Archival Report

Biological Psychiatry

Anxiety, Stress, and Fear Response in Mice With Reduced Endocannabinoid Levels

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ABSTRACT

BACKGROUND: Disruption of the endocannabinoid system through pharmacological or genetic invalidation of cannabinoid CB_1 receptors has been linked to depression in humans and depression-like behaviors in mice. The two main endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol (2-AG), are produced on demand from phospholipids. The pathways and enzymes involved in endocannabinoid biosynthesis thus play a major role in regulating the activity of this system. This study investigates the role of the main 2-AG producing enzyme diacylglycerol lipase α (DAGL-α).

METHODS: We generated and used knockout mice lacking DAGL-α (*Dagla*$^{-/-}$) to assess the behavioral consequences of reduced endocannabinoid levels in the brain. We performed different behavior tests to determine anxiety- and depression-related behavioral changes in *Dagla*$^{-/-}$ mice. We also analyzed expression of genes related to the endocannabinoid system via real-time polymerase chain reaction and used the mitotic marker 5-bromo-2′-deoxyuridine to analyze adult neurogenesis.

RESULTS: *Dagla*$^{-/-}$ animals show an 80% reduction of brain 2-AG levels but also a reduction in cortical and amygdalar anandamide. The behavioral changes induced by *Dagla* deletion include a reduced exploration of the central area of the open field, a maternal neglect behavior, a fear extinction deficit, increased behavioral despair, increased anxiety-related behaviors in the light/dark box, and reduced hippocampal neurogenesis. Some of these behavioral changes resemble those observed in animals lacking the CB_1 receptor.

CONCLUSIONS: Our findings demonstrate that the deletion of *Dagla* adversely affects the emotional state of animals and results in enhanced anxiety, stress, and fear responses.

Keywords: Anxiety, Cannabinoids, *Dagla*, Depression, Fear extinction, Stress

http://dx.doi.org/10.1016/j.biopsych.2015.03.033

The endocannabinoid system (ECS) is a retrograde feedback signaling system that plays an important, pro-homeostatic role in the central nervous system. On release into the synaptic cleft, the endogenous cannabinoids can bind and activate presynaptic cannabinoid receptors (1). Diacylglycerol lipase (DAGL) enzymes are responsible for the generation of 2-arachidonoyl glycerol (2-AG), one of the main endocannabinoids. Two isoforms, termed DAGL-α and DAGL-ß, have been described, which are encoded by the *Dagla* and *Daglb* genes, respectively. Whereas DAGL-α is expressed at high levels during neuronal development and in the adult central nervous system, DAGL-β is less abundant in the adult brain (2). During brain development, both enzymes are present on axonal tracts and—together with the cannabinoid receptor CB_1—on growth cones of growing neurons, implicating an important function of the ECS in axon guidance and path-finding (2,3). In the adult brain, DAGL-α expression is restricted to the postsynaptic dendritic compartment (2). Several reports indicate a function of DAGL-α also during adult neurogenesis in the subventricular zone and hippocampus (4,5). DAGL-α is also the main enzyme involved in the production of 2-AG in the context of the endocannabinoid-mediated retrograde synaptic suppression, because depolarization-induced suppression of inhibition is completely absent in *Dagla*$^{-/-}$ mice (6). For a brief period from 2006 until 2008, rimonabant, a CB_1 antagonist, was clinically used in Europe under the trade name Acomplia (Sanofi-Aventis, Paris, France) to treat obesity. However, the compound caused serious adverse psychiatric side effects including anxiety and depression (7), which led to a withdrawal from the market. These clinical findings validated previous results from rodent studies showing that the genetic or pharmacological blockade of CB_1 increases depression- and anxiety-like behavior (8,9). Depression is a highly prevalent psychiatric disorder with a lifetime risk of approximately 20% and is associated with high levels of morbidity and mortality (10). Additionally, it has been determined to be a risk factor for many illnesses, including obesity and cardiovascular and neurodegenerative diseases (11–13). A major environmental factor, which elicits anxiety disorders and depression, is stress (14,15). Repeated homotypic stress leads to an elevation of 2-AG levels in cortical brain regions, which is supposedly mediated through an increased synthesis of 2-AG (16). These mechanisms are thought to be involved in the pathophysiology of depression and fear extinction. In this study, we analyzed the behavioral phenotype of mice with a disruption of *Dagla* and report the development of increased stress- and

