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Diacylglycerol lipase alpha in astrocytes is involved in maternal care and affective behaviors

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Abstract

Genetic deletion of cannabinoid CB1 receptors or diacylglycerol lipase alpha (DAGLa), the main enzyme involved in the synthesis of the endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG), produced profound phenotypes in animal models of depression-related behaviors. Furthermore, clinical studies have shown that antagonists of CB1 can increase the incidence and severity of major depressive episodes. However, the underlying pathomechanisms are largely unknown. In this study, we have focused on the possible involvement of astrocytes. Using the highly sensitive RNAscope technology, we show for the first time that a subpopulation of astrocytes in the adult mouse brain expresses *Dagla*, albeit at low levels. Targeted lipidomics revealed that astrocytic DAGLa only accounts for a minor percentage of the steady-state brain 2-AG levels and other arachidonic acid derived lipids like prostaglandins. Nevertheless, the deletion of *Dagla* in adult mouse astrocytes had profound behavioral consequences with significantly increased depressive-like behavioral responses and striking effects on maternal behavior, corresponding with increased levels of serum progesterone and estradiol. Our findings therefore indicate that lipids from the DAGLa metabolic axis in astrocytes play a key regulatory role in affective behaviors.

KEYWORDS

2-arachidonoylglycerol, astrocyte-specific knockout, depression, maternal behavior, targeted lipidomics, tripartite synapse

1 | INTRODUCTION

The endocannabinoid system (ECS) mediates an inhibitory feedback mechanism between the post- and the presynapse, modulating important aspects of synaptic plasticity such as depolarization-induced suppression of inhibition and long-term depression (Freund et al., 2003;

Kano et al., 2009). The most abundant endocannabinoid (eCB) in the brain, 2-arachidonoylglycerol (2-AG), is produced by the cleavage of diacylglycerol (DAG) precursors from membrane lipids. The synthesis of 2-AG is catalyzed by the rate-limiting enzyme diacylglycerol lipase alpha (DAGLa) (Bisogno et al., 2003; Gao et al., 2010; Jenniches et al., 2016; Tanimura et al., 2010) in the postsynaptic compartments

of synapses. Its precursor DAG also serves as a second messenger and plays a role in multiple metabolic processes and lipid signaling pathways (Eichmann & Lass, 2015). 2-AG activates presynaptic G-protein coupled cannabinoid receptor type 1 (CB1), which in turn results in an inhibition of further transmitter release. Homosynaptic and heterosynaptic mechanisms underlying this retrograde signaling process have been identified (Chevaleyre & Castillo, 2003; Neu, Földy, & Soltesz, 2007). Additionally, the 2-AG pool provides an important store for arachidonic acid that gives rise to prostaglandins (Nomura et al., 2011).

Studies on mice with a constitutive *Dagla* deletion showed an increased behavioral despair, responsiveness to stress, and anxiety-like behavior. The phenotype of these mice is similar to those lacking CB1 (Jenniches et al., 2016; Shonesy et al., 2014). Results of human studies have also indicated that genetic variants of genes encoding for constituents of the ECS are associated with affective disorders (Hillard & Liu, 2014; Stahl et al., 2019). Pharmacological blockade of the CB1 by rimonabant increased the risk for major depressive episodes, resulting in the withdrawal of this drug from the market in 2007. These observations demonstrate that mice with disrupted eCB signaling are valid models for studying depression. In humans, it has also been demonstrated that patients suffering from depression show reduction in circulating endocannabinoids (Hill, Miller, Carrier, Gorzalka, & Hillard, 2009). Additionally, there is evidence that general disturbances in the arachidonate lipidome might be involved in affective behaviors, since nontargeted lipidomics in patients with major depression have shown changes in the DAGLa substrate diacylglycerol (Liu et al., 2016). Furthermore, depression in humans is associated with decreased levels of the arachidonate metabolite prostaglandin D2 (PGD2) (Chu, Wei, Zhu, Shen, & Xu, 2017).

Behavioral effects of eCB signaling have mostly been attributed to neuronal mechanisms (Kano et al., 2009). However, astrocytes, the most abundant glial cell type in the brain, also express CB1, albeit in much lower levels than neurons (Metna-Laurent & Marsicano, 2015). The activation of astroglial CB1 by 2-AG was shown to mobilize Ca^{2+} from intracellular stores (Navarrete & Araque, 2008), which in turn triggered the release of the gliotransmitters glutamate and D-serine, and potentiated the transmission in synapses of adjacent neurons (Henneberger, Papouin, Oliet, & Rusakov, 2010). At the behavioral level, this neuron-astrocyte crosstalk contributed to spatial memory formation (Robin et al., 2018).

DAGLa was also shown to be expressed in spinal cord astrocytes (Garcia-Ovejero et al., 2009; Hegyi et al., 2018). In primary cultured cortico-hippocampal astrocytes, Dagla expression and in vitro levels of endocannabinoids in WT and KO mice have been characterized (Viader et al., 2016). These findings indicate that astrocytes may possess a fully functional ECS, which could be relevant in the regulation of tripartite synaptic plasticity involving astrocytes and neurons (Belluomo, Matias, Pernègre, Marsicano, & Chaouloff, 2015; Han et al., 2012; Navarrete, Diez, & Araque, 2014; Walter, Dinh, & Stella, 2004). However, it is currently unknown how much astrocytes in the adult brain contribute to the steady-state 2-AG levels, thereby affecting arachidonate remodeling, and it is also nknown how this affects behavior.

Dysfunctions of astrocytes have been reported to be highly correlated with major depressive disorder. Post-mortem brains of patients with major depressive disorder exhibit a decreased number or density of astrocytes, deregulation of astrocytic markers and astrocytic hypertrophy (Cotter et al., 2002; Nagy et al., 2015; Torres-Platas et al., 2011). Morphological and numerical alterations in astrocytes have also been frequently observed in animal models of depression (Rajkowska, Hughes, Stockmeier, Miguel-Hidalgo, & Maciag, 2013; Sanacora & Banasr, 2013). Dysregulations in astrocytes also seem to play a role in the pathologies of other affective disorders, like autism spectrum disorders or bipolar disorder (Petrelli, Pucci, & Bezzi, 2016; Rajkowska, Halaris, & Selemon, 2001).

Based this evidence, we hypothesized that astrocytes express functional *Dagla* and that dysregulation of astrocytic eCB production impacts affective behaviors. To investigate this, we characterized *Dagla* expression in neurons and astrocytes, generated astrocyte-specific and inducible *Dagla* knockout (GLAST-CreERT2-Dagla KO) mice via the Cre/loxP system, and assessed lipidomic changes and resulting behaviors in models of anxiety and depressive-like behavior.

2 | METHODS AND MATERIALS

2.1 | Animals and animal husbandry

Heterozygous GLAST-CreERT2 [Slc1a3tm1(cre/ERT2)Mgoe] mice from The Jackson Laboratory, carrying a tamoxifen-inducible form of Cre (CreERT2) in the locus of the astrocyte-specific glutamate aspartate transporter (GLAST) (Mori et al., 2006) were crossed with Dagla f/f mice [B6.Dagla tm1Zim] to produce astrocytic Dagla knockout (GLAST-Dagla KO). Seven to nine weeks old GLAST-Dagla KO mice and Dagla f/f littermates were intraperitoneally injected twice daily with 1 mg tamoxifen (T-5648; Sigma, Schnelldorf, Germany) dissolved in 50 μL corn oil (90%) and ethanol (10%) for five consecutive days (Mori et al., 2006). Experiments were started 4 weeks later. Cre-negative Dagla f/f littermates were used as controls. Heterozygous Rosa-Tomato reporter mice (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J; https://www.jax.org/strain/007914) were crossed with the GLAST-CreERT2- line. All mouse strains were maintained on a C57BL/6J genetic background.

The floxed *Dagla* gene, the wild type allele, and the loss-of-function *Dagla* locus, as well as the Cre locus were identified by polymerase chain reaction (PCR) using appropriate primers (Cre1_fwd CATTT GGGCC AGCTA AACAT, Cre2_fwd GCATT TCTGG GGATT GCTTA, Cre1_rev CCCGG CAAAA CAGGT AGTTA, Cre2_rev TGCAT GATCT CCGGT ATTGA, KO_fwd TAGCT TAGCC CCCAT GTGAC, KO_rev CCCAG TAGCC ACAGA ACCAT, WT_fwd GAGAT GGGTC CACCT CCTT).

2.2 | Immunohistochemistry

Mice were anesthetized with isoflurane and transcardially perfused with 20 ml of cooled PBS, followed by 60 ml of 4% formaldehyde.

