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Differential Roles of Diacylglycerol Lipase (DAGL) Enzymes in Nicotine Withdrawal

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1. INTRODUCTION

Smoking continues to be a major worldwide health problem. Nicotine, a natural alkaloid of tobacco, is largely responsible for initiation and maintenance of tobacco dependence (Benowitz, 2010). Although many cigarette smokers report a desire to quit smoking, few are successful despite the current availability of pharmacotherapies, due to the limited effectiveness of these treatments for many patients (George and O'Malley et al. 2004). Increased understanding of the neurobiological systems involved in nicotine intake and reward may uncover new and more efficacious therapies for nicotine cessation. Preclinical animal models and clinical studies reveal that the endocannabinoid system (ECS) regulates nicotine reinforcement and dependence and thus offers several potentially viable therapeutic targets. For example, the cannabinoid receptor type-1 (CB1) antagonist/inverse agonist rimonabant prolonged abstinence rates in smokers expressing motivation to quit (Le Foll et al. 2008) and reduced nicotine self-administration and preference in rodents (Merritt et al. 2008; Cohen et al. 2005). Multiple rodent studies indicate an important role for 2-arachindonoylglycerol (2-AG), a primary endogenous CB1 ligand, as a mediator for nicotine-dependent behaviors. Nicotine-induced increases of 2-AG formation in the ventral tegmental area likely contribute to nicotine-induced behavioral responses (Buczynski et al. 2013). Furthermore, chemical inactivation of monoacylglycerol lipase (MAGL), the primary 2AG catabolic enzyme, reduced nicotine conditioned place preference (Muldoon et al. 2020) as well as both somatic and affective withdrawal signs in nicotine dependent mice (Muldoon

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et al. 2015) through a CB1-dependent mechanism of action. Collectively, these results suggest an important role of 2-AG signaling during nicotine reinforcement and dependence. Paradoxically, an inhibitor of 2-AG biosynthesis enzymes, diacylglycerol lipase-α (DAGL-α) and DAGL-β, was reported to reduce nicotine self-administration in rats (Buczynski et al. 2016). However, the distinct roles of DAGL-α and DAGL-β during nicotine withdrawal has not been established. In this report, we used DAGL-α and DAGL-β transgenic mice to examine the relative contribution of each of these enzymes during nicotine withdrawal.

2. MATERIAL AND METHODS

2.1. Animals

Subjects consisted of adult male and female DAGL-α KO and WT mice and DAGL-β KO and WT mice on a mixed 99% C57BL/6 and 1% 129/SvEv background. The derivation of these mice was conducted by Hsu et al. (2012), and heterozygous DAGL-α and β mice breeding pairs were originally generated in the Cravatt laboratory and transferred to Virginia Commonwealth University. All mice (age 8–10 weeks) were pair-housed under a 12 h light/12 h dark cycle (06:00 to 18:00 h), at a constant temperature (22°C) and humidity (50%–60%), with food and water available ad libitum. The study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out following the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All mice were observed for general well-being and their weight was measured daily.

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt [(−)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] was purchased from Sigma-Aldrich (St Louis, MO), and was dissolved in physiological saline (0.9% sodium chloride). All doses of nicotine refer to the free-base form.

2.3. Nicotine dependence protocol

Male and female mice were anesthetized by inhaling isoflurane/oxygen vapor mixture (1–3%). Alzet osmotic minipumps (model 2000; Alzet Corporation, Cupertino, CA) were then implanted subcutaneously on the dorsum. The minipumps were kept at a constant flow rate to deliver 24 mg/kg/day of nicotine bitartrate or saline for 14 days (1 mg/kg nicotine was released per hour) which is known to maintain stable nicotine levels and elicit nicotine withdrawal signs upon removal (Damaj et al. 2003; Jackson et al. 2008).

