from D2 compared with vehicle-treated cells ($p = 7.51e\text{-}08$) and no morphological changes in response to prolonged DEX in B6 cultures (Figure S1d,g).

Prolonged DEX treatment did not affect the proportion of mature OLs in cultures from either strain

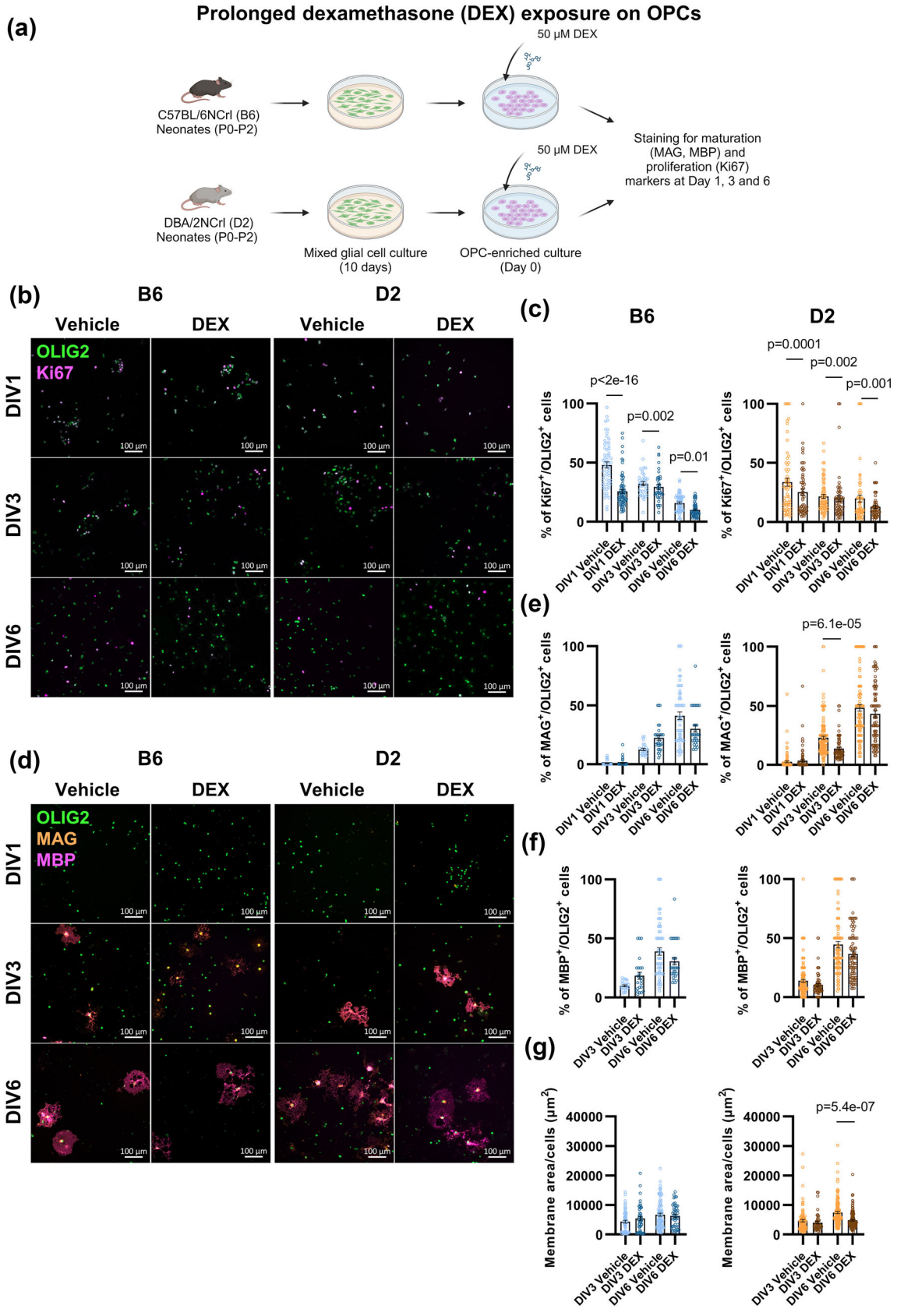

FIGURE 3 Legend on next page.