The brains were postfixed in 4% formaldehyde (only for co-stainings with tdTomato) for 1 hr, incubated in 20% sucrose/PBS for approximately 3 days, and frozen in dry ice-cooled isopentane. Tissues were stored at −80°C until further processing and cut in a cryostat with a chamber temperature of −22°C. Slices were collected on SuperFrost Plus adhesion slides (Thermo Fisher) and stored at −20°C. For DAGLa staining, brains were cut in 40 μm thick slices (Bregma −0.94 to −3.34 mm). For immunostainings with cell type specific markers and tdTomato, brains were cut into 16 μm slices (Bregma −1.46 until −2.46, every eighth section collected on a slide). After immunostaining, brain slices were briefly washed in ddH₂O and embedded in DAPI Fluoromount-G(R) media (Southern Biotechnology Associates. Inc., Birmingham, AL). Fluorescence images were obtained with the Leica SP8 confocal microscope, with 20x (NA 0.07, pixel size: 0.001 mm x 0.001 mm) or 40x (NA 1.1, pixel size: 0.142 μm x 0.142 μm) objective lenses. Phosphate buffer (PB, 0.2 M, pH 7.6) was used as a diluent and wash solution for DAGLa staining (DGLa-Rb-Af380; Frontier Institute Co., Ltd., Hokkaido, Japan). Triton (0.3%) was used for permeabilization (10 min). Nonspecific antibody binding was blocked with 5% normal goat serum and 0.2% Triton (1 hr at RT), DAGLa antibodies were incubated overnight at RT (1:400 in blocking solution). Secondary goat anti-rabbit Alexa-Fluor647 (Life Technologies, Darmstadt, Germany) was incubated for 2 hr at RT (1:500 in blocking solution). For tdTomato immunohistochemistry phosphate-buffered saline (PBS; 0.01 M, pH 7.4) served as a general diluent and wash solution. Sections were permeabilized by 0.5% Triton X-100 in PBS for 1 hr. Unspecific binding was blocked by 3% BSA for 2 hr prior to primary antibody incubation (24 hr at 4°C). Microglia were stained with rabbit anti-Iba1 antibody (1:2000 in 0.3% BSA/PBS; Wako, Richmond, VA), astrocytes with rabbit-anti-S100ß (1:2000, Abcam, Cambridge, GB) and chicken anti-GFAP (1:2000 Abcam, Cambridge, GB), neurons with rabbit anti-NeuN (1:500 Merck, Millipore, Burlington, USA). Secondary antibodies were anti-rabbit Alexa-Fluor 488 (1:1000), donkey anti-rabbit Alexa-Fluor 647 (1:2000) or goat anti-chicken Alexa-Fluor 488 (1:2000).

2.3 | mRNA expression analysis by TaqMan

RNA from frozen brain was extracted with Trizol® Reagent (Life Technologies, Bleiswijk, Netherlands) and ceramic beads (Peqlab, Darmstadt, Deutschland) in a shaking homogenizer and centrifuged at 12000*g* for 10 min at 4°C. 1-bromo-3-chloropropane (BCP, 1:10) was added, vortexed for 30 s, incubated for 3 min at RT and centrifuged (12,000 g, 10 min, 4°C). The RNA-containing upper phase was collected in fresh tubes. RNA was precipitated with isopropanol, washed twice with 75% EtOH and dried at RT. The pellet was dissolved in 20–50 μL RNase-free water. RNA concentration and purity were determined using a spectrophotometer (NanoDrop by Thermofisher, Waltham, MA USA). All samples were digested with DNase I (Roche Diagnostics, Mannheim, Germany). RNA was reverse transcribed using desoxynucleotide mix (10 mM, Sigma-Aldrich), Oligo(dt)₁₂₋₁₈ primer, 5x first strand buffer, 0.1 M DTT and Reverse Transcriptase Super

Script II ® (Life Technologies). mRNA levels were determined using TaqMan® Gene expression assays (Applied Biosystems, Darmstadt, Germany; *Dagla*: Mm00813830_m1), *Gapdh (*Mm00446968_m1) was used as the housekeeping gene and analyzed in the LightCycler®480 (Roche Diagnostics, Mannheim, Deutschland). Expression was calculated with relative quantification (2^{-ddCt}) setting.

2.4 | RNAscope assay and image analysis

The RNAscope method was used to evaluate the expression of *Dagla* in the cells of interest (www.acdbio.com/RNAScope). For this purpose, mice (n = 3–4 per genotype) were killed by decapitation, brains were quickly removed, flash frozen in dry ice-cooled isopentane and stored at −80°C. Brains were cryosectioned at a thickness of 10 μm and mounted on SuperFrost Plus slides (Thermo Fisher, Germany). The exact Bregma coordinates were identified from every twelth slide according to Paxinos Brain atlas (Franklin & Paxinos, 2008). RNAscope Multiplex Fluorescent Reagent kit was used according to manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA). The probes for *Dagla* mRNA (cat.no 478821) were multiplexed with the probes detecting a specific pan-astrocytic marker *Aldh1l1* (cat.no 405891-C2) (Zhang & Barres, 2010) and a well-established neuronal marker *Rbfox3* (cat.no 313311-C3). Confocal images of sections were obtained using a Leica TSC SP8 with 40x magnification (291 μm² area) and analyzed using Fiji software (version 2.0.0-rc-69; NIH, Bethesda, MD, USA). Cell nuclei were manually encircled and selected as regions of interest from an image with DAPI, *Aldh1l1* and *Rbfox3* signals. Nuclei with at least 2 dots of *Aldh1l1* and no *Rbfox3* signal were designated as astrocytes and nuclei with at least 2 *Rbfox3* and no *Aldh1l1* particles as neurons. The same number of astrocytes and neurons were counted on a picture (n = 24–30 cells per animal, depending on the brain region). The numbers of *Dagla* particles per selection (astrocytes or neurons) were then quantified. Micrographs from at least two consecutive sections from an animal were analyzed per brain region. In every assay, sections from constitutive Dagla KO mice (Jenniches et al., 2016; Shonesy et al., 2014a) were used as controls for nonspecific binding of the *Dagla* probe.

2.5 | Behavioral experiments

All experiments were conducted in adult mice (2–3 months in the beginning and 4–5 months at the end of testing), starting from the least stressful (nestlet test) and ending with the most stressful experiment (forced swim test). There was a 1 week interval between the tests. All experiments were approved by the North Rhine-Westphalia State Environment Agency (AZ: 84–02.04.2017.A234).

Pup retrieval: Mice were habituated for 30 min. Then the entire litter as well as the male were transferred to a new cage while the Cre-positive mother was kept in the home cage. After 5 min three pups (PN 2–6 days) were returned to the home cage away from the nest at the opposite end of the cage. The latency to sniff the first pup

and retrieve all three pups to the nest was measured. If the female did not retrieve all pups within 5 min the test was terminated resulting in a latency of 300 seconds. The entire test was recorded on video. Pup retrieval test was performed with experienced mothers that had previously mothered at least one litter. Please note that Dagla f/f control dams were also treated with tamoxifen.

Nestlet test: Nest building performance is often used to assess stress-related behavior (Gaskill, Karas, Garner, & Pritchett-Corning, 2013); (Arras, Rettich, Cinelli, Kasermann, & Burki, 2007). For the test, a nestlet (https://www.plexx.eu/products/nestlets) was weighed and placed into the home cage. After 4 hr, loose parts of the nestlet were removed and the weight of the remaining nestlet was determined. The percentage of the nestlet that was shredded was calculated using start- and the end-weight of the nestlet. Nest scores followed the procedure described by Deacon et al. (2006).

Sucrose preference test: Mice were single housed and could freely choose between a water bottle and a bottle filled with 1% sucrose solution. Sucrose preference was evaluated for 3 days by weighing of the bottles. In addition, body weight and food intake of mice were measured daily. Water and sucrose intake were measured every 24 hr and the position of bottles was switched daily to exclude a side preference of a mouse. Sucrose preference was calculated as sucrose solution consumed divided by the total amount of liquid consumption.

Open-field test: Mice were allowed to move freely in an open-field box (44 cm x 44 cm), moderately illuminated with a light intensity of around 130 lx, for 30 min. Decreased time spent in center (25% of entire box) was considered as measure of anxiety. Freezing behavior or decreased movements were also considered as an indication of anxiety. Analysis was executed by "Ethovision XT" software (Noldus Information Technology Inc, Wageningen, Netherlands). After each mouse, boxes were cleaned with ethanol.