2.4. Spontaneous nicotine withdrawal

On the evening of day 14, minipumps were removed and withdrawal signs were observed and scored on day 15 (i.e., 18–24 h after minipump removal). An observer blinded to the experimental treatment conducted the behavioral testing in a battery of *in vivo* assays optimized to detect consistent results with minimal within-group variability (Jackson et al. 2008). Mice were first evaluated for 5 min in the light-dark box (LDB) test for avoidance of the illuminated chamber [i.e., the total time spent in the light compartment 5 min test period was recorded using a video monitoring technique and ANY-MAZE software (Stoelting Co., Wood Dale, IL)] and for locomotor activity via recording of number of transitions between

the light and dark side. Following the LDB test, somatic withdrawal signs were measured during a 20-min observation. The total number of signs was summed, which included paw and body tremors, head shakes, backing, jumps, curls, and ptosis. Hyperalgesia was evaluated in the hot-plate test (52°C) immediately following the somatic sign observation period. Finally, mice were assessed in a two-bottle choice sucrose preference (2% sucrose concentration) test. Animals were exposed to two 30 ml sipper tubes, one with tap water and the other with 2% sucrose solution and the measurements were taken at 24 h after removal of nicotine or saline minipumps for 24 hours. Sucrose preference (percentage) was calculated as follows: preference = [sucrose solution intake (ml)/total fluid intake (ml)] × 100. For more information on methods please see Hamouda et al. 2021.

2.5. Data analysis

Data were analyzed using the GraphPad software, version 9.3 (GraphPad Software, Inc., La Jolla, CA). All tests were analyzed using 2-way ANOVA with genotype (KO versus WT) and treatment (saline versus nicotine) as the independent variables. The Tukey post hoc test was used when appropriate. Data were initially analyzed using 3-way ANOVA with sex as the third factor, but sex was subsequently collapsed because no significant main effect of sex or interactions of sex were found (Table 1). Data are expressed as the mean ± S.E.M, and P values less than 0.05 were considered significant.

3. Results

3.1. Somatic and affective nicotine withdrawal signs are DAGL-α-dependent

Spontaneous withdrawal was induced in DAGL-α WT and KO mice via removal of nicotine minipumps and withdrawal signs, including somatic, affective, and hyperalgesia were observed. As shown in Fig. 1A, a two-way ANOVA analysis of the LDB time showed a significant Treatment x Genotype interaction [$F(1,36) = 8.513$; $p= 0.006$]. Post-hoc analysis (Tukey) revealed that removal of nicotine minipumps led to increased duration of time spent in the light by DAGL-α KO mice compared with WT mice ($p=0.0036$) However, nicotine treatment did not show an effect on WT saline vs WT nicotine mice ($p=0.2704$). Analyses of the total number of transitions between boxes revealed no significant differences among groups when analyzing for Treatment x Genotype interaction [$F(1,36) = 1.845$; $p= 0.1828$], indicating that locomotor activity changes are not likely to be responsible for any differences seen (Fig 1E). As indicated by a significant Treatment x Genotype interaction [$F(1,36) = 41.11$, $p<0.0001$], DAGL-α deletion prevented spontaneous withdrawal signs following nicotine minipump removal (Fig 1B). In contrast, DAGL-α deletion did not affect thermal hyperalgesia following nicotine minipump removal, as indicated by a significant effect of Treatment [$F(1, 36) = 14.06$; $p=0.0006$], but not Genotype [$F(1, 36) = 0.5035$; $p=0.4826$] or Treatment X Genotype interaction [$F(1, 36) = 0.06587$, $p=0.7989$] (Fig 1C). Finally, 24 hours after minipump removal, DAGL-α WT and KO mice were assessed for sucrose preference for approximately 24 hours (Fig 1D). As revealed by a significant Treatment x Genotype interaction [$F(1, 36) = 6.778$, $p=0.0133$], the nicotine-treated DAGL-α WT mice showed a significant decrease in sucrose preference compared with the other three groups, while the nicotine-treated DAGL-α KO mice showed nearly identical sucrose preference

as the saline-treated DAGL-α KO mice (Tukey post hoc p=0.9891). Sex was subsequently collapsed as there were no significant sex differences found in these analyses (Table 1).

