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Research report

Behavioral profile of rats submitted to session 1-session 2 in the elevated plus-maze during diurnal/nocturnal phases and under different illumination conditions

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Abstract

The elevated plus-maze (EPM) model usually employs nocturnal species (e.g. rats and mice) and the tests are almost exclusively performed during the diurnal phase (lights on), leading some laboratories to perform experiments with animals under a reversed light cycle to overcome this problem. However, it is questionable whether the artificial reversal of the light cycle for short periods guarantees modifications in all the physiological parameters found in normal subjects. The present study evaluated the session 1-session 2 (S1-S2) EPM profile in rats during their normal diurnal or nocturnal phase using different illumination conditions. Prior exposure to the EPM decreased open arm exploration for all groups in S2, regardless of the circadian phase and illumination condition; however, this behavior was decreased in subjects tested during the nocturnal phase, when compared to the diurnal phase. Risk assessment (RA) behavior was decreased under high illumination for both circadian phases in S1 and increased in the first minute of S2, when compared to the last minute of S1. Although open arm exploration and RA behavior were decreased under high illumination, when compared to low illumination conditions in both circadian phases, general locomotor activity was only decreased during the nocturnal phase. The results are discussed in terms of circadian variations in the behavioral profile and as a possible source of variability in pre-clinical models of anxiety.

Keywords: Anxiety; Elevated plus-maze; Risk assessment; Circadian rhythm; Illumination conditions; Rat

1. Introduction

Circadian rhythm synchronizes [37] and influences a large number of physiological and behavioral processes, such as sleep [29], feeding [12,36], drinking [53] and body temperature [55], as well as exploratory [53] and social behavior. Within the central nervous system, altered physiological parameters can also be found in the release of neurotransmitters [2,11,34,51], activation of neurotransmitter receptors and associated secondary messenger systems [1,10,26,28,32,36,57] and in the expression of immediate-early genes [17,20]. In the rat, the nocturnal phase of the circadian rhythm corresponds with the active, inquisitive and responsive period [30].

Recently, this circadian rhythmicity has been evaluated in pre-clinical models of anxiety [16,27,44]. Kelliher et al. [30] pointed out that, probably due to reasons of practicality, these behavioral investigations, whilst employing nocturnal species, are almost exclusively performed during the diurnal phase (lights on) of the circadian cycle. To overcome this situation, some laboratories have kept the subjects under a reversed light cycle, but this procedure may not guarantee that all the normal physiological functions will change along with the circadian cycle. In fact, very little of the rat's behavior profile has been studied to identify the minimum time required to elicit a complete behavioral and physiological adaptation to the natural situation. In the elevated plus-maze (EPM), a commonly used model [23,43,46] for assessing anxiolytic effects of compounds

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acting on γ-amino-butyric acid (GABA) [24,48] and serotonin (5-HT) [19,50] systems, the circadian phase of testing failed to consistently alter behavior in rats kept under a reversed light cycle [27]. However, it has been suggested that the rodent circadian rhythm could be a possible source of variability in the EPM. Indeed, discrepancies between laboratories in elucidating an anxiolytic effect of 5-HT_{1A} agonists/antagonists in the EPM have been explained by circadian variations in drug activity [21,44,45]. For example, Rodgers et al. [44] found that LY297996, a 5-HT_{1A} antagonist, was anxiolytic in the mid-dark phase but not in the mid-light phase. Such behavioral differences agree with the idea of a circadian rhythm in 5-HT_{1A} receptor-mediated effects [12,34].

Organismic variables, such as species, genetic strain [40], gender and age [26], as well as procedural variables, such as housing [33], prior handling [3], prior stress [52] and previous maze experience [6] have been shown to affect baseline behavior in the EPM. Although some results have shown that illumination conditions failed to alter the rat's behavior in the EPM [5,14,39], it may also be considered an aversive stimulus in the EPM [35], since rats tested under low illumination showed more open arm exploration and were generally more active than those tested under high illumination conditions [9,18]. Test illumination conditions may also explain inconsistencies for 5-HT receptor-active compounds [8], since the same dose of the 5-HT_{1A} agonist, 8-OH-DPAT, has produced anxiolysis under high illumination and anxiogenesis under low illumination conditions [22].

Taking into account these concerns, the purpose of this study was to evaluate the behavioral profile of rats, kept under a normal light cycle, submitted to the EPM in both session 1 (S1) and session 2 (S2), during the diurnal or nocturnal phase and under different illumination conditions.