O-maze: The circular O-maze (40 cm height; 46 cm diameter) was divided into four equal quadrants, two open and two closed quadrants, illuminated with approximately 200 lx in a sound-isolated room. Mice were always placed into the same open quadrant and their movements were recorded for 5 min. Analysis of time spent in different areas, distance travelled and velocity was executed by "Ethovision XT" software. Additionally, stretched posture while leaving the closed areas (an indication for higher anxiety and cautious behavior), and looking down behaviors (an indication for low anxiety) were counted. After each trial, the O-maze was cleaned with ethanol.

Porsolt forced swim test: Mice were placed in a glass cylinder (28 cm height, 20 cm diameter) filled with water (25–26°C) for 6 min. During the last 4 min of the test, immobility time of mice was measured. Immobility time or floating behavior is interpreted as despair behavior. After the test, mice were dried with paper tissue. The entire test was recorded on video.

Vision and olfaction tests: For the vision test, mice were held from the base of their tail and were slowly moved down to the grid of their home cages from approximately 20 cm distance. If the mouse stretched the forelimbs before whiskers were touching the grid, mice received a score of 3 (meaning that the mice have intact vision). If the

mouse stretched them when whiskers were already touching the grid it received a score of 2. If the nose was touching the grid, it was scored with 1. To test olfaction, mice were habituated to a cotton swab for 30 minutes in their home cages. Afterwards, a cotton swab dipped in water was offered to the mouse for 2 min. The time a mouse was spending actively with the cotton swab was measured. Subsequently, another cotton swab that was previously rubbed on the body of a mouse pup (Figure 5D) or scented with urine of the other gender (Supplemental Figure 6) was offered to the test mouse for 2 min and again the time sniffing was measured. Mice spending more time with the cotton swab smelling of pups were designated able to smell (Zou, Wang, Pan, Lu, & Xia, 2015).

Home cage activity measurements: Mice were habituated to single-housing and the inverted dark/light cycle (12 hr light from 9 p.m. to 9 a.m.) for at least 7 days before home cage activity was recorded using an infrared sensor attached to the cage lid. Movements in the home cage were recorded every 30 seconds and were analyzed by the Mouse-E-Motion system (Infra-e-motion GmbH, Henstedt-Ulzburg, Germany). Measurements shown in the graphs were summed up to 1 hr.

2.6 | Extraction and measurements of endocannabinoids

After completing the behavioral tests, mice were killed by decapitation. Hypothalamus, prefrontal cortex, striatum, hippocampus, and retrosplenial cortex were dissected using a brain matrix (Zivic Instruments, Pittsburgh, PA) and punchers (diameter 1 mm, Fine Science Tools; Heidelberg, Germany) within 3 minutes. At the University of Bern, extraction and quantification of the concentration of endocannabinoids and related lipid components was carried out using the targeted lipidomics LC–MS/MS procedure previously described by Chicca et al., 2017 (Chicca et al., 2017). Additionally, SAG was quantified in the same method using deuterated 1-stearoyl-2-arachidonoyl-sn-glycerol-d8 (SAGd8) as internal standard (SAG: 662 - > 341, 667 - > 327. SAGd8 670 - > 341).

To validate our measurements, the experiment was repeated using an independent setting at the University of Mainz. The first measurement is reported in Figure 3, the repetition in Supplemental Figure 2. At the University of Mainz, quantification was carried out using LC–MS/MS procedure as previously described by (Lomazzo et al., 2015).

2.7 | Measurements of female reproductive hormones

Female GLAST-Dagla KO and control mice were decapitated during the behavioral estrus stage (proestrus and estrus) 3 weeks after tamoxifen injections. The ovarian cycle was first determined visually and validated by vaginal lavage (Byers 2012; McLean 2012). Blood was collected in a reaction tube and was allowed to clot for at least

30 minutes. After centrifugation for 10 minutes at 2000 g, serum was collected. Levels of FSH, LH, oxytocin, prolactin, estradiol and progesterone were measured using the Multiplexed hormone detection assay by LUMINEX technology (MPTMAG-49 K, RMNPMAG-83 K and MSHMAG-21 K by Merck/Millipore). Assays were performed on a magpix device. A few values that were at least 10-times higher than other values in the same group were excluded from the analysis.

2.8 | Data analysis

Data are presented as mean ± SEM. The numbers of animals/samples are indicated in the figure legends. Statistical significance was assessed using Student's t test, Mann–Whitney test (for nonparametric data) or 2-way ANOVA (Sidak post hoc test). Significant outliers were identified and excluded using Grubbs' test.
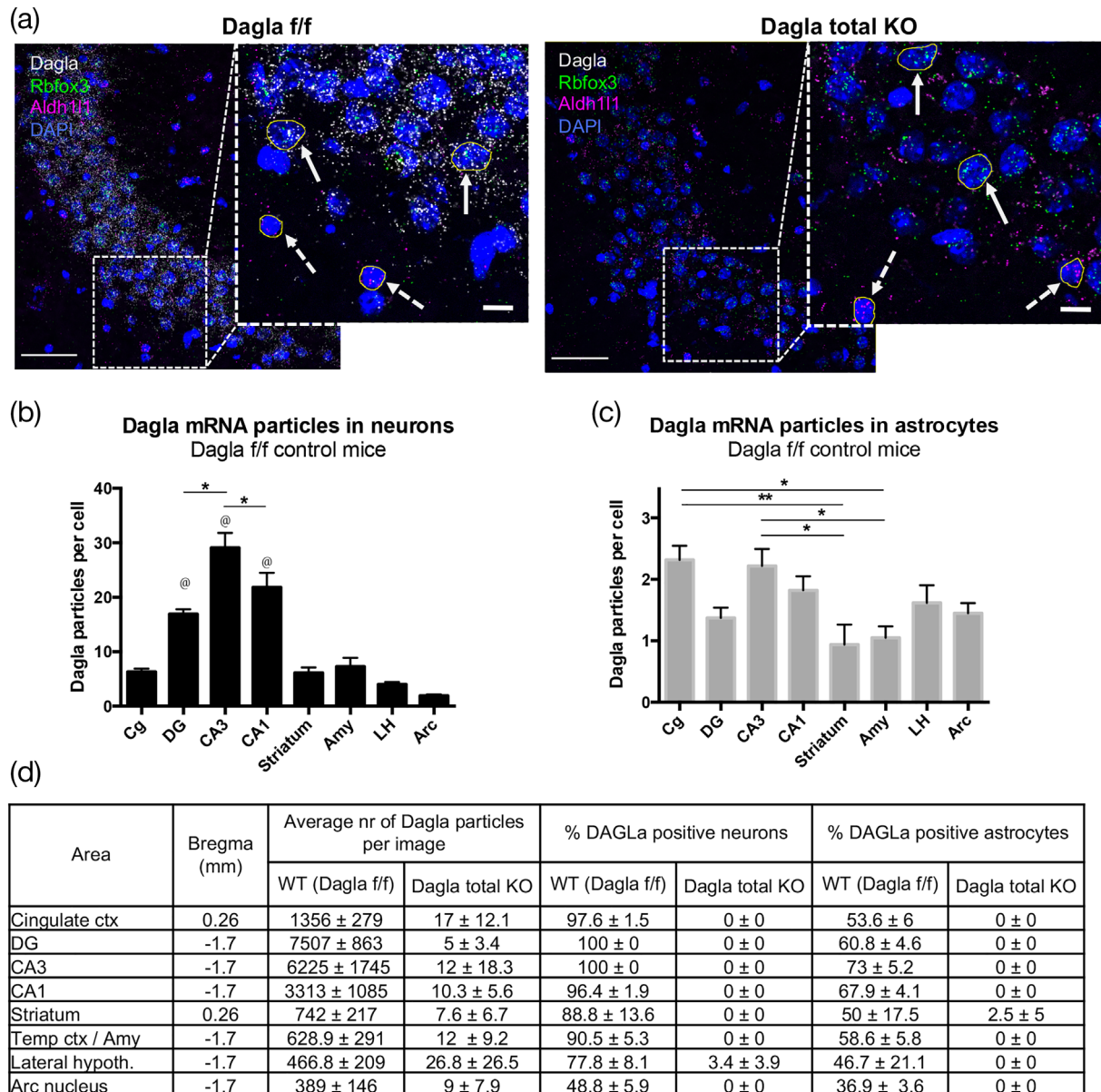

