The link between learning performance, immobility in the forced swim test, and hippocampal glia

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ABSTRACT

Aim: To obtain maximal translational insights from animal models of depression, we need to know the meaning of behavioral parameters of animal models. The extent of construct and face validities of behavioral despair in the form of behavioral immobility in forced swim test (FST) is disputed. In this study, learning performance in a dual solution T-Maze and immobility on the 2nd day of FST was compared to shed light on this debate. Furthermore, we aimed to inspect the relationship between hippocampal glial densities and behaviors observed.

Method: Twelve adult male Sprague Dawley rats were tested in the dual-solution T-Maze and in FST. Subsequently, hippocampal slices were obtained, astrocyte and microglia cells were stained, and the densities were calculated for each subject.

Results: The rats utilized different learning strategies to solve the T-Maze. But irrespective of strategy, the rats that exhibited an overall efficiency in their learning performance, remained immobile for longer durations on the 2nd day of the FST. No significant relationship was detected between hippocampal microglia and behavioral indices in T-Maze and FST. However, we detected a significant positive correlation with CA1 astrocyte density and T-Maze learning and dentate gyrus CA1 astrocyte density and headshake behavior in FST.

Conclusions: The subjects showing a better cognitive performance in the T-Maze were immobile longer in the FST. This observation raises doubts about immobility as depression index and posits that it might reflect better learning. Our results also suggest that hippocampal glia cell types are differentially involved in cognition and affect.

Key words: Forced swim test, behavioral despair, learning, t-maze, glia, hippocampus.
Introduction
Depressive disorders are characterized by mood disturbances that affect an individual’s daily functioning and constitute a large burden for individuals and societies they live in. According to the World Health Organization (2018) report [1], major depressive disorder, the most frequent form of depressive disorders constitutes the number one cause of disability. A recent report by Kessler et al. (2012) [2] indicates a 16.6% lifelong prevalence of major depressive disorder in the United States. Given the chronic harm depressive disorders have in the modern world, it becomes of paramount importance to generate valid and reliable preclinical models of depressive disorders [3], undoubtedly an ardent task [4].

Forced swim test (FST) is a widely used animal model to gauge depression-like behavior in rodents [5]. The test consists of two trials separated apart by 24 hours. In these trials, the animals are placed in a water filled cylinder from which escape is not possible. The first trial, which typically lasts for 15 minutes, is considered to be the stress induction phase while the second trial typically lasts for 5 minutes. Immobility is considered to be an indice of behavioral despair reflecting a depressive behavioral phenotype; in this state the rodents do not exhibit any movement other than keeping their heads above water. Even during periods of extended immobility, the rodents can exhibit various struggling behaviors to different degrees [6].

It has been repeatedly shown that antidepressant treatment in between the two trials reduced behavioral immobility, findings bolstering the predictive validity of the forced swim test [7]. In a similar vein, exposure to enriched environments, electroconvulsive shock, and REM deprivation also reduce immobility durations in the second test [8]. Complementing this picture, increasing stress levels through increased water depth [9], increased time spent in water [10], and prior stressors [6, 11] make the animals more susceptible to behavioral despair.

However, forced swim test as a preclinical depression model is not immune to serious criticisms. First of all, depression in humans is characterized by a plethora of cognitive, behavioral, and emotional symptoms and FST falls short of capturing this complexity [5]. A serious challenge to FST comes from the antidepressant response’s time profile of reduced mobility; while antidepressants take 2-4 weeks to have their antidepressive effects in humans [12], a single day of antidepressant treatment in between the two trials suffices to reduce immobility in FST [3]. The findings alluded to pose serious challenges to the construct and predictive validity of FST as a preclinical model of depression [13, 14].

Phenomenologically speaking, increased immobility seen in the second trial might as well represent a passive coping strategy. There is evidence that this reduced mobility might reflect a learning process where animals learn that active coping behaviors like climbing and swimming do not result in the removal of the stressor [10, 14, 15, 16, 17, 18, 19]. While remaining immobile does not speed up the removal of the stressor, it is more energy preserving compared to active coping behaviors.