2. Materials and methods

2.1. Subjects

The subjects were 68 male Wistar rats weighing 250–300 g, aged 13–15 weeks at the time of testing, housed in groups of five to six per cage (50 × 30 × 15 cm), kept in a room (vivarium), under a normal light cycle (12:12 h light:dark phase; lights on at 06:00 h), in a temperature controlled environment (23 ± 1 °C) and with free access to food and water. The subjects were reared in the above conditions from weaning and 48 h before the experiment, they were moved to an adjacent room under the same light cycle and regimen conditions as in the vivarium. The experimental sessions were conducted during the onset of the diurnal (between 07:00 and 11:00 h) or nocturnal (between 19:00 and 23:00 h) phase.

2.2. Apparatus

The EPM was made of wood and consisted of two opposite open arms, 50 × 10 cm (surrounded by a 1 cm high Plexiglas ledge) and two enclosed arms, 50 × 10 × 40 cm, elevated to a height of 50 cm above the floor. The junction area of the four arms (central platform) measured 10 × 10 cm. The floor of the maze was painted with impermeable dark epoxy resin, in order to avoid urine impregnation.

2.3. Procedures

The experiments were carried out in a room with low (44 lux) or high (600 lux) illumination conditions (measured on the central platform), the latter being the same light level as in the main laboratory during the day. Behavior was recorded by videocamera. A monitor and a video-recording system were installed in an adjacent room. A trained observer scored the parameters from the videotape. After each trial, the maze was cleaned with ethanol solution (10% v/v).

Subjects submitted to the EPM, in both S1 and S2 (48 h later), during each circadian phase, were randomly allocated ($n = 16–18$ per group) to a corresponding or contrasting illumination condition, thus forming four groups: DH (submitted to the EPM during the diurnal phase under high illumination conditions); DL (diurnal phase under low illumination conditions); NL (nocturnal phase under low illumination conditions); and NH (nocturnal phase under high illumination conditions). Rats in the two contrasting groups (DL and NH) were only exposed to the level of test illumination at the moment of being submitted to the EPM.

2.4. Full session profile analysis

In both sessions, the parameters analyzed were the frequency of open and enclosed arm entries and the amount of time spent by the rats on the central platform, open and enclosed arms (four paws inside the arm). These data were used to calculate percentage open entries [%OE; open entries/(open + enclosed entries) × 100] and percentage time spent in open arms (%OT; open arm time/300 × 100). In addition, the frequency of attempts to reach the open arms (protected stretched attend posture with the head and one, two or three paws positioned inside the open arms and retraction to the original position), performed by rats from the central platform or from the enclosed arms, was recorded as tries. Risk assessment (RA) behavior was interpreted according to the formula: RA = [frequency

Table 1
Two-factor (group × session) repeated measures ANOVA results from full session analysis showing significant main effects and interactions

Parameter/factor	Group (df = 3,64)	Session (df = 1,64)	Group × session (df = 3,64)
% Open entries	$F = 16.4$; $P < 0.00001$	$F = 67.2$; $P < 0.00001$	NS
% Open time	$F = 12.8$; $P < 0.0001$	$F = 86.4$; $P < 0.0001$	NS
Risk assessment	$F = 8.8$; $P < 0.0001$	$F = 8.3$; $P < 0.01$	NS
Enclosed arm entries	$F = 3.2$; $P < 0.05$	$F = 5.4$; $P < 0.05$	NS

Rats were submitted to the EPM during the diurnal/nocturnal phase and under different illumination conditions. df, Degrees of freedom; NS, not significant.

of tries/(300 − time spent in open arms) × 60]. Thus, it was possible to estimate the frequency of tries per minute, performed by rats from protected areas of the maze.

2.5. Minute by minute profile analysis

The parameters analyzed were based on the same parameters described above. Each 60-s interval (time bin) was used to calculate %EO and %TO. RA behavior performed from protected areas in the EPM, for each time bin, was calculated using the formula: RA = [frequency of tries/(60 − time spent in open arms) × 60].

2.6. Statistics

Data obtained from rats submitted to the EPM in both sessions were analyzed by two-factor (group × session) repeated measures analysis of variance (ANOVA), followed by Newman–Keul's tests. A further statistical approach (minute by minute) was used to identify changes in behavioral profiles within sessions. Data obtained from minute by minute analysis were analyzed by three-factor (group × session × time bin) repeated measures ANOVA, followed by Newman–Keul's tests. The level of statistical significance adopted was $P < 0.05$. All statistical analyses were performed using the software Statistica® (StatSoft Inc., Tulsa, OK).