Area	Bregma (mm)	Average nr of Dagla particles per image		% DAGLa positive neurons		% DAGLa positive astrocytes	
		WT (Dagla f/f)	Dagla total KO	WT (Dagla f/f)	Dagla total KO	WT (Dagla f/f)	Dagla total KO
Cingulate ctx	0.26	1356 ± 279	17 ± 12.1	97.6 ± 1.5	0 ± 0	53.6 ± 6	0 ± 0
DG	-1.7	7507 ± 863	5 ± 3.4	100 ± 0	0 ± 0	60.8 ± 4.6	0 ± 0
CA3	-1.7	6225 ± 1745	12 ± 18.3	100 ± 0	0 ± 0	73 ± 5.2	0 ± 0
CA1	-1.7	3313 ± 1085	10.3 ± 5.6	96.4 ± 1.9	0 ± 0	67.9 ± 4.1	0 ± 0
Striatum	0.26	742 ± 217	7.6 ± 6.7	88.8 ± 13.6	0 ± 0	50 ± 17.5	2.5 ± 5
Temp ctx / Amy	-1.7	628.9 ± 291	12 ± 9.2	90.5 ± 5.3	0 ± 0	58.6 ± 5.8	0 ± 0
Lateral hypoth.	-1.7	466.8 ± 209	26.8 ± 26.5	77.8 ± 8.1	3.4 ± 3.9	46.7 ± 21.1	0 ± 0
Arc nucleus	-1.7	389 ± 146	9 ± 7.9	48.8 ± 5.9	0 ± 0	36.9 ± 3.6	0 ± 0

FIGURE 1 *Dagla expression* analysis in neurons and astrocytes in Dagla f/f and total Dagla KO mice by RNAscope in situ hybridisation. (a) Representative images (scale bar 50 μm) of an RNAscope in situ hybridisation assay detecting transcripts of diacylglycerol lipase alpha (*Dagla*, white), *Rbfox3* (green, neuronal marker) and *Aldh1l1* (magenta, astrocytic marker) in Dagla f/f and Dagla total KO CA3 regions of hippocampus. The magnified inset (scale bar 10 μm) shows examples of encircled cells identified as neurons (bold arrows) or astrocytes (dotted arrows). Note that the white dots indicating *Dagla* expression are lacking in the total KO control (b, c) Quantification of *Dagla* mRNA particles in neurons and astrocytes of Dagla f/f control mice in different brain regions: cingulate cortex (Cg ctx), dentate gyrus (DG), cornu ammonis 3,1 (CA3,1), striatum, amygdala (Amy), lateral hypothalamus (LH) and arcuate nucleus (Arc). (b) RNAscope analysis showed that *Dagla* mRNA expression in neurons varies in different brain regions (F = 34.45, p < .0001). Hippocampal regions (DG, CA3, CA1) significantly differed from all other brain regions, indicated by @. (c) The number of *Dagla* particles expressed in astrocytes in the above-mentioned brain areas (F = 4.382, p < .0008). (D) Quantification of *Dagla* expression in neurons and astrocytes in Dagla f/f and total Dagla KO mice. Mean ± *SEM*: 4–5 animals/group [Color figure can be viewed at wileyonlinelibrary.com]

(a)
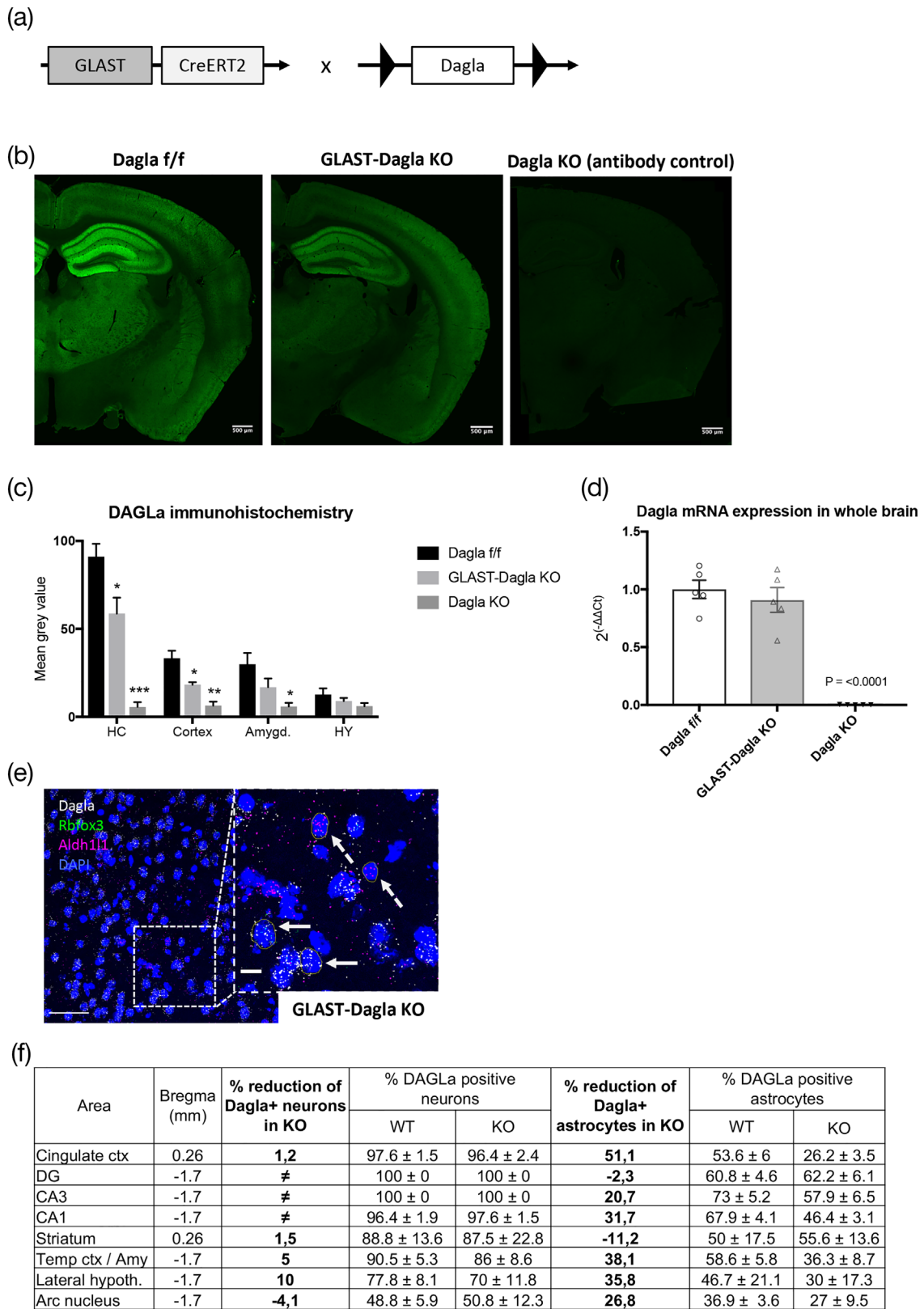
(b) Dagla f/f   GLAST-Dagla KO   Dagla KO (antibody control)

(c) DAGLa immunohistochemistry

(d) Dagla mRNA expression in whole brain

(e)

(f)

Area	Bregma (mm)	% reduction of Dagla+ neurons in KO	% DAGLa positive neurons		% reduction of Dagla+ astrocytes in KO	% DAGLa positive astrocytes	
			WT	KO		WT	KO
Cingulate ctx	0.26	1,2	97.6 ± 1.5	96.4 ± 2.4	51,1	53.6 ± 6	26.2 ± 3.5
DG	-1.7	≠	100 ± 0	100 ± 0	-2,3	60.8 ± 4.6	62.2 ± 6.1
CA3	-1.7	≠	100 ± 0	100 ± 0	20,7	73 ± 5.2	57.9 ± 6.5
CA1	-1.7	≠	96.4 ± 1.9	97.6 ± 1.5	31,7	67.9 ± 4.1	46.4 ± 3.1
Striatum	0.26	1,5	88.8 ± 13.6	87.5 ± 22.8	-11,2	50 ± 17.5	55.6 ± 13.6
Temp ctx / Amy	-1.7	5	90.5 ± 5.3	86 ± 8.6	38,1	58.6 ± 5.8	36.3 ± 8.7
Lateral hypoth.	-1.7	10	77.8 ± 8.1	70 ± 11.8	35,8	46.7 ± 21.1	30 ± 17.3
Arc nucleus	-1.7	-4,1	48.8 ± 5.9	50.8 ± 12.3	26,8	36.9 ± 3.6	27 ± 9.5

FIGURE 2   Legend on next page.

2.9 | Data availability

Data are available from authors on request.

3 | RESULTS

3.1 | *Dagla* expression in astrocytes and its impact on the arachidonate-associated lipidome

The RNAscope technology was used to assess *Dagla* mRNA expression with cellular resolution (Figure 1). The cellular origin of *Dagla* expression was determined by co-labeling with neuronal (*Rbfox3*) and pan-astrocytic (*Aldh1l1*) markers (Figure 1a). In Dagla f/f control mice, the number of *Dagla* mRNA particles was up to 10 times higher in neurons than in astrocytes (Figure 1b,c). The number of *Dagla* transcripts in neurons (Figure 1b) and astrocytes (Figure 1c) also varied among brain regions. Furthermore, we found that 50%–100% of the neurons expressed *Dagla* transcripts, depending on the brain region, whereas only 40%–75% of the astrocytes expressed *Dagla* transcripts (Figure 1d). The specificity of *Dagla* detection was validated using brain tissue from total *Dagla* KO mice (Figure 1d). In these tissues, the *Dagla* signal was virtually nonexistent, thus confirming the high specificity of the RNAscope technology.