3.2. Nicotine withdrawal thermal hyperalgesia is DAGL-β-dependent

DAGL-β WT and KO mice were assessed in the spontaneous nicotine withdrawal procedure described above. In the LDB test, a main effect of nicotine Treatment [$F(1,36) = 20.06$, $p<0.0001$], but no Treatment X Genotype interaction [$F(1,36) = 0.1822$; $p= 0.6720$] or main effect of Genotype [$F(1,36) = 3.491$; $p= 0.0699$], indicated that nicotine minipump removal resulted in significant decrease in time spent on the light side irrespective of genotype (Fig. 2A). Likewise, DAGL-β deletion did not affect the total number of spontaneous somatic withdrawal signs elicited by nicotine minipump removal (Fig. 2B). There was a main effect of Treatment on somatic signs[$F(1,36) = 87.1$, $p<0.0001$]; however, there was no effect of Genotype [$F(1,36) = 0.95$; $p= 0.33$] or Treatment X Genotype interaction [$F(1,36) = 1.990$; $p= 0.1670$]. In contrast, in the hot plate test, significant effects were found for both treatment [$F(1,36) = 8.430$, $p= 0.0063$] and genotype [$F(1,36) = 5.036$, $p= 0.0311$], though the treatment X genotype interaction failed to achieve statistical significance [$F(1,36) = 1.840$; $p= 0.1834$; Fig. 2C]. Finally, DAGL-β deletion did not affect the sucrose preference test. While a significant main effect of Treatment was found [$F(1,36) = 21.32$, $p<0.0001$], there was no effect of Genotype [$F(1,36) = 0.01311$; $p= 0.9095$] or Treatment X Genotype interaction [$F(1,36) = 0.007477$; $p= 0.9316$; Fig. 2D].

4. Discussion

The results of this study suggest that the two 2-AG biosynthetic 2-AG enzymes DAGL-α and DAGL-β play distinct roles in the mouse spontaneous nicotine withdrawal model. Whereas DAGL-α deletion prevented somatic and affective signs of nicotine withdrawal, hyperalgesia resulting from nicotine minipump removal appears to show some DAGL-β dependency. These findings are consistent with the observations that DAGL-α is highly expressed on neurons and DAGL-β is predominantly expressed on macrophages (Hsu et al. 2012).

Studies show that pharmacological inhibition of both DAGL enzymes via the inhibitor DO34 has no effect on body temperature, antinociception, or locomotor activity (Wilkerson et al., 2017). Similarly, we found no differences in locomotor activity in both types of DAGL KO mice. In our study, somatic signs of nicotine withdrawal see a clear effect of genotype and treatment in DAGL-α KO mice, with a complete reduction in signs when compared to WT, while there was no difference in DAGL-β KO mice when compared to WT. Likewise, we found an interaction between genotype and treatment in DAGL-α mice in the light-dark box test following nicotine withdrawal with a decrease of time spent in the light side in nicotine treated mice, and a reversal in KO mice. However, there was a lack of statistical significance on the effect of treatment in WT mice. While C57BL/6J mice in nicotine withdrawal typically see a decrease in time spent in the light side in this test (Stoker at al. 2008), this was not the case in DAGL-α WT mice on a C57BL/6J background. This could possibly be due to slight genetic differences when comparing a DAGL- α WT mouse to a regular C57BL/6J mouse. On the other hand, there is also evidence that the deletion of

DAGL-α increases anxiety-like and anhedonia-like behaviors in mice (Shonesy et al, 2014), which could have influenced the results.

Our results suggest that DAGL-α is required not only for somatic and anxiety-like signs but also for anhedonia-like aspects of nicotine withdrawal. Previous papers from our lab show that nicotine-treated mice in withdrawal show lower sucrose preference, which starts to recover after 48 hours and is vanished by day 5. However, this effect is eliminated in α6 and β2 KO mice, which shows that the changes in sucrose preference can be modulated by genotype and is not a random phenomenon (Alkhlaif et al. 2017). Other papers have shown that pharmacological inhibition of both DAGL enzymes through DO34 has no effect on sucrose preference (Winters et al. 2021), however one study found a decreased sucrose preference in DAGL-α KO female mice, but not male mice (Shonesy et al. 2014). Conversely, our study suggests that the decrease in sucrose preference in mice undergoing nicotine withdrawal is reversed in DAGL-α genotypic deletion while not in DAGL-β deletion in both sexes, implicating a role of DAGL-α in nicotine withdrawal induced anhedonia-like behaviors in mice.

We found that genetic deletion of DAGL-α had no effect on nicotine withdrawal induced hyperalgesia while there was a reversal of this hyperalgesia in DAGL-β KO mice. Conversely, one study showed an increase in latency in the hot plate test in DAGL-α KO naïve mice (Powell et al. 2015), however our control mice showed no genotypic differences among WT and KO mice. Consistent with the findings reported here, pharmacological inhibition of DAGL-β reduced hyperalgesia responses resulting from intraplantar injection of pro-inflammatory lipopolysaccharide (LPS), sciatic nerve injury, or paclitaxel administration (Wilkerson et al. 2017), as well as spontaneous hyperalgesia that occurs in the Berkeley mouse model of Sickle cell disease (Khasabova et al. 2023). Likewise, genetic deletion of DAGL-β ameliorated LPS-induced hyperalgesia. The current study extends these findings to show that inactivation of DAGL-β mitigates nicotine withdrawal-induced hyperalgesia, suggesting a broader role for this enzyme in nociceptive processing. These findings are consistent with the robust expression of DAGL-β in glia and peripheral blood cells, suggesting an important role for these cell types in this aspect of nicotine withdrawal (Damaj et al. 2003).

