RESEARCH ARTICLE

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The Effect of Inhibition of Perisynaptic Astrocyte Glycogen Utilization on Depression-Like Behavior

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ABSTRACT

Objective: Under physiological conditions, astrocytes produce lactate to meet the increased synaptic energy demand due to neuronal activity. In the light of the findings showing that this process is disrupted in the pathophysiology of major depression, the aim of this study is to investigate the effect of pharmacological inhibition of perisynaptic astrocyte glycogen utilization on anxiety-like and depression-like behaviors in female and male mice.

Methods: In this study, DAB (1,4-dideoxy-1,4-imino-D-arabinitol), which is an inhibitor of glycogen breaking enzyme glycogen phosphorylase, was intrahippocampally administered to 15 female and 14 male Swiss albino mice, while 15 female and 12 male Swiss albino mice received intrahippocampal saline injections. Three and five days after the injections, the anxiety-like and depression-like behaviors of the mice were assessed by locomotor activity, open-field test, light-dark box test, tail suspension test and sucrose preference test.

Results: Three days after injection, neither depression-like nor anxiety-like significant behavioral changes were detected in the male experimental group mice compared to the control group; but an increase in locomotor activity (p=0.05) and time spent in the open-field (p=0.01) were observed on the fifth day. In evaluations of the female experimental group mice on the third and fifth days, depression-like and anxiety-like behaviors were found similar to the control group, as seen in the male mice. The only significant difference in the experimental group female mice was found in the sucrose preference test, which revealed an increased tendency to prefer sucrose (p=0.003) compared to the control group.

Conclusion: The inhibition of glycogen use in the hippocampus by DAB did not affect anxiety-like and depression-like behaviors 3 and 5 days after injection in both female and male mice. The increase in the time spent in the open-field by male experimental group mice was associated not with anxiety, but with increase in the locomotor activity. The fact that no significant difference was observed in the light-dark box test, which is another test used to evaluate anxiety, supported this opinion. The increase seen in the sucrose preference test in female experimental group mice was not interpreted as an increase in hedonic behavior because prevention of glycogen breakdown in the hypothalamus might have homeostatically increased sugar-craving and therefore resulted in an increase in sucrose preference. Different set of tests better targeting the energy and glucose metabolism and applied at farther time points than surgery are recommended for future studies.

Keywords: Glycogen, Depression-like Behavior, Anxiety-like Behavior

INTRODUCTION

Several findings suggest that metabolic processes, including those mediated by glycogen stored in astrocytes, may play an important role in depression pathophysiology. Postmortem examinations of the brains of patients with major depressive disorder (MDD) revealed a decrease in glial cell density in the subgenual prefrontal cortex, orbitofrontal cortex, dorsolateral prefrontal cortex, anterior cingulate cortex, and amygdala (Ongur et al. 1998, Rajkowska et al. 1999, Cotter et al. 2001, Bowley et al. 2002). Studies in the last thirty years have also disclosed astrocytes as major players coupling synaptic activity with energy metabolism through the astrocyte-neuron lactate shuttle (ANLS) and glycogenolysis (Box 1). It is generally agreed that glycogen is the source of a significant amount of lactate released from astrocytes into the extracellular environment, especially in response to increased energy demand (Pellerin et al. 2007). The fact that brain glycogen is located mainly in perisynaptic astrocytic processes, where energy demand is high but mitochondria are scarce, supports this proposition. According to this view,