We next deleted *Dagla* in astrocytes using an inducible Cre-driver line (GLAST-CreERT2) that is specifically active in astrocytes. The *Dagla* deletion in this GLAST-Dagla KO mouse line (Figure 2a) was first assessed by immunohistochemistry (Figure 2b,c). The DAGLa signal in GLAST-Dagla KO mice was similar, but weaker than in Dagla f/f controls. Quantification of the immunohistochemistry signal revealed a significant reduction of DAGLa expression in hippocampus and cortex of GLAST-Dagla KO mice. The specificity of the DAGL antibody was confirmed with constitutive Dagla KO mice that completely lack DAGLa expression in all cells. These mice did not show any DAGLa staining (Figure 2b). The DAGLa signal intensity in constitutive Dagla KO mice was significantly lower compared to Dagla f/f control mice in all brain regions except for hypothalamus that generally showed low DAGLa signal (Figure 2c). Next, an RT-PCR analysis was performed on whole brain lysates (Figure 2d). No significant change in *Dagla* mRNA was found in inducible GLAST-Dagla mice. These results indicate that astrocytic DAGLa only contributes a relatively small percentage to the overall *Dagla* expression in the brain.

We also analysed inducible GLAST-Dagla KO mice by in situ hybridization with RNAscope (Figure 2e,f). We found a significant reduction of the number of *Dagla* positive astrocytes in most brain regions (Figure 2f). Exceptions were the dentate gyrus and the striatum, where the number of *Dagla* positive astrocytes was not reduced. The most pronounced reduction of *Dagla* mRNA was observed in astrocytes of the cingulate cortex (51%), temporal cortex (38%) and the lateral hypothalamus (36%). Decreased *Dagla* expression was also observed in CA1 (32%) and CA3 (21%) regions of the hippocampus, as well as in the arcuate nucleus of the hypothalamus (27%). Importantly, we did not observe any reduction in the number of *Dagla* positive neurons (Figure 2f). The specificity of the astrocytic expression of Cre in GLAST-CreERT2 mice was further confirmed using RosaTomato reporter mice (Supplementary Figure 1). The reporter gene expression was induced in up to 80% of all astrocytes, whereas only few neurons were tdTomato positive.

Next, we performed targeted lipidomics to assess the possible contribution of DAGLa in astrocytes to the arachidonate associated lipids. The levels of 2-AG, its precursor 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), as well as arachidonic acid (AA), prostaglandins and other lipids involved in the ECS were determined in different brain regions (Figure 3, Supplementary Figures 2, 3). Female, but not male GLAST-Dagla KO mice showed a slight, but significant decrease (main genotype effect) in the levels of 2-AG and its metabolite arachidonic acid (AA) (Figure 3b,c), which is in good agreement with the more prominent role of DAGLa in neurons, compared to astrocytes, in the production of 2-AG. Female GLAST-Dagla KO mice showed slightly reduced corticosterone (CORT) levels in the brain (a significant genotype effect) (Supplementary Figure 3D). In order to assess the reliability of our experiment, the measurements of 2-AG, AEA and AA levels were repeated in an independent lab (3 weeks after tamoxifen injections). The results from both labs were very similar (Supplementary Figure 2A-C).

3.2 | Exploratory and anxiety-related behaviors

Body weight and food intake did not differ between GLAST-Dagla KO and their corresponding Dagla f/f littermates (Supplementary Figure 4). There was also no difference in open-field exploratory activity between GLAST-Dagla KO mice and Dagla f/f controls (Figure 4a),

FIGURE 2 Validation of inducible GLAST-Dagla KO efficacy by immunohistochemistry (IHC), RT-PCR and RNAscope in situ hybridisation. (a) Genetic scheme of animal model used in the study. (b) Representative images of diacylglycerol lipase alpha (DAGLa) immunostainings from brains of Dagla f/f control mice, tamoxifen-induced GLAST-Dagla KO, and constitutive Dagla KO control mice. DAGLa staining was reduced in GLAST-Dagla KO brains and completely absent in constitutive Dagla KO brains (Scale bar: 500 μm; green: DAGLa; n = 3 per group). (c) Quantification of DAGLa signal intensity by IHC. Signal intensity was significantly reduced in hippocampus (HC) and cortex of GLAST-Dagla KO brains compared to Dagla f/f control brains. In HC, cortex, and amygdala, total Dagla KO brains showed a strong reduction in the DAGLa signal intensity, whereas the DAGLa signal intensity was generally low in WT hypothalamus and not significantly different in KO mice (one-way ANOVA results: HC: $F_{2,6}$ = 39.09, p = .0004; cortex: $F_{2,6}$ = 20.64, p = .0020; amygd.: $F_{2,6}$ = 6.118, p = .0356; HY: $F_{2,6}$ = 1.851, p = .2365). (d) *Dagla* mRNA levels in whole brain lysates normalized to *Gapdh*. RT-PCR results showed no significant reduction of *Dagla* mRNA expression in GLAST-Dagla KO brain (n = 5 per group). (e) Representative images (scale bar 50 μm) of an RNAscope in situ hybridisation assay detecting transcripts of *Dagla* (white), *Rbfox3* (green, neuronal marker) and *Aldh1l1* (magenta, astrocytic marker) in cingulate cortex of GLAST-Dagla KO mice. The magnified inset (scale bar 10 μm) shows examples of encircled cells identified as neurons (bold arrows) or astrocytes (dotted arrows). (f) Quantification of *Dagla* KO efficiency in neurons and astrocytes in different brain regions of GLAST-Dagla mice in comparison to Dagla f/f littermates (n = 3–4 per group) [Color figure can be viewed at wileyonlinelibrary.com]
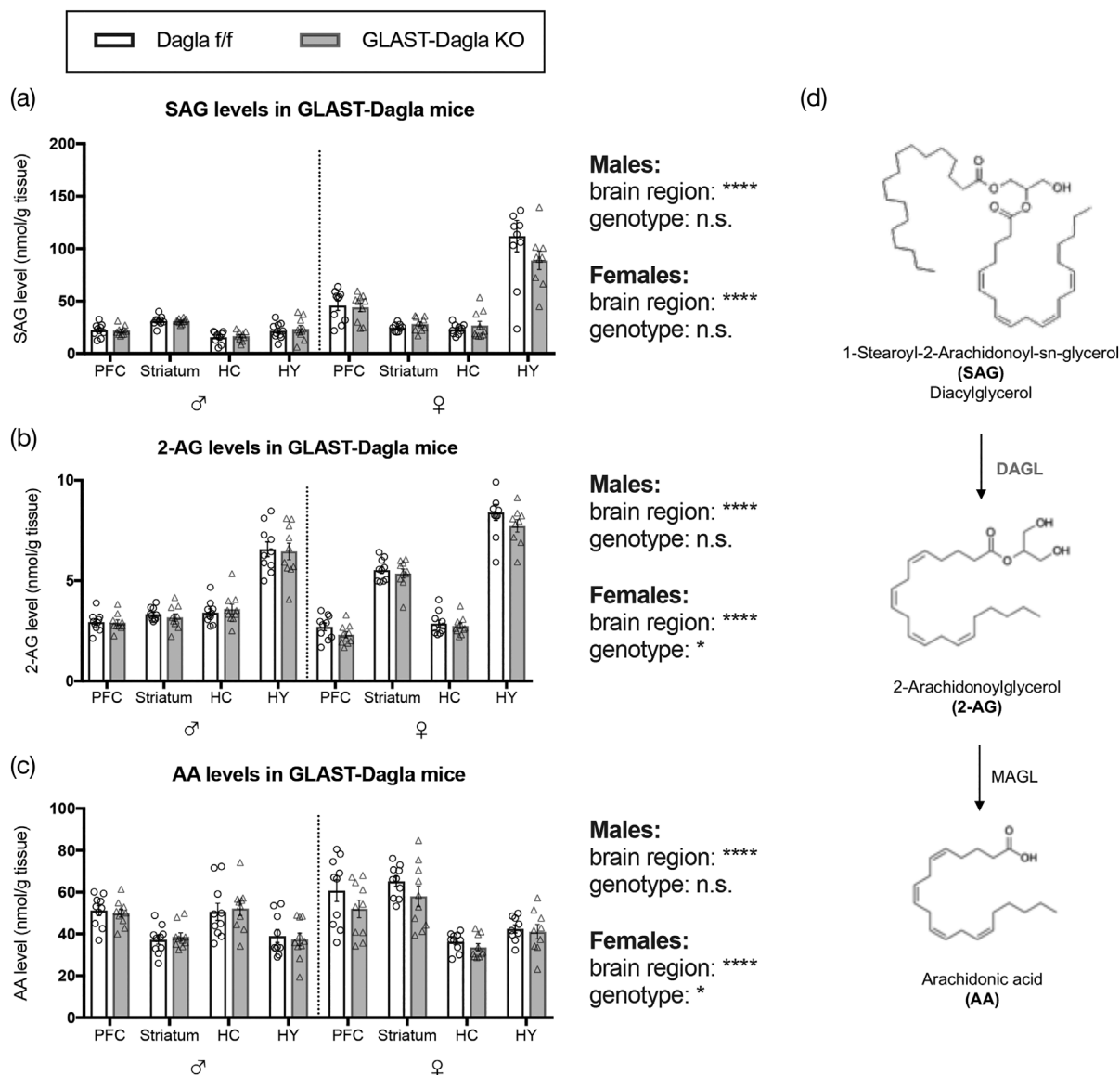
FIGURE 3 Targeted lipidomics by LC–MS/MS (a) The substrate of DAGLa, 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), was not significantly changed in any of the tested brain regions (prefrontal cortex (PFC), striatum, hippocampus (HC) and hypothalamus (HY) in male or female GLAST-Dagla mice. (b) Female, but not male GLAST-Dagla mice showed a significant reduction in 2-arachidonoyl glycerol (2-AG) levels (females: genotype effect: $F_{1,71} = 4.19$, $p = .0444$; brain region effect: $F_{3,71} = 243.8$, $p < .0001$). Post hoc analysis was not significant for any of the tested brain regions. (c) Female, but not male GLAST-Dagla mice showed an overall genotype effect in arachidonic acid (AA) levels (females: genotype effect: $F_{1,70} = 4.069$, $p = .0475$; brain region effect: $F_{3,70} = 25.84$, $p < .0001$), but no area specific differences between genotypes in the post hoc tests. (d) Schematic representation of the synthesis and metabolism of 2-AG by the enzymes DAGL and monoacylglycerol lipase (MAGL). Values represent mean ± *SEM*; $n = 10$ animals/group