FIGURE 3 Prolonged oligodendrocyte progenitor cell (OPC) dexamethasone exposure affects OPC proliferation, oligodendrocyte (OL) maturation and myelin production. (a) OPC cultures obtained from D2 and B6 mice were exposed to 50-μM dexamethasone (DEX) until fixation. (b) Representative fluorescence images of OLs isolated from B6 and D2 mice ($OLIG2^+$ cells) engaged in proliferation ($Ki67^+$/$OLIG2^+$ cells) when treated with DEX or vehicle. (c) Proportion of proliferating OLs ($Ki67^+$/$OLIG2^+$ cells) in B6 (DIV1 vehicle: $N = 2$ cell batches, 61 images, 1510 cells; DIV1 DEX: $N = 2$ cell batches, 72 images, 1922 cells; DIV3 vehicle: $N = 2$ cell batches, 39 images, 1346 cells; DIV3 DEX: $N = 2$ cell batches, 39 images, 2322 cells; DIV6 vehicle: $N = 2$ cell batches, 61 images, 6976 cells; DIV6 DEX: $N = 2$ cell batches, 57 images, 2806 cells) and D2 (DIV1 vehicle: $N = 2$ cell batches, 63 images, 881 cells; DIV1 DEX: $N = 2$ cell batches, 59 images, 853 cells; DIV3 vehicle: $N = 2$ cell batches, 69 images, 2092 cells; DIV3 DEX: $N = 2$ cell batches, 59 images, 1526 cells; DIV6 vehicle: $N = 2$ cell batches, 56 images, 1585 cells; DIV6 DEX: $N = 2$ cell batches, 57 images, 1268 cells) cultures treated with DEX or vehicle. (d) Representative fluorescence images of OLs obtained from B6 and D2 mice and stained for maturation markers after DEX or vehicle treatment. (e) Proportion of pre-myelinating OLs (MAG^+/$OLIG2^+$ cells) in B6 (DIV1 vehicle: $N = 2$ cell batches, 48 images, 1248 cells; DIV1 DEX: $N = 2$ cell batches, 48 images, 1495 cells; DIV3 vehicle: $N = 2$ cell batches, 23 images, 419 cells; DIV3 DEX: $N = 2$ cell batches, 30 images, 380 cells; DIV6 vehicle: $N = 2$ cell batches, 58 images, 401 cells; DIV6 DEX: $N = 2$ cell batches, 32 images, 160 cells) and D2 (DIV1 vehicle: $N = 3$ cell batches, 103 images, 4164 cells; DIV1 DEX: $N = 3$ cell batches, 90 images, 2448 cells; DIV3 vehicle: $N = 3$ cell batches, 134 images, 2530 cells; DIV3 DEX: $N = 3$ cell batches, 68 images, 1142 cells; DIV6 vehicle: $N = 3$ cell batches, 94 images, 791 cells; DIV6 DEX: $N = 3$ cell batches, 88 images, 826 cells) cultures treated with DEX or vehicle. (f) Proportion of mature OLs (MBP^+/$OLIG2^+$ cells) in B6 (DIV3 vehicle: $N = 2$ cell batches, 23 images, 419 cells; DIV3 DEX: $N = 2$ cell batches, 29 images, 378 cells; DIV6 vehicle: $N = 2$ cell batches, 58 images, 401 cells; DIV6 DEX: $N = 2$ cell batches, 22 images, 160 cells) and D2 (DIV3 vehicle: $N = 3$ cell batches, 134 images, 2530 cells; DIV3 DEX: $N = 3$ cell batches, 138 images, 1142 cells; DIV6 vehicle: $N = 3$ cell batches, 93 images, 797 cells; DIV6 DEX: $N = 3$ cell batches, 88 images, 826 cells) cultures treated with DEX or vehicle. (g) Area of myelin membranes formed by mature OLs in B6 (DIV3 vehicle: $N = 3$ cell batches, 58 images, 93 cells; DIV3 DEX: $N = 2$ cell batches, 55 images, 62 cells; DIV6 vehicle: $N = 2$ cell batches, 86 images, 167 cells; DIV6 DEX: $N = 2$ cell batches, 47 images, 63 cells) and D2 (DIV3 vehicle: $N = 3$ cell batches, 112 images, 172 cells; DIV3 DEX: $N = 3$ cell batches, 51 images, 58 cells; DIV6 vehicle: $N = 3$ cell batches, 145 images, 478 cells; DIV6 DEX: $N = 3$ cell batches, 104 images, 293 cells) cultures treated with DEX or vehicle. Bar graphs show mean ± SEM. Each dot represents an average of all cells in one microscope image.