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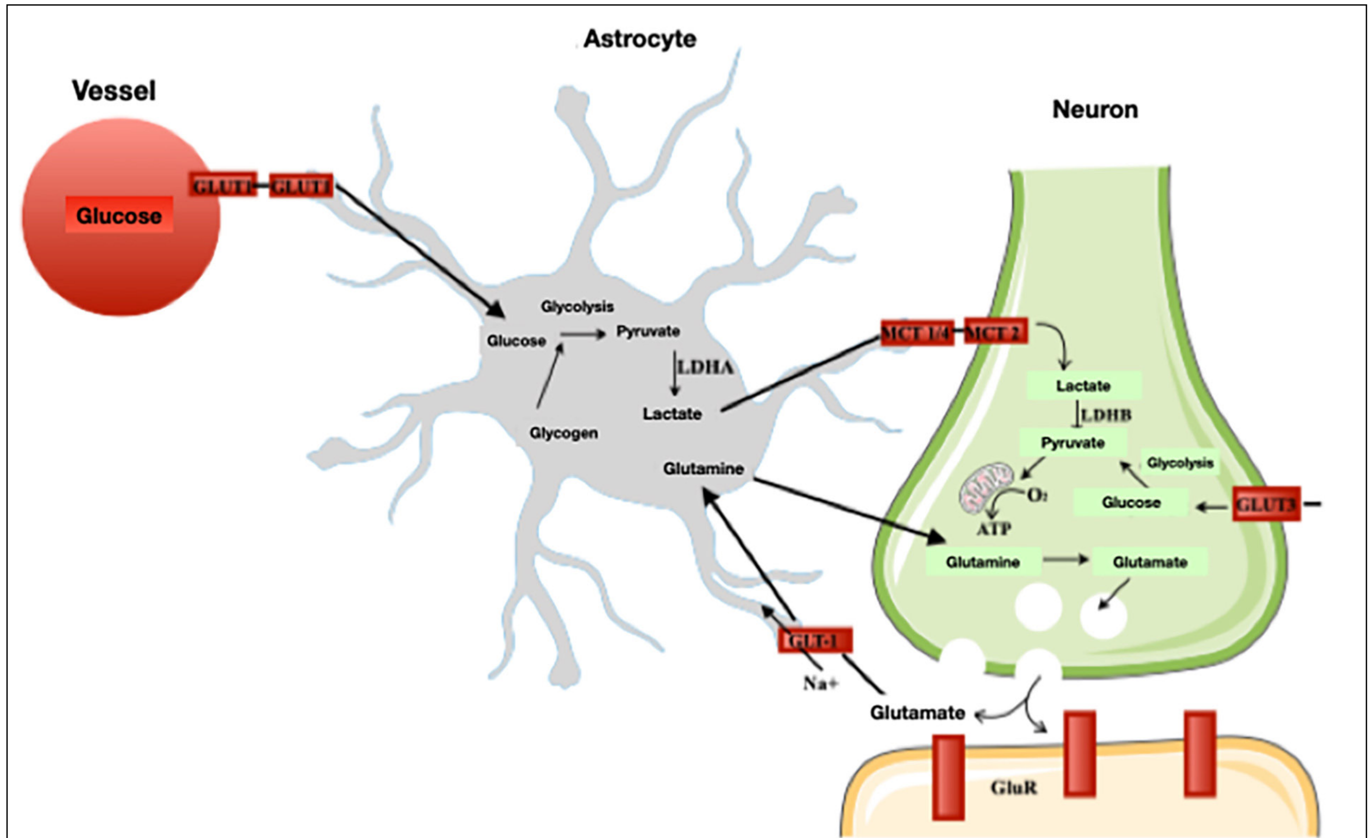

Box 1. Astrocyte-Neuron Lactate Shuttle (ANLS)
Nervous system is responsible for a significant portion of energy consumption in the body. In vivo and in vitro biochemical and imaging studies showed that this metabolic need is predominantly localized at the synapses, therefore synaptic activity needs to be matched with local energy supply (Magistretti and Allaman 2015). In this context, the metabolic coupling between astrocytes and neurons to meet the increased energy need with excitatory synaptic transmission is explained with the astrocyte-neuron lactate shuttle (ANLS) model. According to the model, glutamate, the concentration of which increases in the extracellular space during glutamatergic neurotransmission, is taken up by specific glutamate transporters in astrocytic processes by a sodium ion (Na+)-dependent mechanism. The consequent increase in intracellular Na+ concentration in astrocytic processes activates Na+/K+ ATPase, resulting in ATP consumption. In addition, glutamate in the astrocytes is converted to glutamine by glutamine synthase in order to be recycled back to neurons, and ATP is consumed in this cycle. These energy-consuming processes triggered by excitatory synaptic activity, stimulate glucose uptake from the blood by the glucose transporter GLUT1 located at the astrocytic end-feet on the vessels, and facilitate the conversion of glucose to lactate via the glycolytic pathway to be sent to the synapses. Lactate, which is taken in by the neuronal presynaptic terminals and dendrites via monocarboxylate transporter (MCT) is converted to pyruvate and used for energy production by entering the tricarboxylic acid cycle (Pellerin and Magistretti 1994) (Figure prepared using images from Servier Medical Art. Servier Medical Art is licensed under the Creative Commons Attribution 3.0 Unported License.).
Studies on brain glycogen metabolism have shown that the entry of potassium ions (K+) that accumulated in the extracellular space during neural activity into the astrocyte occurs depending on astrocytic glycogenolysis, and that the uptake of extracellular potassium decreases when energy production through glycogenolysis is inhibited (Xu et al. 2013). Similarly, studies in astrocyte cell cultures have revealed that the uptake of extracellular glutamate by astrocytes is dependent on astrocyte glycogen despite the presence of glucose (Sickmann et al. 2009). All these findings show that glycogenolysis in astrocytic end-feet plays an important role in the maintenance of synaptic neurotransmission by buffering the extracellular K+ ion and glutamate concentration that increases as a result of synaptic activity.

glucose from the circulation is first stored as glycogen, and, when needed, energy is produced nonoxidatively from the glucose moieties that are liberated from glycogen. This insight has been helpful in explaining the PET and fMRI findings pointing to a greater use of glucose than oxygen during increased synaptic activity (aerobic glycolysis, Warburg effect) (Vaishnavi et al. 2010).