as evaluated by the total distance moved and the activity in the center of the open field. There were also no sex differences.

No significant differences were observed in any parameter tested in the zero-maze test with inducible GLAST-Dagla KO mice (Figure 4b).

3.3 | Depressive-like behavior

Depressive-like behavior was assessed using the nestlet, sucrose preference and forced swim tests (Castagné, Moser, Roux, &

Porsolt, 2011; Katz, 1982; Porsolt, Le Pichon, & Jalfre, 1977). In the nestlet test, Dagla f/f control mice shredded nearly the entire nestlet in 4 hr, a significantly higher percentage of the nestlet than that shredded by female GLAST-Dagla KO mice (Figure 5a). Even after 60 hr, the nest quality of female GLAST-Dagla KO mice was still not as good as that of Dagla f/f littermates (Supplementary Figure 5).

In the sucrose preference test, inducible GLAST-DAGLa KO females drank less sucrose as a percentage of total fluid intake, and this was interpreted as anhedonic behavior. This phenotype
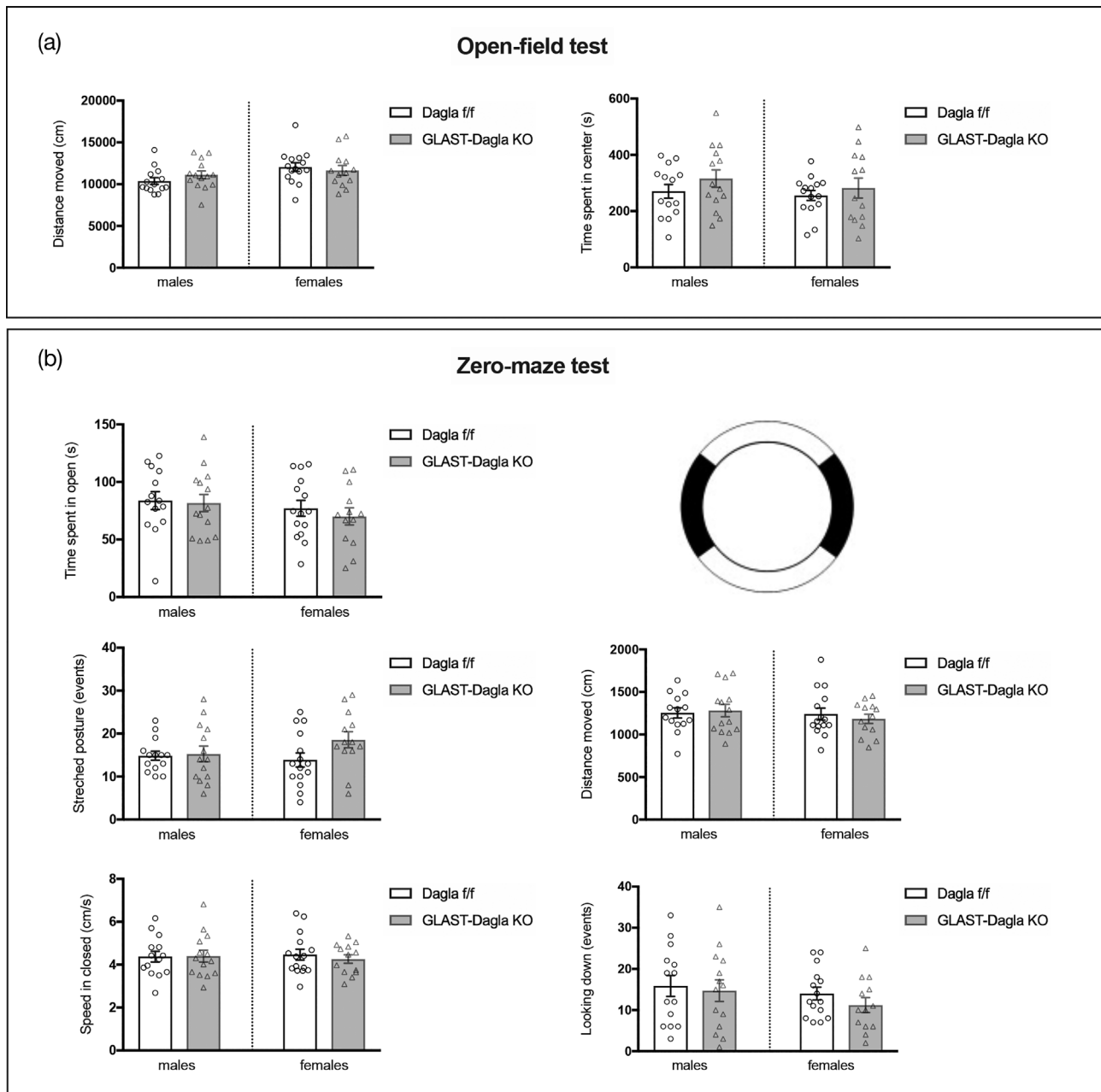
FIGURE 4 Analysis of anxiety-related behavior. (a) Neither male nor female GLAST-Dagla mice showed changes in open-field test behaviors; in the time spent in center (males: $p = .2646$; females: $p = .4879$) or in distance moved (males: $p = .2336$; females: $p = .6191$) during 30 minutes of testing. (b) Male and female GLAST-Dagla KO mice did not show any differences in their behavior in the zero-maze test compared to tamoxifen-injected Dagla f/f controls. Time spent in the open compartment (males: $p = .8469$; females: $p = .4958$), distance moved (males: $p = .7717$; females: $p = .5489$), mean velocity in the closed compartment (males: $p = .9421$; females: $p = .5448$), looking down and stretched posture behaviors were not changed compared to controls. Mean ± *SEM*; $n = 14$ animals/group

was not observed in males (Figure 5b). We did not find a genotype effect in the forced swim test, although female GLAST-Dagla KO mice just failed to show a significantly increased immobility time (Figure 5c; females: $p = .057$). Together, these results indicate a mild sex-dependent depressive-like behavioral phenotype in female GLAST-Dagla KO mice lacking *Dagla* expression in astrocytes.