2.7. Ethics

All procedures were approved by our Institutional Ethics Committee and were in accordance with NIH Animal Care Guidelines.

3. Results

3.1. Full session changes in behavior profile

Table 1 illustrates the two-factor (group × session) repeated measures ANOVA results from full session analysis showing significant main effects and interactions. Briefly, the analysis revealed significant main effects (but no interactions) of the group and session factors for all parameters evaluated in the EPM.

Data illustrated in Figs. 1 and 2 represent the effects of the circadian phases and illumination conditions in rats submitted to the EPM in both S1 and S2. Comparisons using the Newman–Keul's test revealed that prior exposure to the EPM decreased ($P < 0.05$) open

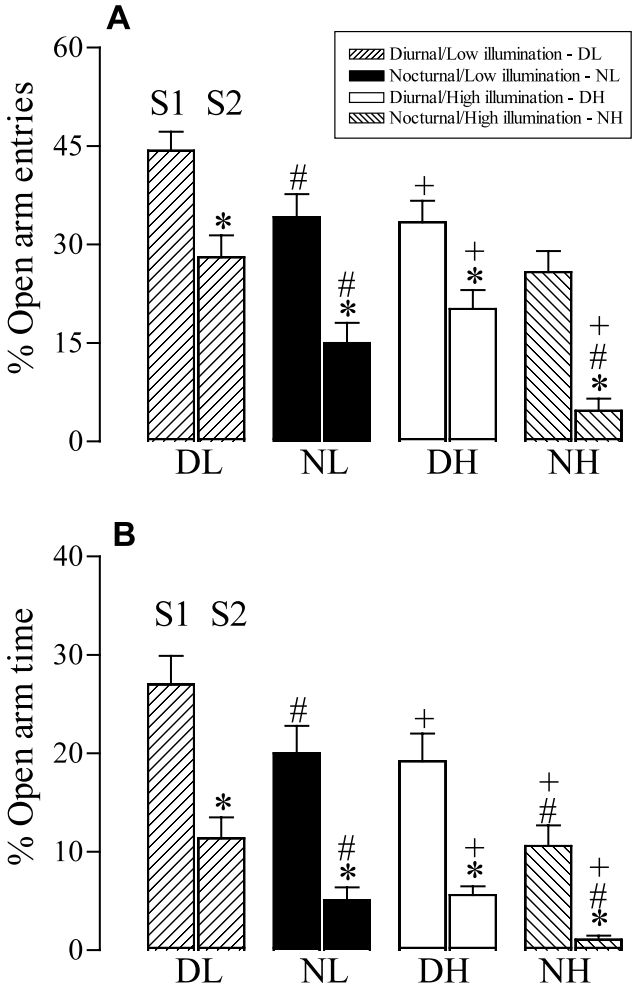

Fig. 1. Circadian phase and illumination condition effects on the percentage of entries (A) and of time spent (B) in open arms, in rats submitted to the elevated plus-maze (EPM) in both session 1 (S1) and session 2 (S2), revealed by two-factor (group × session) repeated measures ANOVA followed by post-hoc Newman–Keul's test ($P < 0.05$). Data are presented as mean ± S.E.M. *Statistical difference from respective group in S1; # statistical difference between circadian phases; + statistical difference between illumination conditions.