(Figure 3d,f). However, the size of MBP^+ area surrounding OLs was reduced by 43% in D2 cultures upon 6 days of prolonged DEX exposure compared with vehicle treatment ($p = 5.4e\text{-}07$), and no differences were found in B6 cultures (Figure 3d,g).

Compared with the vehicle treatment, 1 day of prolonged DEX exposure led to 68.8% ($p = 0.04$) and 47.8% ($p = 0.01$) higher cell viability as measured by the number of $CC3^+$ cells in B6 and D2 cultures, respectively (Figure S2d). Therefore, the difference in the number of differentiated OLs observed in D2 cultures cannot be explained by differences in cell viability induced by prolonged DEX exposure. These findings imply that the influence of DEX on OL differentiation and maturation is independent of its effect on cell survival. Overall, our results suggest that prolonged glucocorticoid exposure inhibits OL differentiation but promotes myelin production in D2 cultures only, with these effects being dependent on the stage of cellular maturity.

3.4 | Prolonged dexamethasone exposure of mature OLs

Chronic stress in vivo may modulate existing myelin (de Faria et al., 2021; Miyata et al., 2016). Therefore, we investigated the effects of prolonged glucocorticoid treatment on mature, myelin-producing, OLs and assessed the influence of the genetic background of the cells. For this purpose, we let OPCs differentiate and mature for 6 days and exposed them to 50-μM DEX for 2 or 4 days (Figure 4a). Prolonged DEX treatment of mature OLs did not affect their number nor the size of MBP^+ area (Figure 4b,e). These data indicate that prolonged glucocorticoid exposure does not affect the number of mature OLs nor the amount of myelin they produce in vitro.

4 | DISCUSSION

In this study, we established an in vitro model consisting of primary OLs isolated from B6 and D2 mouse strains. In naïve conditions, the genetic background of the cells modulated the proliferation and differentiation programmes of OPCs. Acute CORT and prolonged DEX exposures influenced these programmes in a strain-specific manner. In addition, we identified timing effects of glucocorticoids on OPC proliferation and differentiation, suggesting that genetic mechanisms affect the sensitivity of OLs to glucocorticoids depending on their developmental stage. Prolonged glucocorticoid exposure did not influence the amount of myelin produced by mature OLs.