Substantial work indicates an important role for DAGL-α in endocannabinoid-mediated short-term synaptic plasticity (Gao et al. 2010; Schurman et al. 2019). The results of the present study link a role of this enzyme to physical, anxiogenic, and anhedonic effects of nicotine withdrawal. The observations that DAGL-α blockade results in reduced CNS levels of 2-AG (Murataeva et al. 2014) and loss of endocannabinoid-mediated short-term synaptic plasticity (Sidhpura and Parsons 2011), implicate the possibility that reduced CB1 receptor activation may have mediated the findings in the present study. Indeed, genetic deletion or pharmacological inhibition of the CB1 receptor attenuates nicotine conditioned place preference or nicotine seeking behavior (Muldoon et al. 2020). However, the findings that blockade of either the CB1 receptor (Muldoon et al. 2015; Castañé et al. 2002) or the CB2 receptor (Ignatowska-Jankowska et al., 2013) do not affect somatic signs of nicotine withdrawal, suggesting that DAGL-α deletion prevented the occurrence of these behaviors in the nicotine spontaneous withdrawal model through other signaling mechanisms. One

such non cannabinoid receptor mechanism of action is through reduced production of pro-inflammatory eicosanoids within the central nervous system. In particular, 2-AG plays a role in the production of arachidonic acid and its bioactive lipid metabolites (Nomura et al. 2011). Thus, decreased 2-AG production by DAGL-α deletion may result in a dampening of downstream down-stream lipid mediators may facilitate somatic and affective aspects nicotine withdrawal.

A limitation of this study is the use of constituent KO mice when investigating the role of these enzymes in behavioral assays. Results typically need to be interpreted with caution because of the possible compensation or adaptations usually seen with global KO mice. However, no compensation in these DAGL-α and DAGL-β KO models were reported (Reisenberg et al. 2012). Future studies with pharmacological inhibitors of these enzymes will be important to evaluate.

The genotypic differences in this study are unlikely to result from differences in nicotine metabolism, as hyperalgesia (in DAGL-α KO mice) and affective/somatic behaviors (in DAGL-β KO mice) are not affected during nicotine withdrawal, however further pharmacokinetic studies are needed to rule this out. Moreover, our findings do not suggest differences between males and females in the effects of DAGL inactivation (Table 1). Overall, these findings indicate separate roles for DAGL-α and DAGL-β signaling in facilitating the behavioral responses in nicotine withdrawal.

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Highlights:

- Modulation of the endocannabinoid system nicotinic dependence behaviors in rodents
- DAGL-α deletion prevents somatic and affective signs of nicotine withdrawal
- DAGL- β deletion prevents hyperalgesia during nicotine withdrawal