The current paper addresses how immobility in the forced swim test relates to behavioral and anatomical indices of cognitive functioning in rodents. In our behavioral experiments, we investigated the relationship between the learning performance in a dual solution t-maze task where animals were reinforced with food for entering the target arm and the propensity to exhibit immobility in the forced swim test. For
the anatomical parameters, we focused on the hippocampus as it is an integral brain region for the formation of cognitive maps which is imperative for spatial learning and navigation [20, 21] and shows distinct anatomical and functional alterations in affective disorders as a member of the limbic circuitry [22, 23, 24]. We focused on hippocampal microglia and astrocytes as recent studies point out to the important involvement of these cell types in the formation of neural circuits and synaptic plasticity [e.g. 25, 26]. Our findings indicate that immobility on the second forced swimming test day is associated with quicker acquisition of the T-maze task. Interestingly, the pace of T-maze acquisition was related to higher astrocyte counts in the CA1 region of the hippocampus bolstering the anatomical validity of our learning paradigm. Given our results, the ubiquitous observation that depression in humans is largely related to cognitive dysfunction poses a challenge to the validity of the forced swim test as a model of depression.

**Materials and methods**

**Animals and housing**

Twelve male Sprague Dawley rats weighing 250-350 gr. obtained from Kobay Experimental Animals Laboratory Inc (Ankara, Turkey) were used in this study. The rats were randomly assigned to three cages, with four rats per cage (40 cm x 34 cm 17 cm). All rats were housed in an artificially-lit room with access to water and food ad libitum at a temperature of 18-24 °C. The standardized 12:12 hr light-dark cycle (lights on 07:00 AM) was maintained throughout the study.

All experiments were performed with the approval of the Kobay DHL Inc. Animal Research and Ethics Committee (protocol number: 268).

**Behavioral testing**

The behavioral sessions were carried out in an adjacent room during the daytime between 10:00 a.m. and 4:00 p.m. There were three resting days between two experiments. After a handling procedure had been applied once a day for a week, the rats were subjected to the T-Maze procedure. Then, the rats were exposed to the Forced Swim Test (FST) procedure for two consecutive days. On the same day as the FST procedure ended, transcardial perfusion and fixation were performed to remove the brains for the histological applications.

**T-Maze**

Tolman’s T-Maze [27] procedures were carried out to assess rats’ spatial learning abilities and spatial navigational strategies following a food deprivation period. For this, the rats’ diet was restricted to 4 gr/day, starting one week before the experiment. The body weight of each subject was gradually reduced to 90% of the basal weight before the start of the training phase and the weights were stabilized at this level throughout the experiment by recording the weight of the rats every day. A plus-shaped maze was used in the experiment, which was placed 90 cm above the floor (arms: 45 cm length, 12.5 cm width, 7 cm height; center area: 12.5 cm), constructed of black-colored MDF material. A styrofoam panel was used to block the entrance to one arm, rendering a T-shaped maze. At the end of each arm, a perforated food cup (1 cm in diameter) was fixed 3 cm away from the distal end of the arm filled with the food reward (Nestlé CORN FLAKES™) that provided ubiquitous smell of the food from each arm so that smell of the food reward did not provide a navigational cue during the experiment. Separate food wells, which did not allow for visual localization of the reward were attached on top of each perforated food cup at
the end of the maze arms, and these were used for discriminatory baiting as further explained below.

The experiment consisted of three different phases: habituation phase on the first day, training phase and probe trial on the second day. In the habituation phase, each rat explored freely all the four arms of the maze containing the food reward for 10 minutes. By doing so, rats were acclimated to the maze and discovered that if they roam around the maze and travel to the end of the arms, they can find the food reward while getting over their natural neophobicity.

The training phase and probe trial were carried out respectively on the second day. While on habituation day, all of the arms were baited with the food reward; on the second day only one of the four arms randomly selected for each animal was baited. It was expected from the subjects in the training phase to learn the rewarded arm of the maze, regardless of the strategy they use. To illustrate, if the subject was positioned from the south arm, the opposite north arm was blocked with the panel. A bait was placed where the subject can reach either the east arm or west arm (see: Figure 1). In addition, the same amount of bait covered with a perforated container was placed in every arm as to eliminate the effect of the food odor. Each rat had to turn 90° when they reach the center of the maze to the left (i.e., to the west arm if the start arm was the south arm) or to the right (i.e., to the east arm if the start arm was the south arm) once to reach the bait. If the rat turned to the reward arm when released into the maze the rat was allowed to consume a piece of bait. Contrary, if the rat turned to the wrong arm the rat was removed from the maze within three seconds. The floor and walls were cleaned between each trial with a cloth dampened with alcohol diluted with water to wipe out possible clues such as feces, urine, or food bits on the maze surface.