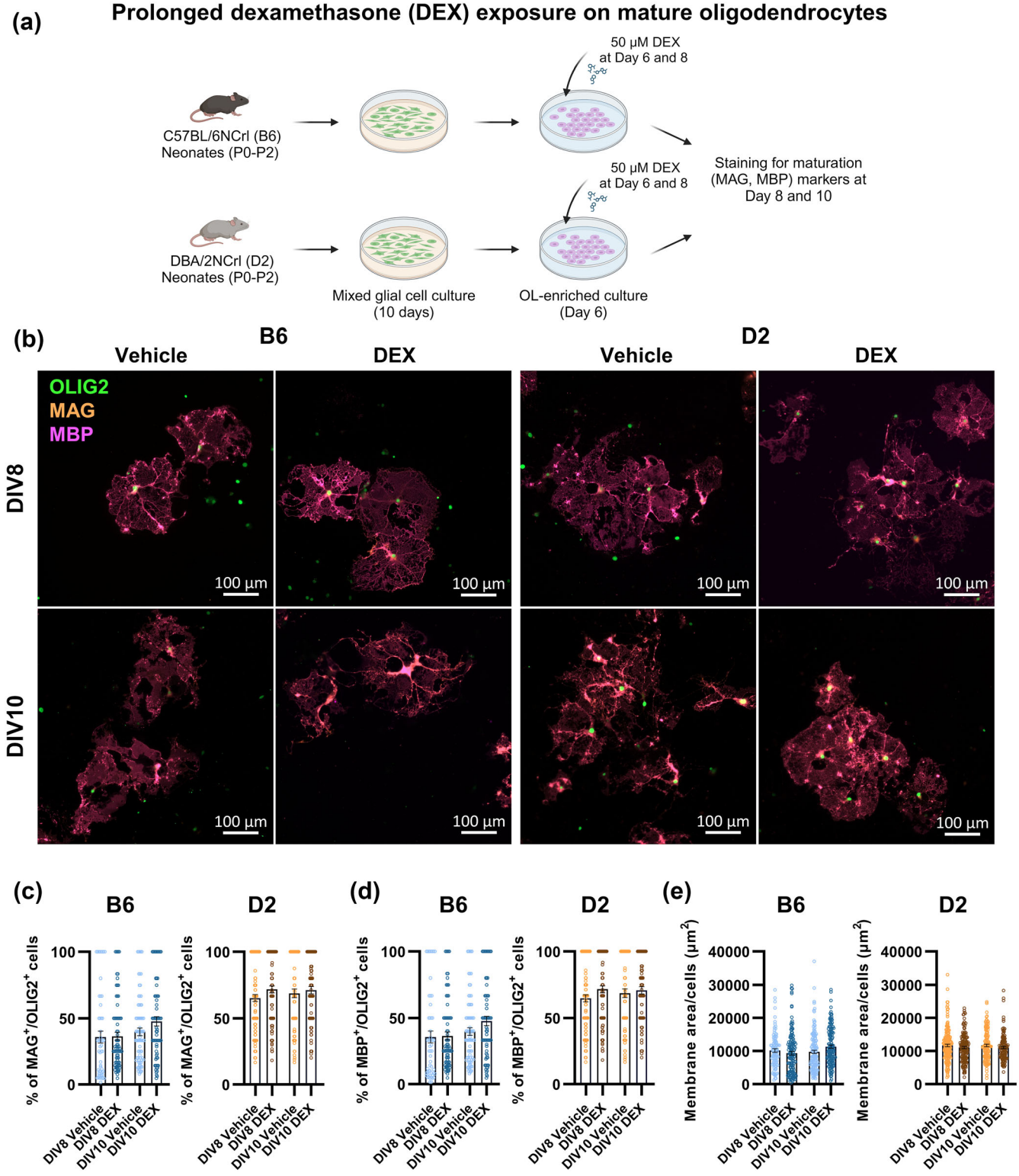

FIGURE 4 Legend on next page.

FIGURE 4 Prolonged dexamethasone exposure on mature oligodendrocyte (OL) does not affect OL maturation or myelin production. (a) Mature OLs cultured for 6 days were exposed to 50-μM dexamethasone (DEX) until fixation. (b) Representative fluorescence images of OLs obtained from B6 and D2 mice and stained for different maturation markers after DEX or vehicle treatment. (c) Proportion of pre-myelinating OLs ($MAG^+/OLIG2^+$ cells) in B6 (DIV8 vehicle: $N = 2$ cell batches, 57 images, 1437 cells; DIV8 DEX: $N = 2$ cell batches, 78 images, 597 cells; DIV10 vehicle: $N = 2$ cell batches, 75 images, 521 cells; DIV10 DEX: $N = 2$ cell batches, 70 images, 395 cells) and D2 (DIV8 vehicle: $N = 2$ cell batches, 108 images, 515 cells; DIV8 DEX: $N = 2$ cell batches, 95 images, 477 cells; DIV10 vehicle: $N = 2$ cell batches, 78 images, 449 cells; DIV10 DEX: $N = 2$ cell batches, 80 images, 422 cells) cultures treated with DEX or vehicle. (d) Proportion of mature OLs ($MBP^+/OLIG2^+$ cells) in B6 (DIV8 vehicle: $N = 2$ cell batches, 57 images, 1437 cells; DIV8 DEX: $N = 2$ cell batches, 78 images, 597 cells; DIV10 vehicle: $N = 3$ cell batches, 75 images, 521 cells; DIV10 DEX: $N = 2$ cell batches, 70 images, 395 cells) and D2 (DIV8 vehicle: $N = 2$ cell batches, 269 images, 515 cells; DIV8 DEX: $N = 2$ cell batches, 95 images, 477 cells; DIV10 vehicle: $N = 2$ cell batches, 78 images, 449 cells; DIV10 DEX: $N = 2$ cell batches, 80 images, 422 cells) cultures treated with DEX or vehicle. (e) Area of myelin membranes formed by mature OLs in B6 (DIV8 vehicle: $N = 3$ cell batches, 97 images, 263 cells; DIV8 DEX: $N = 3$ cell batches, 140 images, 278 cells; DIV10 vehicle: $N = 3$ cell batches, 142 images, 406 cells; DIV10 DEX: $N = 3$ cell batches, 140 images, 321 cells) and D2 (DIV8 vehicle: $N = 3$ cell batches, 180 images, 618 cells; DIV8 DEX: $N = 3$ cell batches, 147 images, 523 cells; DIV10 vehicle: $N = 3$ cell batches, 142 images, 527 cells; DIV10 DEX: $N = 3$ cell batches, 97 images, 529 cells) cultures treated with DEX or vehicle. Bar graphs show mean ± SEM. Each dot represents an average of all cells in one microscope image.