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 ISSN: 0006-3223

depression-related behaviors in *Dagla*$^{-/-}$ mice. Therefore, we generated *Dagla*$^{-/-}$ mice via the Cre/loxP System, which allows us to generate constitutive as well as different cell type-specific *Dagla*$^{-/-}$ mice.

METHODS AND MATERIALS

Animals

The *Dagla* gene was mutated by inserting two loxP sites (ENSMUSG00000035735 flanking exon 1 allowing Cre-mediated excision of the exon, as depicted in Figure 1A. The recombinant and wild-type alleles were identified by means of Southern analysis after Stu I digestion with a specific probe (primer_fw: 5′-AGGAGGATTGGCCTCTGTT-3′; primer_rev: 5′-TGGACGTCGTGACTTATGGA-3′) as 1.9 kb (mutant) or 3 kb (wild-type) fragments, respectively. To obtain constitutive *Dagla*$^{-/-}$ mice, homozygous *Dagla* floxed mice (C57BL/6J genetic background) were bred with Pgk1-Cre mice (17) and subsequently to C57BL/6J animals. Male and female constitutive *Dagla* deficient mice (*Dagla*$^{-/-}$, see Supplemental Information) and wild-type controls (WT) on a C57BL/6J genetic background were group-housed (4–5 animals per cage) in standard cages (light phase for 12 hours, light on at 09:00 AM, except for the home cage activity measurements), with free access to food and water. *Dagla*$^{-/-}$mice were maintained by homozygous breeding. Behavioral experiments were performed with mixed-sex groups and approved by a Local Committee for Animal Health, the North Rhine-Westphalia State Environment Agency.

Behavioral Analysis

All behavioral experiments were performed during the light-phase at least two times with different cohorts of animals.