During the training phase the rats were given a maximum of 35 trials for the training to learn the location of the baited arm in the T-Maze. When the rats got nine out of the 10 consecutive attempts correctly, the probe trial was initiated. The starting arm, where the rat had been placed in the training phase, was rotated 180° to the opposite arm that became the new starting arm for the single probe trial. The rats were classified into the “allocentric” or “egocentric” group on the basis of their performance during the probe trial: the rats who turned to the same reward arm as in the training and reached the bait despite the 180° rotation of the start arm were labeled as allocentric/place learners, the rats who turned to the previously unbaited opposite arms were labeled as egocentric/response learners.

In the training phase, the maximum number of consecutive correct choices and the number of trials that each rat took to achieve the criterion (nine of 10 correct) were counted by two independent observers from the recorded videos. Also, the total duration to complete the probe trial was calculated for each rat. These measurements were used to quantify the learning speed of the rats as an indicator of cognitive performance.

Figure 1. T-maze training of rats during the experiments where rats underwent habituation (Left panel), training (Middle panel), and testing (Right panel) trials. Note that the blocked arms oppose each other during training and testing.
Forced Swim Test (FST)
FST procedure was initiated three days after when the rats were returned ad libitum access to food. In accordance with the standard protocol of the FST (Porsolt et al., 1997), a Plexiglass cylinder tank (50 cm in height, 20 cm in diameter) was filled with 30 cm tap water (25°C) so that the tails of the rats did not touch the bottom of the container. A digital camera (Logitech C270) was positioned to record the setup. All rats were acclimated to the test room for half an hour before starting the trials. On the first day, each rat was kept in the water filled cylinder tank with no possible escape for 15 minutes as the first phase of the experiment. The test phase was applied to each rat 24 h later, on the second day in which the duration of water exposure was reduced to 5 minutes. Throughout the experiment, the tank was cleaned and refilled with fresh tap water at 25°C. The rats were kept in a single cage and it was ensured that they remained dry immediately after the trials. The rats were considered immobile when moved only enough to keep their heads afloat above the water. Two independent observers counted the total duration of immobility on the second day of the FST, the number of diving and head shaking behaviors for each rat by examining the recorded videos.

Histological procedure: Preparation of brain slices
Following the conclusion of the behavioral experiments, animals were deeply sedated with an i.p. injection of urethane 1.25 gr/kg and perfused transcardially with 0.1M phosphate buffer (PB) solution followed by 4% paraformaldehyde (PFA) in 0.1M PB. The removed brains (N=12) were kept in the fixation overnight and were preserved with 30% sucrose in 0.1M PB for later processing. The brains were cut into 100 μm thick coronal sections containing Dentate Gyrus and CA1 subregions of the dorsal hippocampus with vibratome based on The Rat Brain in Stereotaxic Coordinates by Paxinos and Watson (2007) [28].