Considering the key role of astrocytes in meeting synaptic energy demands, the functional significance of glial loss reported in MDD patients deserves closer evaluation. In fact, stress, which is widely used to model depression in animals, has been shown to increase the extracellular glutamate concentration in the prefrontal cortex, triggering astrocytic glycogenolysis, lactate production and release to be used by active excitatory synapses (Moghaddam 1993, Hascup et al. 2010, Musazzi et al. 2010, Satoh and Shimeki 2010, Iwata. et al. 2016). Consistent with these studies, acute exposure to psychological stressors such as restraint stress or footshock stress has been shown to increase extracellular lactate concentration in the hippocampus, basolateral amygdala, and medial prefrontal cortex (Elekes et al. 1996, Uehara et al. 2003, Uehara et al. 2005, Uehara et al. 2007, Uehara et al. 2013), whereas chronic exposure to stress or corticosterone led to a reduction in hippocampal glycogen levels (Zhang et al. 2015). In addition, suppressing glycogen synthase gene expression in the hippocampus has been reported to decrease

astrocytic size and increase depression-like behavior in rats (Zhao et al. 2017).

Neuromodulators that have a role in MDD pathophysiology and antidepressant drug action, namely noradrenaline, glucocorticoids, serotonin (5HT) and adenosine can regulate glycogen turnover by binding to their respective receptors on astrocytes. Noradrenaline at basal levels stimulates glycogen synthesis by binding to α2-adrenergic receptors. However, it facilitates glycogen degradation via β1-adrenergic receptors in response to increased synaptic activity (Hertz et al. 2007, O'Donnell et al. 2012). Glucocorticoids, on the other hand, facilitate glycogenolysis in astrocytes (Tombaugh et al. 1992) and inhibit the stimulant effect of noradrenaline on glycogen synthesis via type 2 glucocorticoid receptors (Allaman et al. 2004). Serotonin, which plays a role in the mechanism of many antidepressant actions, increases glycogenolysis through the 5HT-2A and 5HT-2B receptors (Quach et al. 1982, Darvesh and Gudelsky 2003). Glycogen is used as a reservoir in the astrocyte-neuron metabolic coupling, and the energy is delivered to the synapses in the form of lactate, as mentioned above. Several studies suggest that lactate may exhibit antidepressant properties and increase resilience to stress. Acute intraperitoneal administration of lactate has been shown to reduce the time spent immobile in the forced swimming test, a measure of behavioral despair in rodents, to an extent similar to the tricyclic antidepressant desipramine (Carrard et al. 2018). Moreover, intraperitoneal administration of lactate four hours prior to social defeat stress has been shown to result in stress resilience and reduced anxiety levels (Karnib et al. 2019).

In this study, we tested the hypothesis that disruption of glycogenolysis, thus synaptic energy delivery from perisynaptic astrocytic processes to neurons would increase anxiety-like and depression-like behaviors. For this purpose, we inhibited glycogen phosphorylase- the enzyme catalyzing the release of glucose-1-phosphate, the rate-limiting step of glycogenolysis (Newgard et al. 1989), by bilateral intrahippocampal DAB (1,4-dideoxy-1,4-imino-d-arabinitol) injections (Walls et al. 2008) and assessed its effects on depression-like and anxiety-like behavior.

METHODS

DAB was administered intrahippocampally to the experimental group, while the control group received intrahippocampal saline injections. To observe the relatively short-term effects of intrahippocampal DAB/saline, we performed a set of behavioral tests three days after the injections, which included locomotor activity/open field test, light-dark box test, tail suspension test and sucrose preference test, respectively. Two days after performing the aforementioned set of behavioral tests, the same tests were performed in the same order to evaluate the behavioral effects of the injection on the fifth day.

Animals and Housing Conditions

Swiss albino mice (n=30 female, n=26 male) weighing between 25 to 40 g were kept in transparent plexiglass cages in a room with a temperature of 22±3°C and humidity of 50-60%. The animals were housed under diurnal lighting conditions (12 hours darkness and 12 hours light) and fed ad libitum.

The experimental procedures in this study were all carried out in accordance with the institutional guidelines and approved by the Hacettepe University Animal Experiments Ethical Committee (2018/42).

Intrahippocampal DAB/saline injections

Animals were anesthetized with isoflurane for the intrahippocampal injection procedure (4% for induction, 1.5-3% for maintenance). Oxygen (2 L/min) was supplied throughout the procedure and body temperature was kept at 37.0±0.1°C with a homeothermic blanket and a rectal probe. Tissue oxygen saturation and pulse rate were monitored with a pulse oximeter. Following induction of deep anesthesia, mice were placed in the stereotactic frame and bilateral burr holes were drilled in the skull over the hippocampal coordinates (2.5 mm posterior, +/-2 mm lateral, and 3.6 mm deep with reference to the bregma) for injections. One and a half μl of DAB (1.5 mM, dissolved in saline) was injected bilaterally using two Hamilton syringes with a 26-gauge needle connected to a microinjection pump. The microinjectors were lowered into the injection area within 8 to 10 minutes by using a micromanipulator and DAB was injected bilaterally into the hippocampi at a rate of 0.15 μl/min. We waited for 1 minute to allow DAB to diffuse into the tissue and slowly removed the injectors in 5 minutes using a micromanipulator. Control group received same volume of saline.