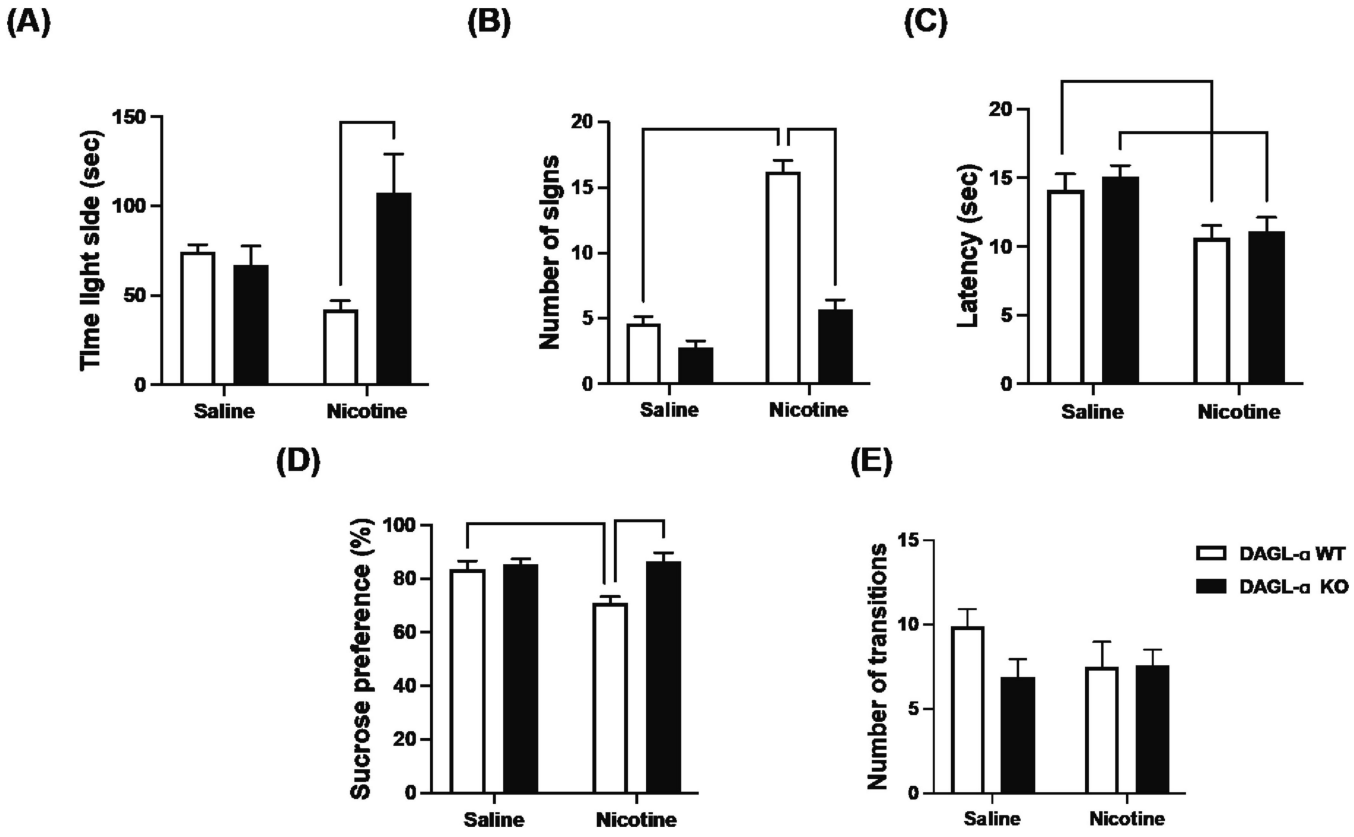

Figure 1. Spontaneous nicotine withdrawal in DAGL-α WT and KO Mice.

DAGL-α WT and KO mice were chronically treated with nicotine (24 mg/kg/day) for 14 days. On day 14, minipumps were removed to induce spontaneous withdrawal. Mice were tested on day 15 for (A) anxiety-like behavior (time spent in light side in light-dark box), (B) somatic signs, (C) hyperalgesia (paw withdrawal latency in hot plate test), and on day 16 for (D) sucrose preference. Locomotor activity (E) was assessed by recording transitions in the light-dark box test. Data are presented as the mean ± SEM of n = 5/sex/group. Statistical significance indicated by *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