3.4 | Maternal care behavior

The pup retrieval test was performed to investigate maternal care and depressive-like behavior. GLAST-Dagla KO dams either failed to retrieve the three pups from the opposite corner of the home cage to the nest or the time required by these dams to complete the task was significantly longer than that of tamoxifen-injected Dagla f/f control
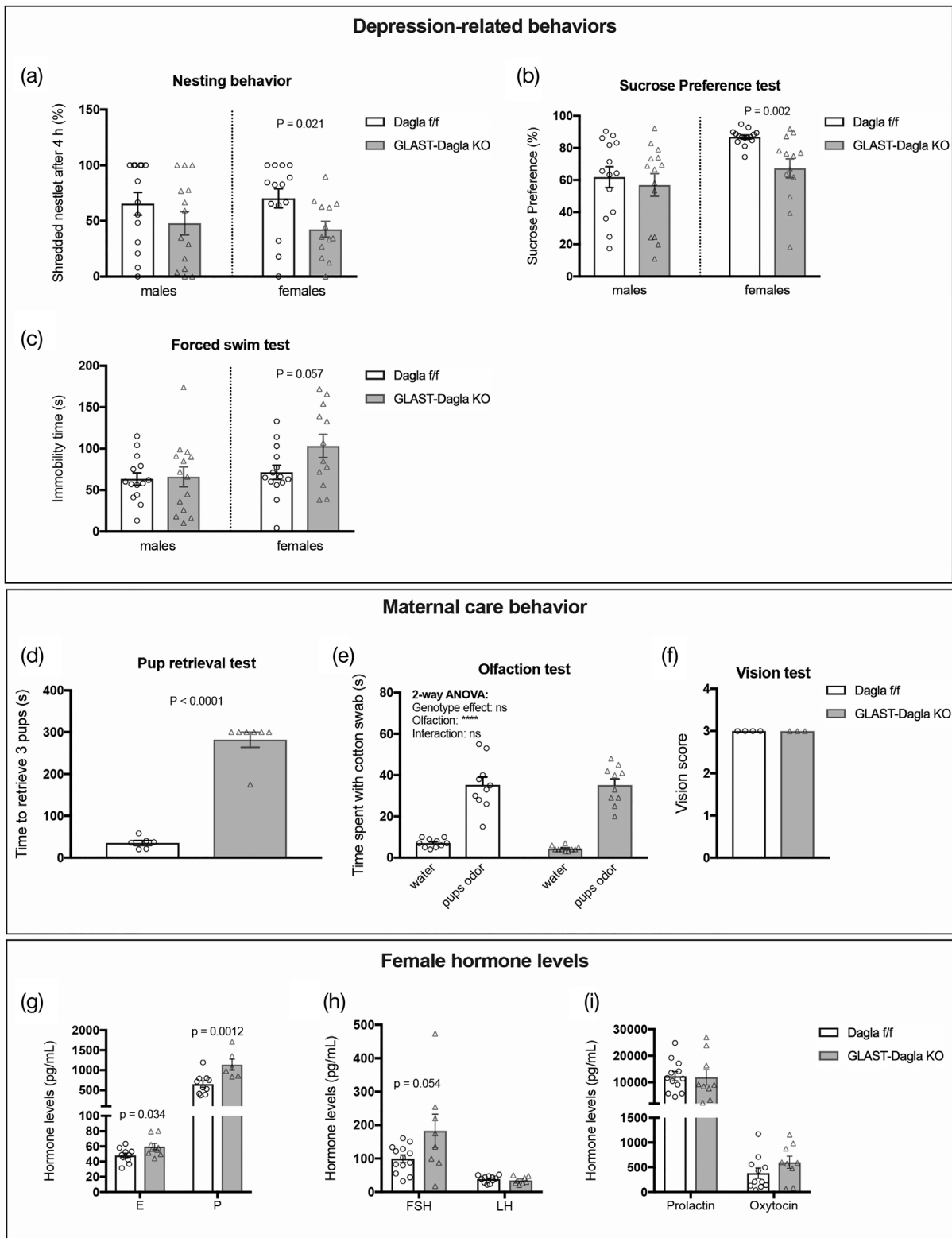
FIGURE 5 Legend on next page.

dams (Figure 5d). Sensory tests with GLAST-Dagla KO dams were conducted to determine if the maternal care phenotype was due to impaired vision or olfaction. Both, female GLAST-Dagla KO mice and controls were able to smell pups (Figure 5e; two-way ANOVA:

$p < .0001$; no genotype effect) as well as urine from the other gender (Supplemental Figure 6). GLAST-Dagla KO and control mice also had normal vision; with all mice receiving a score of 3/3 (Figure 5f). Please note that our previous analysis of constitutive Dagla KO mice

revealed that hearing is not impaired in mice lacking *Dagla* (Jenniches et al., 2016). Noninduced GLAST-Dagla (GLAST-CreERT2 x Dagla f/f) mice retrieved the pups normally (data not shown, *n* = 3).

Our findings therefore strongly indicate that astrocytic DAGLa plays an important role in maternal care behavior by modulating the lipid network.

3.5 | Hormonal changes in female GLAST-Dagla KO mice

Given the interesting sex-dependent behavioral phenotypes in GLAST-Dagla KO mice, circulating female reproductive hormones were analyzed (Figure 5g–i). Thus, blood serum of GLAST-Dagla KO and control mice was collected during the behavioral estrus (proestrus and estrus). Using multiplex assays (Luminex platform), we found that progesterone and estradiol levels were significantly elevated in mice lacking DAGLa in astrocytes (Figure 5g). Also, the levels of follicle-stimulating hormone (FSH) showed a trend to be upregulated (*p* = .054) in female GLAST-Dagla KO mice compared to Dagla f/f control mice (Figure 5h). Oxytocin and prolactin levels (Figure 5i) as well as lutenizing hormone (LH) levels (Figure 5h) were similar in GLAST-Dagla KO and control mice. This finding indicates that DAGLa in astrocytes is involved in the regulation of female reproductive hormones.

4 | DISCUSSION

The enzymes involved in the biosynthesis and degradation of endo-cannabinoids have emerged as important targets for the treatment of psychiatric and neurological disorders (Cristino, Bisogno, & Di Marzo, 2019). In this study we have combined a conditional *Dagla* knockout mouse line, the mRNA detection method RNAscope, and LC–MS/MS to assess the role of astrocytic DAGLa in 2-AG production and associated arachidonate remodeling in the context of neuro-psychiatric disorders. Our results show that Dagla is expressed in astrocytes of the adult mouse brain. Astrocytic DAGLa only produces a small percentage of the overall steady-state brain 2-AG and

associated lipids, but this contributes to the regulation of normal affective behaviors and appears to be critically important for maternal care behaviors.

This finding thus further highlights the importance of endo-cannabinoid signaling in astrocytes, which has only been recognized recently, because the vast majority of the endocannabinoid receptor CB1, like DAGLa, is localized in neurons (Oliveira da Cruz, Robin, Drago, Marsicano, & Metna-Laurent, 2016). Nevertheless, CB1 is clearly expressed in astrocytes in situ (Rodriguez, Mackie, & Pickel, 2001) and despite their low expression levels, these receptors modulate critical aspects of neuron-astroglial interactions, as shown by a number of physiological and genetic studies (Han et al., 2012; Navarrete et al., 2014; Marta Navarrete & Araque, 2008; Robin et al., 2018; Walter & Stella, 2003). Thus, activation of astrocytic CB1 receptors triggers Ca^{2+} transients in astrocytes, stimulates the release of gliotransmitters and regulates carbohydrate and lipid metabolism. Interestingly, studies investigating the localization of CB1 and DAGLa in astrocytes of the spinal cord and the developing mouse brain indicate that both proteins are localized in close proximity (Hegyi et al., 2018; Oudin, Hobbs, & Doherty, 2011; Rodriguez et al., 2001).

Using a highly sensitive expression analysis (RNAscope technology), we show that approximately 40%–70% of the astrocytes positive for the pan-astrocytic marker *Aldh1l1* express *Dagla*. On the other hand, *Dagla* mRNA was detected in almost all neurons analyzed. This indicates that Dagla expression is restricted to a sub-population of astrocytes, but it is currently unclear what the cellular characteristics of this sub-population are. Similar to CB1 receptors, the expression level of *Dagla* in the mouse brain astrocytes was low, only about 10% of that of neurons. mRNA signals were often observed outside the cell body, indicating that DAGLa may belong to proteins that are locally translated in distal processes, as it has been shown for other astro-cytic proteins (Boulay et al., 2017; Sakers et al., 2017). It is thus possible that we have underestimated the amount of *Dagla* transcribed in astrocytes since the fine astrocytic processes could not be identified.