Behavioral Tests

When deciding on the timing of the behavioral tests, we aimed to determine a time frame as far from the anesthesia and surgery as possible to avoid their probable side effects. Therefore we chose to do the behavioral tests on the third and the fifth days after the injection based on the findings of Daniels et al. (2008), which showed that behavioral effects of acute stress was still observed 5 days after stress exposure (Daniels et al. 2008). All the behavioral tests were performed during the light cycle, behavioral experiments being carried out between 13:30-18:30 p.m. on the third day, and between 08:15 a.m.-13:15 p.m. on the fifth day. In order to evaluate behavior within the same circadian period, the mice were divided into five groups and

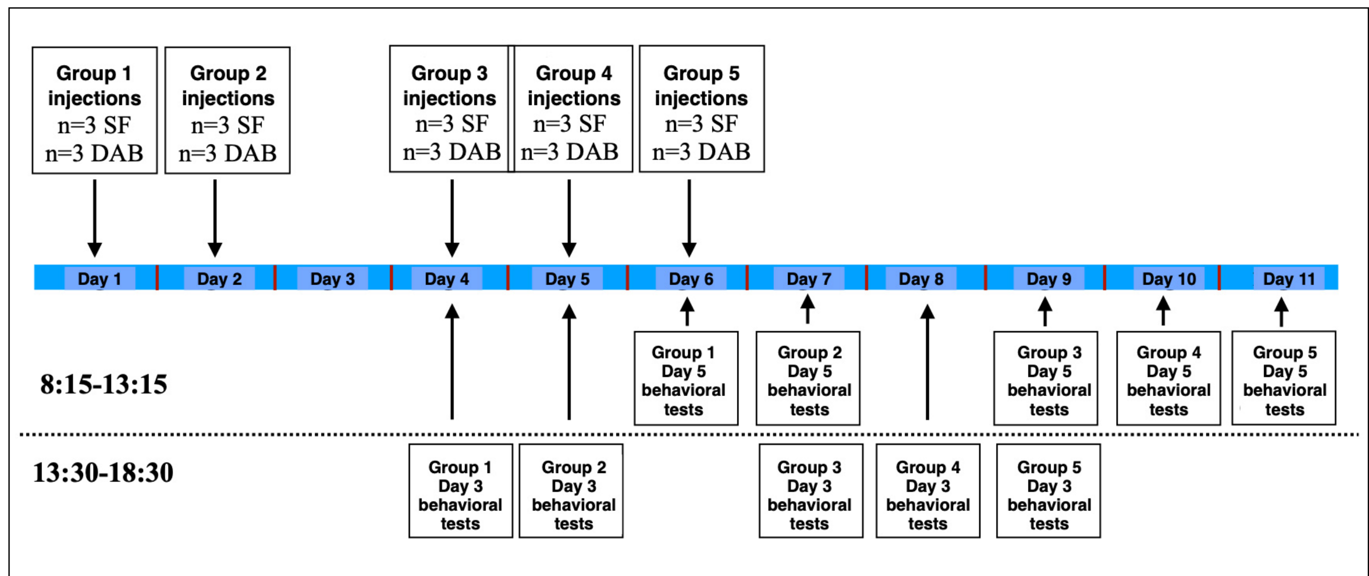

Figure 1. Experimental flow chart. The numbers indicated in the boxes are the number of female mice used in the experiments, the number of male mice is 14 mice injected with DAB (1,4-dideoxy-1,4-imino-d-arabinitol) and 12 mice injected with saline.

the behavioral experiments for each group were initiated on 5 consecutive days (Figure 1). The animals were habituated to the room for 10 minutes before the behavioral testing started (Figure 1). Experimental procedures were carried out 1 month apart for male and female groups.

Locomotor Activity and Open Field Test: The total distance traveled by the mice in a 22.5×22.5×30 cm^3 square box for 10 minutes was tracked with Ethovision XT-8 and used as a measure of locomotor activity. Time spent in the central 11x11 cm^2 area of the same box, i.e. the open field was evaluated as a measure of anxiety-like behavior (Seibenhener and Wooten 2015, Royce 1977). Time spent in the center (open area) was interpreted as low anxiety-like behavior levels, while the time spent at the edges of the box was interpreted as high anxiety-like behavior levels.

Light-Dark Box Test: Animals were put in the dark chamber of a box with a light and a dark compartment, and the latency to the first entry into the light chamber and time spent in the light chamber were recorded as a measure of anxiety-like behavior (Crawley and Goodwin 1980, Takao and Miyakawa 2006).

Tail Suspension Test: Animals were taped from their tails (2-3 mm proximal to the tail tip) to the side of a bench. The time spent struggling and immobile for the last 4 minutes out of 6 minutes was quantified and immobility time was used as a measure of behavioral despair, a depression-like behavior (Steru et al. 1985, Can et al. 2012).

Sucrose Preference Test: Mice were habituated to consuming sucrose by replacing water bottle by two bottles of 1% sucrose solution in their home cages, one day prior to DAB/saline injections. On the third day following the injections, the water bottles were removed from the cages in the morning and the mice were deprived of water for eight hours. The animals were then individually caged and two bottles, one containing tap water and the other containing 1% sucrose solution, were placed in the cages for the test. Half of the bottles with sucrose solution were placed on the right side of the cages, whereas the other half were placed on the left side. After completion of the sucrose preference test, which lasted 12 hours, the consumption of total liquid and sucrose solution was recorded. In normal conditions mice prefer sucrose solution over tap water, therefore sucrose preference index is calculated by dividing sucrose solution intake to total fluid consumption and used as a measure of anhedonia (Brown and Murphy 1973, Liu et al. 2018).