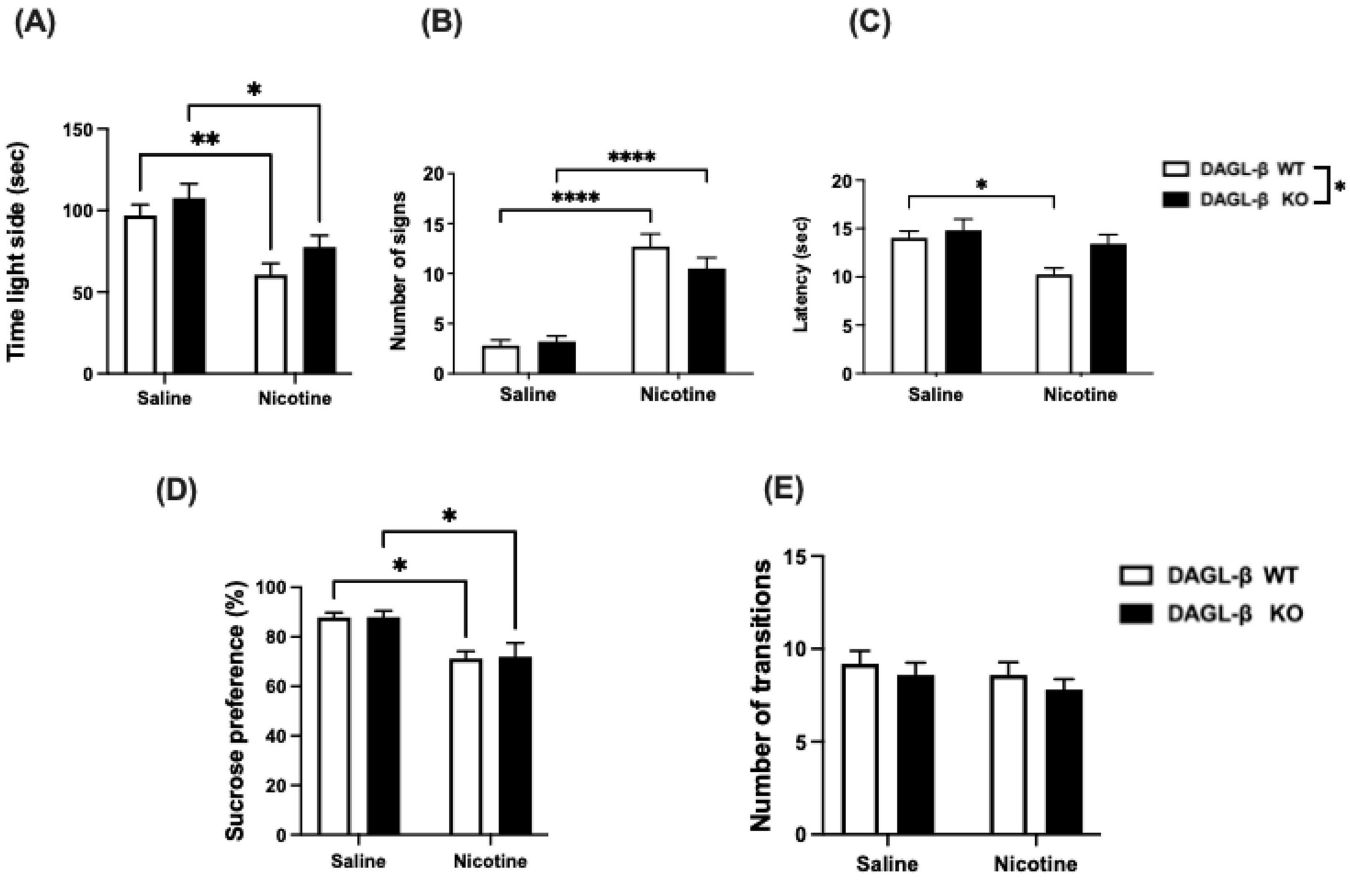

Figure 2. Spontaneous nicotine withdrawal in DAGL−β WT and KO Mice.

DAGL−β WT and KO mice were chronically treated with nicotine (24 mg/kg/day) for 14 days. On day 14, minipumps were removed to induce spontaneous withdrawal. Mice were tested on day 15 for (A) anxiety-like behavior (time spent in light side in light-dark box), (B) somatic signs, (C) hyperalgesia (paw withdrawal latency in hot plate test), and on day 16 for (D) sucrose preference**. Locomotor activity (E) was assessed by recording transitions in the light-dark box test.** Data are presented as the mean ± SEM of n = 5/sex/group. Brackets on legend indicate effect of genotype in hyperalgesia assay. Statistical significance indicated by *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Table 1 –

Results of the Three-way ANOVA analyses with sex as a variable

	Test	F-value	P-value
DAGL-α	Light-dark box	$F_{sex}(1,16) = 0.136$	P=0.716
	Somatic signs	$F_{sex}(1,16) = 2.329$	P=0.146
	Hot plate	$F_{sex}(1,16) = 0.002$	P=0.960
	Sucrose preference	$F_{sex}(1,16) = 0.0002$	P=0.987
	Locomotor activity	$F_{sex}(1,16) = 2.598$	P=0.126
DAGL-β	Light-dark box	$F_{sex}(1,16) = 3.195$	P=0.092
	Somatic signs	$F_{sex}(1,16) = 2.329$	P=0.146
	Hot plate	$F_{sex}(1,16) = 0.024$	P=0.878
	Sucrose preference	$F_{sex}(1,16) = 0.0001$	P=0.968
	Locomotor activity	$F_{sex}(1,16) = 1.347$	P=0.262

n=5/sex/group