To address the significance of astrocytic 2-AG production and associated arachidonate remodeling, we have generated conditional knockout mice lacking DAGLa in astrocytes: inducible GLAST-Dagla KO mice. Our RNAscope results showed that the deletion of *Dagla* in the GLAST-Dagla KO mice was specific to astrocytes, affecting up to

FIGURE 5 Analysis of depression-like and maternal care behavior. (a) Nest-building behavior was unchanged in male GLAST-Dagla KO mice compared to Dagla f/f littermate controls (*p* = .2245). Female GLAST-Dagla KO mice showed significantly decreased nest building. (b) Anhedonia assessed by the sucrose preference test was not changed in GLAST-Dagla KO males (*p* = .6124), whereas female GLAST-Dagla KO mice showed reduced sucrose preference compared to female controls. (c) Female GLAST-Dagla KO mice displayed a tendency for increased immobility in the forced swim test compared to controls, but there was no change in the male group. Mean ± *SEM*; *n* = 14 animals/group. (d) GLAST-Dagla KO dams showed impaired maternal care behavior in the pup retrieval test; the time to retrieve three pups was significantly increased. Six of seven GLAST-Dagla KO mothers did not retrieve the pups during the 5-minute testing period, whereas tamoxifen-injected Dagla f/f control dams retrieved the pups immediately. Please note that noninduced GLAST-Dagla KO mice retrieved the pups normally (data not shown). (e) GLAST-Dagla KO mice spent significantly more time with the scented cotton swab (discrimination between water/pup odor: $F_{1,18}$ = 146.6, *p* < .0001; genotype effect: $F_{1,18}$ = 0.3281, *p* = .5739), suggesting proper olfaction. (f) Vision was not affected by GLAST-Dagla KO; all the mice were graded with the best score (3). (g) The serum levels of estradiol (E) and progesterone (P) were significantly increased in female GLAST-Dagla KO mice compared to tamoxifen-injected Dagla f/f control mice. (h) Follicle-stimulating hormone (FSH) levels were slightly increased in the serum of female GLAST-Dagla KO mice (*p* = .054), whereas levels of lutenizing hormone (LH) were similar between the groups. (i) Serum levels of prolactin and oxytocin were unchanged in female GLAST-Dagla KO mice. Mean ± *SEM*; *n* = 8–10 animals/group; Student's *t*-test

50% of this cell population. This was confirmed with tdTomato reporter mice, which also showed that 37%–79% of S100beta positive astrocytes express Cre, with no expression in neurons.

Subsequently, we measured the levels of lipids involved in the DAGLa cascade from different brain regions in our astrocytic Dagla KO mice to assess the impact of astrocytic *Dagla* on 2-AG production and connected arachidonate remodeling. In female, but not male GLAST-Dagla KO animals, a slight reduction in 2-AG and arachidonic acid (AA) levels was detected. Considering the fact that astrocytes are as numerous as neurons in the brain, these results strongly suggest that the contribution of astrocytes to steady-state brain 2-AG and AA levels is relatively small compared with that of neurons. This finding is in good agreement with the modest expression levels of *Dagla* mRNA in astrocytes in comparison to neurons.

Because astrocytes have recently been proposed to be important in the etiology of depression (Wang, Jie, Liu, Yang, & Gao, 2017), we focused the behavioral analysis of astrocytic Dagla KO mice on affective behaviors. Based on the targeted lipidomics data, we expected only a mild phenotype of GLAST-Dagla KO mice. No anxiety-related phenotype was found indicating that anxiety might be modulated rather by neuronal, than astroglial, *Dagla* deletion (Jenniches et al., 2016; Shonesy et al., 2014b).

Conversely, GLAST-Dagla KO showed alterations in tests for depressive-like behavior. Female, but not male, GLAST-Dagla KO mice, displayed reduced nest-building behavior, anhedonia in the sucrose preference test and a tendentially enhanced immobility in the forced swim test (p = .07). Most strikingly, GLAST-Dagla KO mice showed a profound deficit in the pup retrieval test compared to tamoxifen-injected Dagla f/f control dams. This phenotype was previously observed in complete *Dagla* knockouts (Jenniches et al., 2016). In contrast, Syn-Dagla mice, with a *Dagla* deletion in neurons, retrieved their pups normally (unpublished data). Thus, the maternal care phenotype can be attributed to the lack of *Dagla* in astrocytes. It should be noted that all mice used in behavioral tests, other than that assessing maternal behavior, were derived from noninduced breedings, wherein both *Dagla* alleles were functional in both parents. This breeding scheme excluded the possibility that the maternal care phenotype interfered with the anxiety or depressive-like behavior of the offspring. These findings also suggest that the deletion of *Dagla* from astrocytes did not cause a general disturbance in brain functions, but rather affected specific mouse behaviors.

An alternative possible mechanism for the depressive phenotype in astrocyte-specific KO lines could be a deletion of *Dagla* in neural progenitor cells. All available astrocyte specific promoters, including the GLAST, GFAP and Aldh1l1 promoters, are active in neural progenitor cells of adult mice (Foo & Dougherty, 2013; Mori et al., 2006). It is therefore possible that deletion of *Dagla* in these cells modulates affective behaviors (Gao et al., 2010; Jenniches et al., 2016; Medina & Workman, 2018; Miller & Hen, 2015). To exclude this alternative hypothesis an inducible Cre-deleter strain that is exclusively active in neuronal progenitor cells would be required. To the best of our knowledge, such a mouse strain is currently not available.

In the light of sex differences in the occurrence of depression-related disorders in humans, with higher prevalence of depression in women (Lim et al., 2018), it was noteworthy that only female GLAST-Dagla KO mice displayed depressive-like behavior and a corresponding slight decrease of 2-AG levels, indicating sex differences in the amount and effects of 2-AG produced by astrocytes. Thus, it is conceivable that sex-related differences in the ECS, in addition to other endocrine differences, contribute to the causation of depression.

Measuring female reproductive hormones during behavioral estrus, we found increased estrogen and progesterone levels in female GLAST-Dagla KO mice. Interestingly, studies have shown that THC use inhibits gonadotropin-releasing hormone expression, thus leading to decreased levels of female hormones in cannabis users and deregulation of the ovarian cycle (Brents, 2016). Since the onset of maternal care at birth results from the interactions of multiple hormones, a deregulation of those hormones this might contribute to the impaired maternal care behavior.

The observed maternal care deficits and depression-like behavior also fit in the framework of postpartum depression (PPD) (Di Florio & Meltzer-Brody, 2015; McEvoy, Osborne, Nanavati, & Payne, 2017). GLAST-Dagla KO mice might be useful for studying sexual dimorphism in depressive states.

Although we cannot exclude the possibility that the behavioral phenotypes are due to changes in the arachidonate lipidome, it seems reasonable to speculate that they are primarily caused by an impaired production of 2-AG. Correspondigly, most of the phenotypes observed in this study after *Dagla* deletion in astrocytes, were also observed in CB1 knockout mice (Haller, Bakos, Szirmay, Ledent, & Freund, 2002; Poncelet, Maruani, Calassi, & Soubrié, 2003; Valverde & Torrens, 2012). Furthermore, the pharmacological blockade of CB1 by rimonabant in dams had the same effect on maternal care (Schechter, Pinhasov, Weller, & Fride, 2012) and pharmacological blockade of CB1 receptors increased the occurrence of depressive symptoms in humans (Sam, Salem, & Ghatei, 2011). Therefore, behavioral changes observed in Dagla KO mice could have resulted from the disruption of CB1 receptor activation by the lack of 2-AG. It is interesting to note that reduced basal 2-AG levels have also been demonstrated in patients with depression and posttraumatic stress disorder (Hill et al., 2013, 2009; Hillard & Liu, 2014), which is consistent with the possible involvement of 2-AG in these disorders. On the other hand, non-targeted lipidomics in patients with MDD have also shown changes in the DAGLa substrate diacylglycerol (Liu et al., 2016). Furthermore, depression in humans is associated with decreased levels of the arachidonate metabolite prostaglandin D2 (PGD2). This finding in humans was substantiated by the inhibition of PGD2 biosynthesis in mice, which also produced depression-like behavior (Chu et al., 2017). Thus, changes in the lipid signaling network from DAGLa deletion probably converge to influence depressive-like and maternal behaviors.

To summarize this work, our data demonstrates that Dagla is expressed in a subpopulation of astrocytes and that *Dagla* expression in astrocytes is generally lower than in neurons. Although astrocytic

DAGLa only accounts for a minor fraction of overall brain 2-AG levels and specific knockout of *Dagla* in astrocytes leads to small changes in the arachidonate lipid network, it nevertheless constitutes a hitherto unknown metabolic axis in the ECS. Our data strongly suggest that minor and cell-specific changes in the extended ECS can lead to strong behavioral effects that seem to be regulated by female hormones, contributing to the understanding of psychiatric disorders such as depression.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Data are available from authors upon request.

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