Statistical Analysis

IBM SPSS 20 software was used for statistical analysis. Statistical tests were chosen based on whether the data met parametric assumptions. Locomotor activity, time spent in the light chamber (light-dark box test), time spent in the center (open field test), time spent immobile (tail suspension test), and the ratio of sucrose solution-to-total fluid consumption (sucrose preference test) were assessed by Student t-test. The latency to the first entry into the light chamber in the light-dark box test was evaluated with survival analysis. The effect of locomotor activity change on time spent in the open field was assessed by analysis of covariance (ANCOVA). Changes in locomotor activity, time spent in the open field, and time spent immobile during the tail suspension test at different time points (day 3 and 5) were evaluated by repeated measures ANOVA. A p value <0.05 was considered as statistically

significant. The data are expressed as mean +/- standard deviation in the graphs.

RESULTS

Anxiety-Like Behavior Tests

In the open field test performed on the third day post-injection, no difference was observed in time spent in the center between the control and experimental groups in either sex ($t(26)=0.16$, $p=0.873$ for females; $t(24)=0.10$, $p=0.920$ for males) (Figure 2A). On the other hand, there was an increase in time spent in the center in male mice injected with DAB compared to their respective controls in the open field test performed five days after the injection ($t(20.094)=2.86$, $p=0.01$). There was no statistically significant difference between female control and DAB groups ($t(26)=1.47$, $p=0.155$) (Figure 2B).

On the third day following the injection, locomotor activity was similar in DAB and control groups in both sexes ($t(16.322)=0.71$ for females, $p=0.486$; $t(23)=0.73$, $p=0.472$ for males) (Figure 2C). On the other hand, there was an increase in locomotor activity in male DAB group compared to male control mice five days after the injections ($t(21.456)=2.08$, $p=0.05$). No such difference was observed between the female DAB and control groups ($t(27)=-0.003$, $p=0.997$) (Figure 2D). To rule out the possibility that the increase in time spent in the center in open field test may result from a general increase in locomotor activity, ANCOVA was performed. The time spent in the center was found to be similar between the experimental groups in both female and male mice, when the effect of locomotor activity was controlled ($F(1.24)=1.907$ $p=0.180$ for females; $F(1.22)=2.828$, $p=0.107$ for males).

In repeated measures ANOVA performed to evaluate the effect of repeated behavioral assessments on day 3 and 5 after the injections, there was a significant effect of time in duration in the center in the open field test in both sexes ($F(1.23)=75.366$, $p<0.0001$ for males; $F(1.25)=60.004$, $p<0.0001$ for females respectively). The mean time spent in the center was shorter on the fifth day compared to the third day for both males and females, regardless of the groups. There was no time x group interaction in either sex ($F(1.23)=0.570$, $p=0.458$ for males; $F(1.25)=0.059$, $p=0.810$ for females). Similarly, the effect of time on locomotor activity was significant in both male and female mice ($F(1.23)= 87.522$, $p<0.0001$; $F(1.24)=44.973$, $p<0.0001$, respectively). Locomotor activity was found to be lower on the fifth day compared to the third day for both males and females, regardless of the group. However, there was no time x group interaction in either sex ($F(1.23)=0.903$, $p=0.352$; $F(1.24)=0.779$, $p=0.386$ for males and females, respectively). These findings reveal that both locomotor activity and anxiety-like behavior decrease over time in both sexes, but this effect is not related to the effects of DAB.

In the third day light-dark box test, latency to first entry into the light chamber was similar between the control and DAB groups in both females and males (Kaplan Meier survival analysis; $p=0.197$; $p=0.297$ respectively) (Figure 2E). Time spent in the light chamber also did not show significant differences between the groups in either sex ($t(26)=-1.07$, $p=0.295$ in females; $t(23)=-1.17$, $p=0.254$ in males) (Figure 2F). On the fifth day post-injection, no difference was observed in latency to first entry into the light chamber between the control and DAB groups in both females and males ($p=0.342$, $p=0.457$) (Figure 2G). Time spent in the light chamber was also found to be similar between DAB and their respective control groups in both sexes ($t(20.418)= -1.24$, $p=0.230$ in females; $t(24)=-0.64$, $p=0.528$ in males) (Figure 2H).