Several transcription factors are involved in tightly regulated feedback loops to either maintain proliferating OPCs in their progenitor state, preventing premature differentiation or promoting their differentiation into mature OLs (Elbaz & Popko, 2019). In naïve conditions, we found that the genetic background of the OLs modulated their proliferation, differentiation and maturation programmes. Under these conditions, D2 cultures contained less proliferating OPCs compared with B6 cultures. Furthermore, D2 cultures had more pre-myelinating and mature OLs compared with B6 cultures. Concurring with these observations OLs of D2 cultures had a more complex morphology compared to OLs from B6 cultures, suggesting a more differentiated cell state at DIV3 of OLs isolated from D2 mice. Strain differences in myelination patterns have also been found in adult mouse brain. Hippocampal myelinated fibres of C57BL/6J mice have higher expression levels of myelin genes and proteins, greater size of myelinated fibres and higher conduction velocity compared with DBA/2J mice (Goudriaan et al., 2020), and similarly, B6 non-stressed control mice from a CSDS experiment have higher expression levels of myelin-related genes compared with D2 control mice (Laine et al., 2018). These data suggest that genetic background influences the intrinsic ability of OLs to mature and produce myelin, affecting myelination patterns and network function. Genetic factors also influence cortical myelination patterns in humans (Liu et al., 2019), with heritability estimated at 29–66% depending on the brain region, as assessed from myelin content maps of 873 individuals from the Human Connectome Project.

In response to stress, CORT is released by the adrenal glands triggering various physiological responses associated with acute stress response, preparing the individual to confront the stressful situation. OLs and OPCs express both glucocorticoid and mineralocorticoid receptors (Matsusue et al., 2014; Zhang et al., 2014). The goal of the acute CORT exposure experiment was to examine the long-term effects of a brief CORT exposure on OPCs mimicking a scenario where CORT concentration temporarily increases due to an acute stressor and subsequently returns to baseline levels. One day after the acute CORT exposure, OPC proliferation was inhibited in B6 cultures but promoted in D2 cultures compared with vehicle treatment. Furthermore, acute CORT exposure in B6 cultures inhibited OL differentiation and maturation compared with vehicle treatment over the following days, whereas these programmes were promoted in D2 cultures. Also, OLs from D2, but not B6, cultures formed more myelin sheets 6 days after the initial CORT exposure compared with vehicle treatment. Previous literature on the effects of a brief CORT exposure on OPCs is limited, but in general, glucocorticoids act to modulate the timing of OL development by inhibiting the response of OPCs to mitogens thereby promoting differentiation (Barres et al., 1994). Also, various stressors seem to promote OPC proliferation and differentiation. Three-hour restraint stress in rats leads to increased OPC proliferation in the hippocampus (Long et al., 2021), and contextual fear conditioning induces OPC proliferation and differentiation in the mPFC of C57BL/6J mice (Pan et al., 2020). This finding contrasts with our in vitro findings of decreased maturation of OPCs after acute CORT treatment in B6 cultures (Pan et al., 2020). These discrepancies are likely due to differences between in vivo and in vitro conditions. Our study focused on OPC-enriched cultures without the presence of neurons, which are known to be major drivers of adaptive myelination, through their neuronal activity and secreted neuropeptides (Gibson et al., 2014; Osso et al., 2021). However, our results clearly indicate that the genetic background of OPCs alone is sufficient to modulate acute effects of CORT.