Immunohistochemistry for glial markers
100 μm thick brain sections were washed 3 times with 0.1M PB, and then permeabilized with 10% methanol and 1% sodium borohydrdride for one hour. The sections were blocked in 10% normal goat serum and 0.5% Triton X for 24h for non-specific target binding. Subsequently, the sections were subjected to immunohistochemistry for glial fibrillary acidic protein (GFAP) which is an astrocyte marker, and ionized calcium binding adaptor molecule 1 (IBA-1) protein which is a microglia marker. A series of sections were incubated in rabbit-anti-GFAP+ and primary antibody diluted 1:1000 for 24h. Another section series from the same brains were incubated in the rabbit-anti-IBA1 antibody diluted 1: 1000 for 24h. All the sections washed three times with 0.1M PB were then recovered from the secondary goat-anti-rabbit antibody diluted 1: 500 for 4h, after which the antibodies were conjugated with the horseradish peroxidase (HRP) enzyme. This binding reaction was visualized with NovaRED TM (Vector Labs, Burlingame, CA). The stained sections were dehydrated with ethanol and cleared with xylene. Afterwards, they were coated with DEPEX, fixed on glass microslides and were coverslipped. The pictures of the Dentate Gyrus and CA1 subregions of the dorsal hippocampus were taken with the Motic B210 3MP digital microscope with 4X, 10X, 40X objective lenses. The region of interest (ROI) and the density of astrocytes and microglia cells on the micrographs were calculated via the ImageJ program for further statistical analysis.
Figure 2. Two sections taken with 10X lens including dentate gyrus (DG) and CA1 subregions of the dorsal hippocampus. (A) The section stained for the astrocyte marker glial fibrillary acidic protein (GFAP+) (1: 1000 rabbit-anti-GFAP antibody, 1: 500 goat-anti-rabbit antibody). (B) The following brain section taken from the same brain, IBA-1 staining which is a protein specific to microglial cells was applied (1:1000 rabbit-anti-IBA1 antibody, 1: 500 goat-anti-rabbit antibody).

Statistical analysis
Microsoft Excel and SPSS v.21 were used for the data analysis. To compare the groups, Wann-Whitney U non-parametric tests were used to investigate the relationships between behavioral parameters in T-maze, FST, and anatomical variables, Pearson correlations were carried out.

Results
The initial parameter that was recorded about the subjects concerned their performance on the T-Maze. Based on their choice in the probe trial of the T-Maze (as explained in detail in the methods section), the 12 rats were classified as allocentric/place learners – relying on the hippocampus-based strategy (n = 8), and as egocentric/response learners (n = 4) – relying on the striatum-based strategy for navigation. In addition to the strategy, we have extracted several other parameters from the T-Maze performance of the subjects:

- The number of trials out of the max. 35 training trials that it took the subject to reach to the learning criterion (i.e., how many trials did it take rats to achieve 9 correct choices out of 10 consecutive attempts),
- Max. number correct choices to reach the learning criterion (for e.g., if an animal reached 9/10 criterion by making 2 successive correct choices, followed by an incorrect attempt and then by 7 uninterrupted correct choices; the animal got a score of “7” for this indice),
- The duration (in sec) to complete the probe trial.

A summary of the T-Maze performance of the subjects is given in Fig. 3. From the FST performance, the indices we have extracted were the total duration of immobility, the frequency of active struggling behaviors (pedaling, jumping, diving), the frequency of head-shake behavior on the second day of the FST.

Figure 3. The graphical summary of T-Maze performance of each subject in the experiment. Each
column corresponds to the data from a single rat. Blue squares indicate the correct trials for allocentric/place learners and the green squares indicate the correct trials for egocentric/response learners. Trials with incorrect choices (i.e., failure to locate the baited arm) are indicated with yellow squares for both allocentric and egocentric strategy groups.

Immunohistochemical parameters that were used in the current study were the densities (# cells / 100 µm²) of GFAP+ astrocyte glia cells and IBA-1+ microglia cells in the dentate gyrus and CA1 subregions of the hippocampus.

Comparison of T-Maze performance between allocentric/place learners and egocentric/response learners

To test the relationship between studied parameters in T-Maze, the first groups of rats utilizing different learning strategies in T-Maze were compared. Since the sample size is limited and there are more rats utilizing allocentric learning strategy compared (n = 8) to egocentric learners (n = 4), we ran Mann-Whitney U non-parametric tests.

It was found that the groups did not differ significantly in terms of total number of trials to reach the criterion for learning (U = -0.681, p = 0.57). The groups also were not significantly different from each other in terms of max. number of consecutive correct choices (U = 0.347, p = 0.808). Finally, the duration to complete the probe trial was contrasted between the groups, there was no statistical difference between the allocentric/place and egocentric/response learners (U = -0.818, p = 0.497).