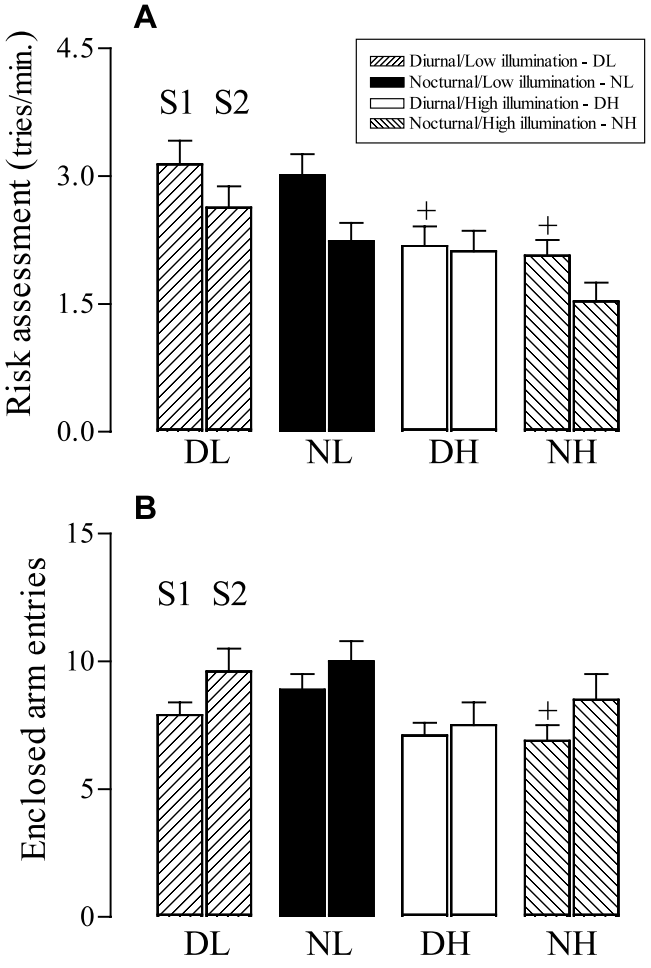

Fig. 2. Circadian phase and illumination condition effects on risk assessment behavior (A) and enclosed arm entries (B), in rats submitted to the elevated plus-maze (EPM) in both session 1 (S1) and session 2 (S2), revealed by two-factor (group × session) repeated measures ANOVA followed by post-hoc Newman–Keul's test ($P < 0.05$). Data are presented as mean ± S.E.M. + Statistical difference between illumination conditions.

arm exploration, represented by %OE and %OT parameters, for all groups in S2, regardless of circadian phase and illumination condition. Nevertheless, NL and DH groups decreased ($P < 0.05$) open arm exploration when compared to the DL group in both S1 and S2 (Fig. 1). In addition, open arm exploration was decreased in the NH group when compared to DH and NL groups in S2 (Fig. 1), while only %OT was decreased in NH group when compared to the former in S1 (Fig. 1B). RA behavior was decreased for both DH and NH, when compared to DL and NL groups in S1 (Fig. 2A), respectively. Enclosed arm entries were reduced in NH when compared to NL group in S1 (Fig. 2B).

3.2. Minute by minute changes in behavior profile

Table 2 illustrates the three-factor (group × session × time bin) repeated measures ANOVA results from minute by minute analysis showing significant main effects and interactions. Briefly, the analysis revealed a significant group × session × time bin interaction for the %OT parameter, indicating that the pattern of behavioral change in each time bin differed as a function of test group and session. ANOVA performed on RA behavior data revealed a tendency ($P = 0.086$) for the group × session × time bin interaction. ANOVA revealed also a session × time bin interaction for all parameters evaluated in the EPM. In addition, significant main effects (but no interactions) of the group, session and time bin factors were detected for all parameters evaluated in the EPM (Table 2).

Data illustrated in Figs. 3 and 4 also represent the behavioral profile of rats submitted to the EPM during the diurnal/nocturnal phases and under low/high illumination conditions. Further comparisons using the Newman–Keul's test revealed that %OE was decreased ($P < 0.05$) in the fifth minute for DL and NL groups (Fig. 3A) as well as from the third to the fifth minutes in the DH group in S1 (Fig. 3B), when compared to the first minute. In addition, the NH group exhibited a decreased %OE in the first minute of S1, when compared to the DH group (Fig. 3B). In both DL and NL groups, %OT was decreased from the third to the fifth minute in S1 (Fig. 3C). In the second minute of S1, a decreased %OT was observed in NL group when com-

Table 2
Three-factor (group × session × time bin) repeated measures ANOVA results from minute by minute analysis showing significant main effects and interactions

Parameters/factor	% Open entries	% Open time	Risk assessment	Enclosed entries
Group (df = 3,64)	$F = 12.8$; $P < 0.00001$	$F = 12.8$; $P < 0.00001$	$F = 8.1$; $P < 0.0001$	$F = 3.2$; $P < 0.05$
Session (df = 1,64)	$F = 79.8$; $P < 0.00001$	$F = 86.4$; $P < 0.00001$	$F = 6.6$; $P < 0.05$	$F = 5.4$; $P < 0.05$
Time bin (df = 4,256)	$F = 14.3$; $P < 0.00001$	$F = 27.1$; $P < 0.00001$	$F = 14.8$; $P < 0.00001$	$F = 36.7$; $P < 0.00001$
Group × session (df = 3,64)	NS	NS	NS	NS
Group × time bin (df = 3,64)	NS	NS	NS	NS
Session x Time bin (df = 4,256)	$F = 6.5$; $P < 0.001$	$F = 13.7$; $P < 0.00001$	$F = 7.2$; $P < 0.00001$	$F = 11.4$; $P < 0.00001$
Group × session × time bin (df = 12,256)	NS	$F = 1.8$; $P < 0.05$	$F = 1.6$; $P = 0.086$	NS

The same conditions apply as in Table 1. df, Degrees of freedom; NS, not significant.