Depression-like Behavior Tests

In this study, the tail suspension test and the sucrose preference test were used to evaluate depression-like behavior in mice. Immobility times in tail suspension test on the third day post-injection were not statistically significant between the control and DAB groups in females and males ($t(24.076)=-1.25$, $p=0.223$; $t(24)=0.37$, $p=0.713$) (Figure 3A). Similarly, on the fifth day post-injection there was no statistically significant difference in immobility times between the groups in both females and males ($t(28)=-1.22$, $p=0.234$; $t(23)=-0.514$, $p=0.612$). (Figure 3B). Repeated measures ANOVA showed a significant effect of time on immobility time in the tail suspension test in both male and female mice ($F(1.23)=45.600$, $p<0.0001$; $F(1.28)=5.433$, $p=0.027$ respectively). The time spent immobile had increased on the fifth day compared to the third day in both males and females, regardless of the experimental group. However, there was no time x group interaction in either sex ($F(1.23)=0.486$, $p=0.493$; $F(1.28)=0.089$, $p=0.767$ in male and female mice, respectively). This set of findings suggest that the time spent immobile in the tail suspension test showed an increase with time in both sexes, being more pronounced in male mice (an average increase of 63 seconds in males and 25 seconds in females). This change could have been observed because the mice learned that there was no escape during the tail suspension test performed on the third day, and thus showed increased behavioral despair in the test performed on the fifth day post-injection.

Sucrose preference index was higher in the female DAB group than the female control group in sucrose preference test performed on the third day post-injection, while no statistically significant difference was observed between the male experimental groups ($t(28)=3.28$, $p=0.003$; $t(24)=1.30$, $p=0.206$) (Figure 3C).