But irrespective of the learning strategy, we have detected a significant positive correlation between the total number of trials it took subjects to reach to learning criterion (i.e., correct choice in 9 out of 10 successive trials) and time it took the subject to complete the probe trial (r (12) = .821, p = 0.002) (Fig. 4). In other words, the rats which learned the location of the baited arm in the T-Maze rapidly also demonstrated faster choice and reward consumption response in the probe trial.

![Figure 4](image_url)

A marginally significant positive correlation was also detected between the total number of trials it took subjects to reach to learning criterion and max. number of consecutive correct choices in training in T-Maze (r (12) = - .575, p = .051).

Comparison of T-Maze and FST behavioral performances:

- To investigate the relationship between studied parameters in T-Maze and FST tasks, firstly, the groups of rats utilizing different learning strategies in T-Maze were compared. Since the sample size is limited and there are
more rats utilizing allocentric learning strategy compared (n = 8) to egocentric learners (n = 4), we ran Mann-Whitney U non-parametric tests. When T-Maze strategy and FST immobility was compared, no significant differences were detected (U = 1.361, p = .214). Moreover, T-Maze learning strategy was not significantly related to frequency of headshake behavior on the second day of FST (U = 0.597, p = .570).

To investigate a possible link between behavioral performance in T-Maze and FST, Pearson bivariate comparisons were carried out between T-Maze parameters (total number of training trials, duration to complete the probe trial and the max. number of consecutive correct choices) with FST parameters (duration of immobility on the 2nd day of FST, headshake frequency on the 2nd day).

The relationship between duration of immobility on the 2nd day of FST, the total number of training trials and the duration to complete the probe trial were not significant (r (12) = -.249, p = .435; r (12) = -.310, p = .354, respectively). But a significant positive correlation was detected between duration of immobility on the 2nd day of FST and the max. number of consecutive correct trials in T-maze (r (12) = .636, p = .026) (Figure 5).

Comparison of T-Maze and FST behavioral performances with the astrocyte and microglia densities in hippocampal subfields:

In the last set of analyses, the behavioral parameters from the T-Maze and FST were contrasted against the microglial densities across DG and CA1 hippocampal subfields. To calculate the GFAP+ and Iba1+ glial cells, at least 3 hippocampal sections/rat clearly stained with the appropriate antibody for the glial marker proteins were imaged under 10X magnification. The digital images of the hippocampus then transferred to ImageJ. The hippocampal DG and CA1 regions were demarcated on the imaged sections and stained cells that were clear in the focal place were counted using “Cell Counter” Image J Program. The density of Iba1+ microglia in DG and CA1 hippocampal subfields were not statistically different in allocentric/place learners and egocentric/response learners (U = 1.134, p = .315; U = -1.443, p = .2, respectively). The density of GFAP+ astrocytes were also not statistically different in the DG between the groups (U = -1.701, p = .109) but a significant difference emerged between the groups in the CA1 subfield (U = 2.193, p = .032): egocentric/response learner subjects had a higher density of GFAP+ astrocytes in CA1 (Mdn$_{ego}$ = 0.0030/100 µm$^2$, Mdn$_{allo}$ = 0.0022/100 µm$^2$) than allocentric/place learners.
When the glial cell densities were compared with the T-Maze performance, the density of Iba1+ microglia were not significantly correlated with the total number of training trials, the max. number of consecutive correct choices and duration to complete the probe trial (For the DG: r = -.514, p = .106; r = -.241, p = .475; r = -.418, p = .230, respectively. For the CA1: r = .499, p = .208; r = -.332, p = .422; r = .611, p = .145, respectively.) The GFAP+ astrocyte density in the CA1 hippocampal subfield, however, was significantly negatively correlated with the total number of training trials (r = -.664, p = .036), and duration to complete the probe trial (r = -.761, p = .017) but not with the maximum number of consecutive correct choices (r = .419, p = .228). But this relationship was subfield specific as we did not observe a significant relationship between DG GFAP+ astrocyte density and the total number of training trials, the max. number of consecutive correct choices and duration to complete the probe trial in T-Maze (r = .218, p = .520; r = .188, p = .579; r = .130, p = .720).