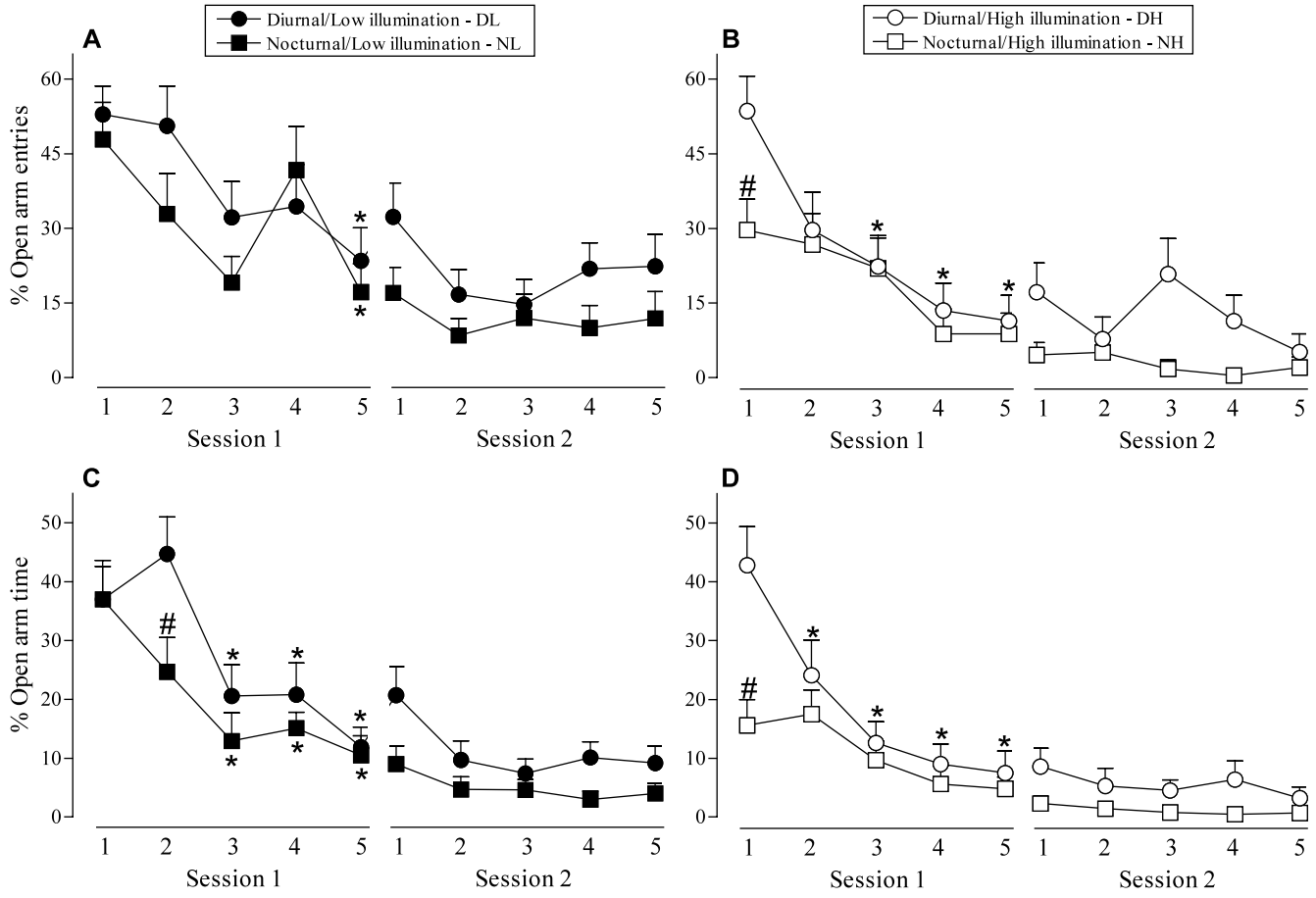

Fig. 3. Minute by minute analysis of the circadian phase and illumination condition effects on the percentage of entries (A) and of time spent (B) in open arms, in rats submitted to the elevated plus-maze (EPM) in both session 1 (S1) and session 2 (S2), revealed by three-factor (group × session × time bin) repeated measures ANOVA followed by post-hoc Newman–Keul's test ($P < 0.05$). Data are presented as mean ± S.E.M. *Statistical difference from first minute in same group and session; # statistical difference between circadian phases.