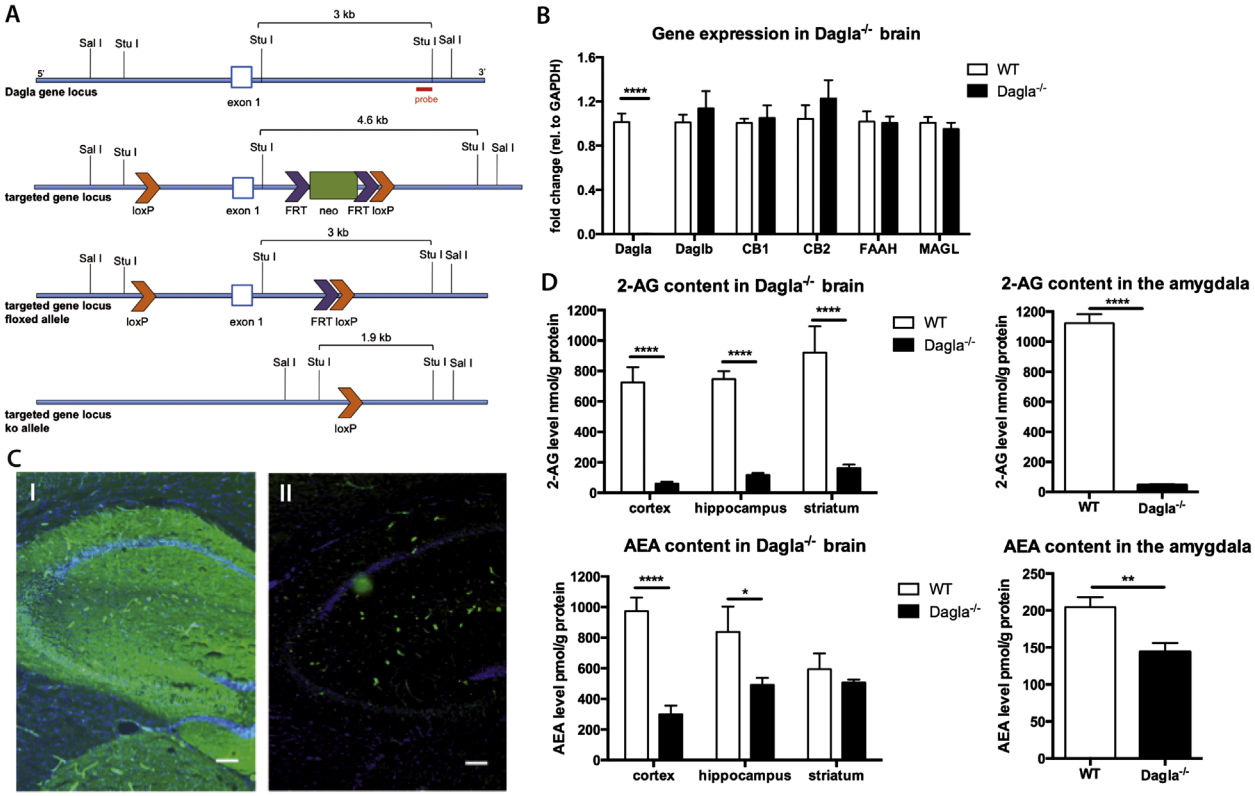

Figure 1. Cloning strategy and validation of *Dagla* knockout mice. **(A)** An 8.4-kb fragment flanking the first exon of the *Dagla* gene was introduced into a minimal vector by homologous recombination. One loxP site (orange) was inserted upstream of exon 1. At the 3′ end of the first exon, a neo-cassette flanked by FRT sites (purple) and with an additional loxP site was inserted into the targeting vector for selection of embryonic stem cells. Subsequently, the neo-cassette was removed by FLP-mediated recombination. To obtain constitutive *Dagla*$^{-/-}$ mice (C57BL/6J genetic background), homozygous floxed *Dagla* mice were cross-bred with the Pgk1-Cre mouse line. **(B)** mRNA levels normalized to GAPDH. Real-time PCR results show a complete loss of *Dagla* mRNA transcript in *Dagla*$^{-/-}$ mice (two-way ANOVA, Bonferroni post-hoc test, values represent mean ± SEM: $n = 5$ animals/group, $^{****}p < .0001$). **(C)** Representative DAGL-α immunostainings of (I) wild-type and (II) *Dagla*$^{-/-}$ mice hippocampi. Staining is completely absent in *Dagla*$^{-/-}$ brain (scale: 100 μm). **(D)** 2-AG level is significantly decreased (by 80–90%) in cortex, hippocampus, striatum, and amygdala of *Dagla*$^{-/-}$ mice. AEA levels are decreased in cortex by 60%, hippocampus by 30%, and amygdala by 25% but not in the striatum of *Dagla*$^{-/-}$ mice. Note that the amount of 2-AG and AEA in the amygdala was measured in a separate experiment. Statistical analysis for eCB measurement in cortex, hippocampus, and striatum: two-way ANOVA, Bonferroni post-hoc test, values represent mean ± SEM: $n = 5$ animals/group, $^{*}p < .05$, $^{**}p < .01$, $^{****}p < .0001$. Statistical analysis for eCB measurement in amygdala samples: Student *t* test, values represent mean ± SEM: $n = 10$ animals/group, $^{*}p < .05$, $^{**}p < .01$, $^{****}p < .0001$. AEA, anandamide; ANOVA, analysis of variance; FAAH, endocannabinoid-degrading enzyme fatty acid amide hydrolase; FLP, flippase; FRT, flippase recognition target; DAGL, diacylglycerol lipase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAGL, monoacylglycerol lipase; PCR, polymerase chain reaction; WT, wild type; 2-AG, 2-arachidonoyl glycerol.

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