Microglia and astrocyte densities in hippocampal subfields were also compared with the FST parameters of immobility duration and headshake frequency. Irrespective of the hippocampal subfields, Iba1+ microglia density was not significantly correlated with either of the immobility duration and headshake frequency (For the DG: r = -.561, p = .072; r = .177, p = .602, respectively. For the CA1: r = -.423, p = .297; r = .276, p = .508, respectively.) In terms of GFAP+ astrocytes, we detected a marginally significant negative correlation with DG GFAP+ cell density and the headshake frequency on the 2nd day of FST (r = -.596, p = .053) but not with duration of immobility (r = .478, p = .137). CA1 astrocyte density was not correlated with either of the headshake frequency or duration of immobility (r = .053, p = .884; r = .296, p = .406).

**Discussion**

The present study investigated the relationship between learning in a T-maze that is open to solution through multiple learning strategies and behavioral despair as measured by immobility on the second FST day. Microglia and astrocyte cell densities were calculated for different hippocampal subfields also as the glial cells as cell types and hippocampus as a brain region is thought to play sophisticated roles in mood and cognition [29]. The results can be briefly summarized as follows. In terms of behavioral efficacy, both egocentric and allocentric strategies were comparable. Overall, the faster the rats reached learning criterion, the faster they also completed the probe trial used to classify them as egocentric and allocentric learners. The egocentric and allocentric learners did not exhibit differences in terms of their immobility scores on FST day 2. Nonetheless, a positive relationship between the number of consecutive correct trials and immobility on FST day 2 emerged from our analysis indicative of a tendency to remain more immobile in animals that learned the T-maze task more effectively. This relationship becomes more meaningful in light of the marginally significant relationship between the learning trials required to reach criterion and the number of consecutive correct trials before animals reached the criterion. As alluded to before also, reaching learning criterion earlier was associated with faster completion of the probe trial. Overall, the behavioral results indicate that the better the animals learn the T-maze, the more immobile they tend to remain on FST day 2.

At the anatomical level, microglia counts in hippocampal CA1 and DG subregions did not
show any relation to learning while astrocyte counts appeared to be higher in the CA1 region of the egocentric learners but not in the DG subregion. Interestingly, CA1 astrocyte counts overall showed a negative correlation with the total number of training trials required to reach learning criterion and duration to complete probe trials. In other words, astrocyte numbers in CA1 are associated with better learning performance. DG astrocyte on the other hand showed a negative correlation with the number of headshakes, an index of struggling behavior in FST [30].

The multiple memory systems hypothesis poses that different memory systems can cooperate or compete for the solution of a particular cognitive task [31]. The T-maze task utilized in the current study could be solved in two ways: 1) by formation of a cognitive map, a strategy largely dependent on the hippocampus and 2) by learning a set of response sequences taking the organism as a reference point that is largely dependent on the striatum. The former strategy is referred to as the allocentric strategy while the latter is referred to as the egocentric strategy [31]. The current study suggests that both strategies can result in comparable behavioral performance as apparent from the lack of differences in the acquisition rates of both egocentric and allocentric learners. Intriguingly, egocentric and allocentric learners did not differ in their level of immobility in FST day 2. This result has some phenomenological implications. Human clinical literature suggests that patients with major depressive disorder have smaller hippocampal volumes [22, 23] along with functional hippocampal alterations [24]. From the perspective of multiple memory systems theory, the compromised system would not be able to compete for expression with the intact system [32]. Complementing this, stress exposure, a major risk factor for depressive disorder, in animals bolsters egocentric strategies [33]. The absence of differences in immobility in FST day 2 casts doubts about the serviceable function of FST as an animal model of depression. Just to the contrary, the rats that possessed higher efficacy in their learning in the T-maze tended to display higher immobility during FST day 2. These results are more fitting with the notion that immobility during FST day 2 reflects a learning phenomenon [10] rather than a state of behavioral despair. Indeed, De Pablo and colleagues [17, 34] have found that post-FST1 anisomycin treatment that interferes with the protein synthesis; hence the consolidation of learning [35] was associated with a reduction in immobility during FST day 2. The so called short term ameliorating effects of antidepressants [36] on FST day 2 might actually be related to the fact that acute antidepressants have the potential to impair cognitive functioning [37].