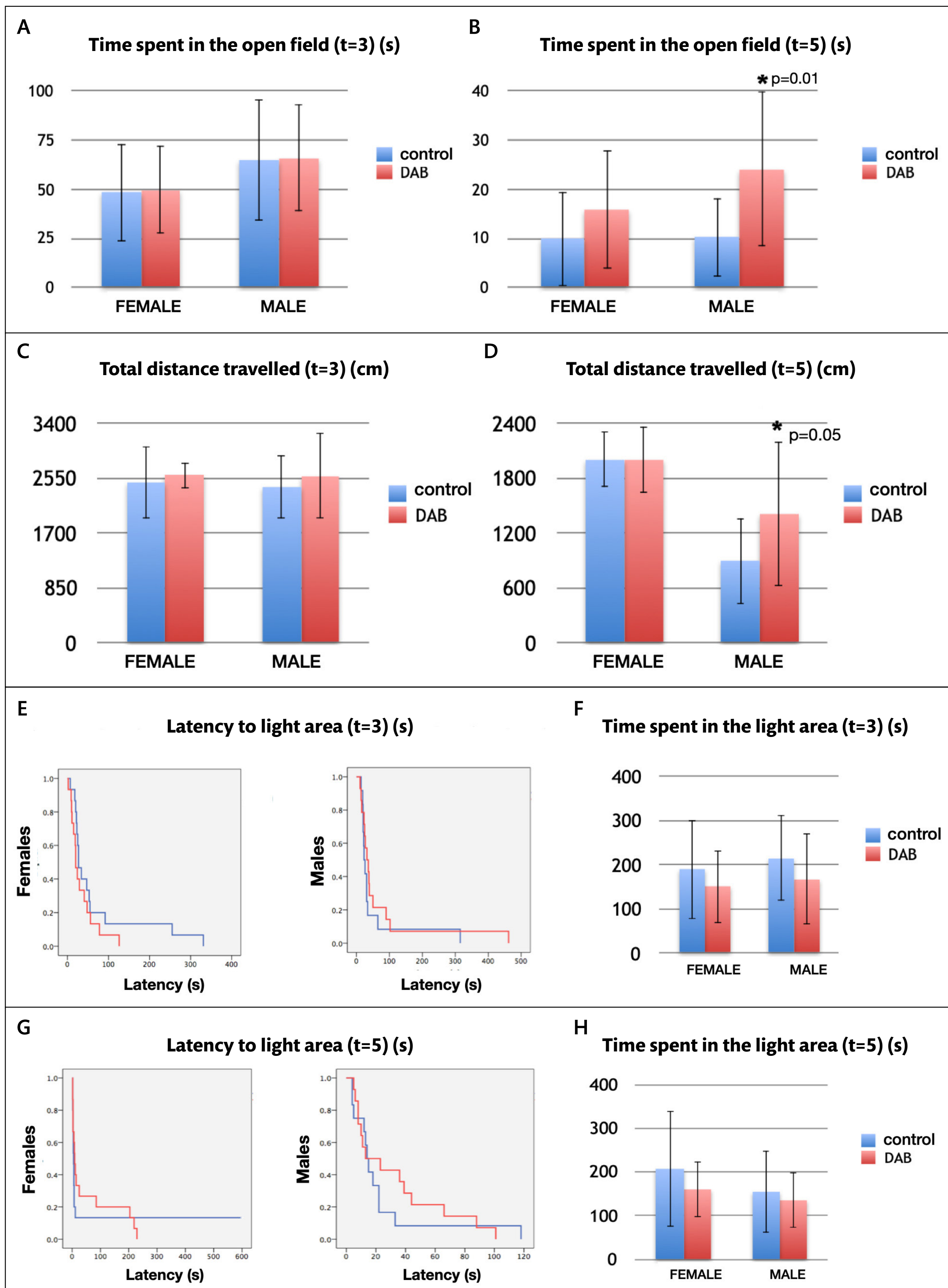

Figure 2. A. The time spent in the open field were similar between the groups on the third post-injection day, (n=14/female group, n=12-14/male group) **B.** Time spent in the open field increased on the fifth day after injection in male DAB mice (n=11-14/group). No significant difference was observed between the control and DAB groups in females (n=14/group). **C.** Third day locomotor activity test, distance traveled was similar between groups (n=13-14/female group; n=11-14/male group). **D.** An increase in locomotor activity was observed in male mice in the DAB group on the fifth day (n=12-14/group). There was no significant difference between the control and DAB groups in females (n=14-15/group). **E.** Third day light-dark box test. The latency to first enter the light chamber was similar between groups (n=14/female group, n=11-13/male group). Blue lines in the graph represent control group, red lines represent DAB group. **F.** Third day light-dark box test. Time spent in the light chamber was not different between groups (n=14/female group; n=12-13/male group). **G.** Fifth day light-dark box test. The latency to first enter the light chamber was similar between the groups (n=13-14/female group; n=11-13/male group). Blue lines in the graph represent control group, red lines represent DAB group. **H.** Fifth day light-dark box test. Time spent in the light chamber did not differ between groups (n=14-15/female group; n=12-14/male group).

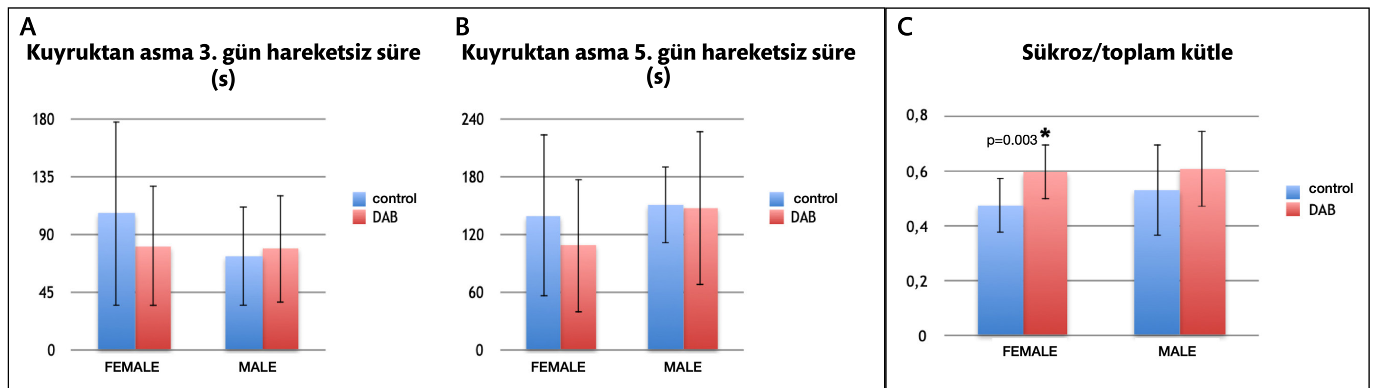

Figure 3. A. Third day tail suspension test, time spent immobile was similar between groups (n=15/female group; n=12-14/male group) **B**. Fifth day tail suspension test, time spent immobile was not different between groups.(n=15/female group; n=11-14/male group) C. Third day sucrose preference test. In female DAB group, the ratio of the sucrose solution consumed (g) to total fluid intake (g) was higher than in the control group mice (n=15/female group). No statistically significant difference was observed in males (n=12-14/male group).

DISCUSSION

Considering that most of the lactate supplied to neurons by astrocytes in order to meet the energy demand by increased synaptic activity comes from glycogen as part of the astrocyte-neuron metabolic coupling (Pellerin et al. 2007), it is possible that decreases in astrocytic glycogen and lactate levels may be involved in depression pathophysiology. Studies showing that chronic high corticosterone levels and chronic stress lead to a decrease in astrocyte glycogen along with an increase in depression-like behavior support this proposition (Zhang et al. 2015, Zhao et al. 2017). It is known that exposure to stress, which is used to model depression, causes an increase in glutamate release and therefore results in a subsequent increase in synaptic activity and energy demand (for a detailed review, see Popoli et al. 2011). Based on these findings, we examined whether inhibition of glycogenolysis in the hippocampus influenced anxiety-like and depression-like behavior in male and female mice.

In the open field test used to evaluate anxiety-like behavior, no significant differences were observed in either sex on day 3, while male DAB group spent longer time in the open field than controls on day 5. The total distance travelled on the fifth day was also longer in DAB group, which suggests that the increase in time spent in the open field may be associated with increased locomotor activity. Indeed, in the ANCOVA performed to test this possibility, the time spent in the open field by females and males was found to be similar between the groups when the effect of locomotor activity was controlled. In the light-dark box test, another test used to evaluate anxiety-like behavior, no significant difference was observed between the control and DAB groups in females and males at both time points, which supports this interpretation. In addition, the repeated measures ANOVA revealed that the time spent in the open field and locomotor activity decreased in both sexes over time, but this decrease was not related to the effects of DAB, as there was no time x group interaction effect. This may be due to habituation of mice to the box in which the test was conducted, resulting in decreased novelty of the box and exploratory behavior in the second encounter.