Chronic stress is a risk factor for anxiety disorders, and mouse models of psychosocial stress have been associated with increased oligodendrogenesis and differences in myelination in stress-associated brain regions (Bonnefil et al., 2019; Laine et al., 2018; Lehmann et al., 2017). Here, we investigated genetic background effects of prolonged glucocorticoid exposure on OPCs and myelin production in vitro. Prolonged 50-μM DEX exposure inhibited OPC proliferation in both B6 and D2 cultures. Our results are in line with much of the literature, demonstrating that glucocorticoids inhibit OPC proliferation, thereby increasing differentiation into mature OLs in vitro (Barres et al., 1994) and in vivo (Alonso, 2000; Schröter et al., 2009), although one study has reported no effects on OPC proliferation (Jenkins et al., 2013). We also found that prolonged DEX exposure reduced the number of pre-myelinating OLs, the size of the myelin sheet area and the complexity of the OL morphology in D2, but not in B6 cultures, compared with vehicle treatment. Previous studies have found several factors that influence how glucocorticoids affect OL maturation and myelin production, including the stage of cellular maturation (Almazan et al., 1986; Joubert et al., 2010; Kumar et al., 1989) and the dose of the glucocorticoid (Miguel-Hidalgo et al., 2019; Warringa et al., 1987). Our data add to this literature by showing that the effect of prolonged glucocorticoid treatment on OL maturation and subsequent myelin formation also strongly depends on the genetic background of the cells.

Several mouse chronic stress models are associated with differences in myelin thickness (Gao et al., 2019; Laine et al., 2018; Liu et al., 2012), but it is not known whether these changes are due to de novo myelination or modification of existing myelin. To investigate the effect of DEX on more mature OLs, we cultured them for 6 days, followed by exposure to prolonged DEX treatment for either 2 or 4 days. We found that glucocorticoid exposure did not affect the number of mature OLs, nor the size of the myelin sheet area in B6 or D2 cultures. Our results agree with recent work demonstrating that OPCs appear to be more sensitive to their environment and crucial for adaptive myelination processes in the adult brain compared with mature OLs (Gibson et al., 2014; Xiao et al., 2016). Alternatively, mature OLs may require a longer glucocorticoid exposure to show an effect on myelin formation. Supporting this notion, prolonged treatment of mature MGCs with either 50-μM CORT or 50-μM DEX for 16 days resulted in lower myelination (Miguel-Hidalgo et al., 2019). Modifications of existing myelin sheaths by mature OLs might involve more subtle changes than alteration of the overall amount of myelin produced that we measured in our model. For example, we recently identified modifications in the nodes of Ranvier morphology in response to CSDS (Koskinen et al., 2023). To summarize, our findings suggest that glucocorticoids may have a greater impact on myelination processes when OPCs are exposed to glucocorticoids as compared with more mature OLs.