Our anatomical results are less straightforward when it comes to interpretation. We did not observe any relationship between CA1 and DG microglia counts with T-maze and FST performances. Whether there are differences in other regions is a question that awaits an answer. Moreover, the absence of effects in this study does not negate that hippocampal microglia play crucial roles when it comes to learning. For instance, brain-derived neurotrophic factors released from microglial cells have been shown to be of critical importance for spatial learning in the hippocampus [25, 26]. On the other hand, the pro-inflammatory responses of microglia, which are hampered by the activation of nicotinic acetylcholine receptors bearing α7 subunits [38, 39], have been shown to impair cognition [40]. These findings actually support the thinking that microglia population might serve as a double edged sword when it comes to
their effects on cognition and this very nature might have masked the meaningful variances in our studies.

While the role of the astrocyte population is a multifaceted, their presence is not associated with an undesired state such as inflammation apart from traumatic brain injury related astrogliosis [41] which does not occur in the intact, normal brain. Some of their conventional roles include providing metabolic support to neurons and pH homeostasis [42] and contribution to the neurovascular demands [43]. Aside from the traditional roles known, recent decades have shown that astrocytes play a role in regulating synaptic communication between neurons, releasing glutamate when necessary [44]. This function is also regulated by acetylcholine [45], a neuromodulator intricately involved in cognitive functions [46]. Pabst and colleagues [47] have found that the optogenetic activation of the septohippocampal pathway recruits hilar interneurons through astrocytes producing long-lasting hyperpolarizations in the pyramidal neurons. In line with the crucial roles of astrocytes, astrocyte numbers in our study was related to more efficacious learning in the t-maze. There are two major culprits to the credibility of this account though. One is the finding that CA1 astrocyte counts were higher in the CA1 of egocentric learners. The other pertains to the absence of a relationship between astrocyte counts and FST day 2 performance which we are tempted to relate to memory of the FST day 1. Expecting a higher CA1 astrocyte count in allocentric learners is a straightforward logic and it is very plausible that astrocytes in CA1 play a crucial role in determining the glucose balance between the hippocampus and the striatum as these seem to be coordinated in accordance with the memory system usage [48]. The latter culprit pertaining to the absence of evidence for astrocyte involvement in immobility during FST 2 might be explained by the fact that FST involves a stressful learning situation as evidenced by the natural tendency of rodents to avoid water especially when it threatens their thermoregulation [5]. In this respect, differences might emerge in the ventral hippocampal regions that are implicated in stressful situations [49] and not studied in the current set of experiments, which constitutes a weakness. Another flaw pertains to our low sample size. Nonetheless, seeing the effects we saw despite the limited sample size points out also to their strength. Future studies implementing bigger sample sizes and utilizing parametric statistics will yield a richer answer to the exact cognitive mechanisms involved in t-maze learning and how this relates to what is being actually learned during FST.

As alluded to before, there is evidence to the contrary to our studies. Treatments used to prevent and/or ail depression in humans such as environmental enrichment, electroconvulsive shock, and REM duration are shown to diminish immobility during FST day 2 [8]. However, false positive results with psychostimulants, negative results with some selective serotonin reuptake inhibitors, lower immobility being related to lower defecation rates as reviewed by Armario in 2021 [5] pose a challenge to the view that FST immobility reflects pure behavioral despair as well as reduced immobility observed after inactivating molecular learning switches using anisomycin [17, 34]. Ateşyakar and colleagues [50] have found low cognitive competence to be associated with increased immobility in FST day 2, a finding that seem to contradict our findings. Nevertheless, the discrepancies might be explained by the availability of different cognitive strategies in solving the cognitive tasks. For instance, the radial arm maze task.
implemented by Ateşyakar and colleagues [50] is typically solved using allocentric strategies rather than egocentric strategies [51] while the T-maze configuration used in our studies provide more liberty to the animals [e.g., 31]. In conclusion, our studies support the notion that FST day 2 immobility is mainly a reflection of memory mechanisms while they do not rule out other factors such as behavioral despair and coping strategies. It is hoped that more detailed future studies will shed light on the exact contribution of these different mechanisms to immobility in FST.

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Ethical statement:
The study was confirmed by Local Ethics Committee (protocol number: 268).

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