Although there was no statistically significant difference in any measure of tail suspension test between the control and DAB groups in both sexes, the sucrose preference test revealed that the female DAB group preferred sucrose solution more than the control group. This finding indicates that, contrary to our hypothesis, inhibition of glycogen use may increase hedonic behavior. However, while our previous research showed that DAB can easily diffuse in the interstitium (Figure 4) (Kılıç 2013), it is also possible that this difference may be due to the diffusion of DAB into the hypothalamus resulting in the induction of homeostatic mechanisms stimulating sugar intake. Therefore, we recommend using other behavioral tests to evaluate hedonic behavior in future studies such as social interaction rather than sucrose preference test, which may alter glycogen metabolism.

We observed no significant difference between the groups regarding anxiety-like and depression-like behavior on both the third and the fifth day. This result does not match previous studies that reveal the role of glycogen and lactate in depression-like behavior (Zhao et al. 2017, Carrard et al. 2018, Karnib et al. 2019). These studies convincingly showed that the decrease in hippocampal astrocytic glycogen levels caused by chronic corticosterone or chronic stress exposure, modelling depression, or suppressing glycogen synthase 1 enzyme levels with RNA interference resulted in increased depression-like behavior in rodents (Zhang et al. 2015, Zhao et al. 2017). Studies demonstrating that acute lactate administration increases stress resilience and/or shows antidepressant properties further support the role of lactate produced by glycogenolysis in depression pathophysiology (Carrard et al. 2018, Karnib et al. 2019). The inconsistency between our findings and the literature may be due to several reasons: Due to the limitation

Figure 4. Unilateral intracerebroventricular DAB injection caused disruption in synaptic ion balance and consequently resulted in the opening of Pannexin 1 (Pan 1) channels. Propium iodide (PI) uptake was used as an indicator of Pan 1 channel opening, and the red dots in the figure indicate the regions with PI uptake (Figure taken from the doctoral thesis of Kıvılcım Kılıç, 2013). Based on the figure, it could be deduced that DAB can easily diffuse into the interstitium and reach brain regions far from the injection site, including the hypothalamus, marked with a blue circle.

of the number of tests that could be performed after acute injections, we chose the most widely used behavioral tests that allow modelling different aspects of anxiety and depression-like behaviors in our experimental design. Therefore, the possible depressogenic or anxiogenic effects of restriction of synaptic energy supply by impairing glycogen utilization that were not evaluated by the behavioral tests used in our study cannot be excluded. As a matter of fact, in the above-mentioned studies, the depression-like behavior was assessed by the duration of immobility in forced swimming test, the duration of social interaction following chronic social defeat stress, and the time spent in open arms in the elevated plus maze (Carrard et al. 2018, Karnib et al. 2019). In addition, our experimental design included surgical intervention unlike the studies mentioned above, hence; despite the fact that the behavioral tests were performed 3-5 days after the injections, factors such as incomplete tissue healing and/or consequent pain may have caused an increase in anxiety-like and depression-like behavior in the control group to an extent that can mask the differences induced by the experiment. In a similar manner, isoflurane anesthesia administered during the surgical procedure may have masked the potential effects of DAB on depression-like behavior or lasting consequences of the recent anesthetic use (e.g. transcriptional) may have confounded behavioral testing. Considering these drawbacks, we plan to perform behavioral experiments as far from the traumatic effects of surgery and anesthesia as possible in future studies. For this purpose, waiting for tissue healing after placing a guide cannula into the hippocampus of the mice, and then performing the behavioral testing after injecting DAB/saline through the cannula without needing anesthesia could eliminate the possible undesirable effects mentioned above.

Studies showing that acute stress increases lactate concentration in various brain regions suggest that lactate supply increases in order to respond to the enhanced energy

demand (Elekes et al. 1996, Uehara et al. 2003, Uehara et al. 2005, Uehara et al. 2007, Uehara et al. 2013). For this reason, it is possible that the single dose administration of the glycogen phosphorylase inhibitor DAB into the hippocampi in this study did not sufficiently limit synaptic energy supply globally in the brain because basal synaptic activity was not promoted by a factor such as stress. The effects of acute DAB injection on the synaptic transmission may have been balanced by compensatory mechanisms and therefore may not have reflected on behavior. In order to make the masked behavioral effects of DAB observable, repetitive DAB injections and/or increasing the synaptic activity, and thus the synaptic energy demand, by acute stress before the DAB injection (in other words, creating a demand that cannot be met by the supply) should be considered in future studies. In addition, the effects of inhibiting glycogen use on depression-like and anxiety-like behavior were evaluated only on the third and fifth days after injection in our study. Considering the possibility that the behavioral effects of synaptic energy use disruption may emerge later, conducting behavioral tests at later time points might be informative as well.

To summarize, in this study, the inhibition of perisynaptic astrocyte glycogen use did not have a significant effect on depression-like and anxiety-like behavior in female and male mice on the third and fifth days after hippocampal administration of the pharmacological inhibitor DAB. In future studies, methods that will eliminate the effects of anesthesia and surgery, and the use of approaches such as stress exposure that will disrupt the energy demand/supply balance should be considered.

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