How may the in vitro results we obtained translate to the in vivo differences in adaptive myelin plasticity and anxiety-like behaviour between B6 and D2 mice (Laine et al., 2018)? We found a striking strain difference, especially in response to acute CORT treatment, which inhibited OPC proliferation, OL differentiation and maturation in B6 cultures and promoted them in D2 cultures, which also formed more myelin sheets than B6 cultures. Prolonged DEX exposure, in turn, reduced the number of pre-myelinating OLs, the size of the myelin sheet area and the complexity of the OL morphology in D2 but not in B6 cultures. Thus, acute and prolonged glucocorticoid exposures may have different consequences that also depend on the genetic background. Of note, the expression levels of the glucocorticoid receptor (*Nr3c1*) gene did not differ between B6 and D2 cultures, suggesting that observed strain differences may be due to differences in the downstream events (Figure S3). The behavioural response to stress differs considerably between the strains, with B6 mice being mostly stress resilient and D2 mice mostly stress susceptible (Laine et al., 2018). Drawing direct comparisons between our in vitro results and in vivo behaviour is challenging. Increased OL differentiation and maturation in an acute stressful situation may either be adaptive or maladaptive depending on the brain region. For an adaptive response, more myelin may not always be better, as shown by our study on CSDS (Laine et al., 2018). For example, B6 susceptible mice had smaller g-ratio (i.e., thicker myelin) compared with either stress resilient or control mice in the BNST after stress. The optimal myelin modification needed to retain resilience in the face of adversity may thus depend on the need of the circuit to maintain spatial connectivity and network function. This is in line with the growing understanding that anxiety disorders are more likely characterized by circuit-level dysfunction than isolated disruptions within a single brain region (Robinson et al., 2019). The importance of myelin plasticity in the modulation of network function is now emerging (Bonetto et al., 2021), but the underlying molecular mechanisms remain poorly understood. Our study provides the first steps towards understanding how OPCs and OLs respond to glucocorticoids and how genetic factors further modulate these responses.

Our study comes with some limitations. First, our in vitro model consisted of enriched, but not pure, cultures of OLs while OLs and myelination-related processes are potentially modulated by other glial cell types

(Hagemeyer et al., 2017; Tognatta et al., 2020). However, the enrichment for OPCs limits the effects originating from other cell types. OL differentiation can proceed independently of heterologous cell–cell interactions, as demonstrated by comparative analysis of OLs isolated from rat brains and those cultured in vitro, suggesting the robustness and autonomy of OL differentiation (Dugas et al., 2006). Thus, our model system provides a platform to investigate the effect of various stress-related substances on OL function. Secondly, we only tested one concentration of CORT and DEX, and future experiments could explore dose effects. Finally, a notable strength of our study is that we analysed the data with a generalized mixed model with the inclusion of the cell batch as a random effect. This approach allowed us to enhance the reliability and accuracy of the results.

5 | CONCLUSION

We have established an in vitro model, which allows investigation of the effects of various stress-associated substances, for example, stress hormones, oxidative stress or inflammation-related molecules, on OL structure and function. Concurring with results from in vivo models showing significant strain differences in myelin plasticity after chronic psychosocial stress (Koskinen et al., 2023; Laine et al., 2018), we found that the genetic background had a significant effect on proliferation, differentiation, maturation and myelin sheet formation of OL lineage cells after glucocorticoid exposure. We believe that in vitro models are needed to facilitate the translation of preclinical findings to genetically heterogeneous human populations for the development of effective treatment strategies for anxiety disorders. In contrast to in vivo stress models where the social interactions between animals can significantly influence the outcome, the major strength of cell cultures lies in the ability to maintain a controlled environment, eliminating many unpredictable factors. It is possible that some individuals are more prone to anxiety disorders due to genetic differences influencing the function of the OL lineage cells and their response to stress hormones, and our model provides a novel platform to study the underlying mechanisms.

AUTHOR CONTRIBUTIONS

Conceptualization: Iiris Hovatta, Adrien Gigliotta; methodology: Rashmi Kothary, Sarah Cummings, Adrien Gigliotta and Iiris Hovatta; formal analysis: Adrien Gigliotta; data curation: Adrien Gigliotta; writing—original draft preparation: Adrien Gigliotta; writing—review and editing: Iiris Hovatta; visualization: Adrien Gigliotta; supervision: Iiris Hovatta; project administration: Iiris Hovatta; funding acquisition: Iiris Hovatta; technical assistance: Sarah Cummings, Jessica Mingardi, Vida Alikhani, Kalevi Trontti and Alessandro Barbon. All authors have read and agreed to the published version of the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

None.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16285.

DATA AVAILABILITY STATEMENT

All data of this study are publicly available from FigShare doi: 10.6084/m9.figshare.24494725.

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