pared to the DL group (Fig. 3C). This measure was decreased in the DH group from the second to the fifth minute in S1 (Fig. 3D). In NH group, we also observed a decreased %OT in the first minute of S1, when compared to DH group (Fig. 3D). Although full session analysis showed a decreased open arm exploration in the NH group in S2, minute by minute analysis failed to show the expected decrease in either %OE or %OT throughout S1, as observed for other groups. No differences regarding circadian phase or illumination conditions were detected with data obtained in S2 (Fig. 3A–D).

Data depicted in Fig. 4 show the Newman–Keul's analysis of the results obtained using RA and enclosed arm entries data. RA behavior was decreased for the NL group from the third to the fifth minute (Fig. 4A), as well as for the DH and NH groups from the second to the fifth minute in S2 (Fig. 4B). Minute by minute analysis revealed an increased RA behavior under high illumination in the first minute of S2, when compared to the last minute of S1 (Fig. 4B). Post-hoc comparisons showed that enclosed arm entries were decreased in the fifth minute in the DL group and from the second to the fifth minute in the NL group in S2 (Fig. 4C). Furthermore, there was a decrease in the first minute of S2 in DL, when compared to the NL group (Fig. 4C). However, note that minute by minute analysis also revealed an increased enclosed arm entries in the first minute of S2, when compared to the last minute of S1 (Fig. 4C,D).

4. Discussion

It has been demonstrated that circadian rhythm influences cognitive, physiological and behavioral patterns [4,15,37,42,56] as well as drug effects [38,41,44,54] in both humans and animals. Moreover, behavioral responses in rodents may be altered by illumination conditions [9,18,22,27,35], which adds to the problem of inter-laboratory variability related to this animal model of anxiety.

In the present study, regardless of the circadian phase and the illumination condition, full session analysis

showed that prior exposure to the EPM decreased open arm exploration for all groups in S2. Our results are in agreement with previous studies showing that, in maze-experienced mice, the %OE and the %OT are decreased, suggesting a qualitative shift in emotional state elicited in S2, realized 24 h later in S1 [24,25]. Although a similar S1–S2 transition behavioral profile was detected in all groups, a comparison within groups revealed various circadian phase–illumination conditions interactions. Subjects from NL group showed a decreased open arm exploration when compared to the DL group, in both sessions, suggesting that under low illumination there is a circadian phase effect. In addition, open arm exploration was decreased in NH group, when compared to subjects from DH and NL groups in S1, suggesting circadian phase and illumination condition effects, respectively. Indeed, our results are in accordance with studies showing that high illumination decreases open arm exploration in rats submitted to the EPM during the diurnal phase [9,18,22,35]. By contrast, Jones and King [27] failed to show any statistical difference in open arm exploration in Sprague–Dawley rats submitted to the EPM. In our view, these discrepancies could be related to some important differences between our study and theirs, such as previous maze experience (naïve versus maze experienced rats in an EPM-like model), circadian phase (normal versus 2 weeks-reversed light cycle), light level (low = 44 vs. 9 lux and high illumination = 600 vs. 297 lux), age (13–15 vs. 10 weeks) as well as housing conditions. Further, as behavioral responses of rats submitted to the EPM may also be influenced by variables related to the maze construction, such as dimension and surface of the maze and height of ledges around the open arms [23], we could not conclude that these contradictory results may be exclusively reflecting a circadian phase effect. Further minute by minute analysis showed a progressive reduction in open arm exploration (mainly %OT) for DL, NL and DH groups, starting around the second or third minute of S1 and persisting throughout S2. These results agree with the idea that after an initial overall exploration, the subjects avoid the open arms starting around the third minute of S1 [25]. The NH group did not exhibit any minute by minute effect, probably due to the combination of high exploration circadian phase with high aversive illumination conditions, resulting in a higher fear-exploratory drive.

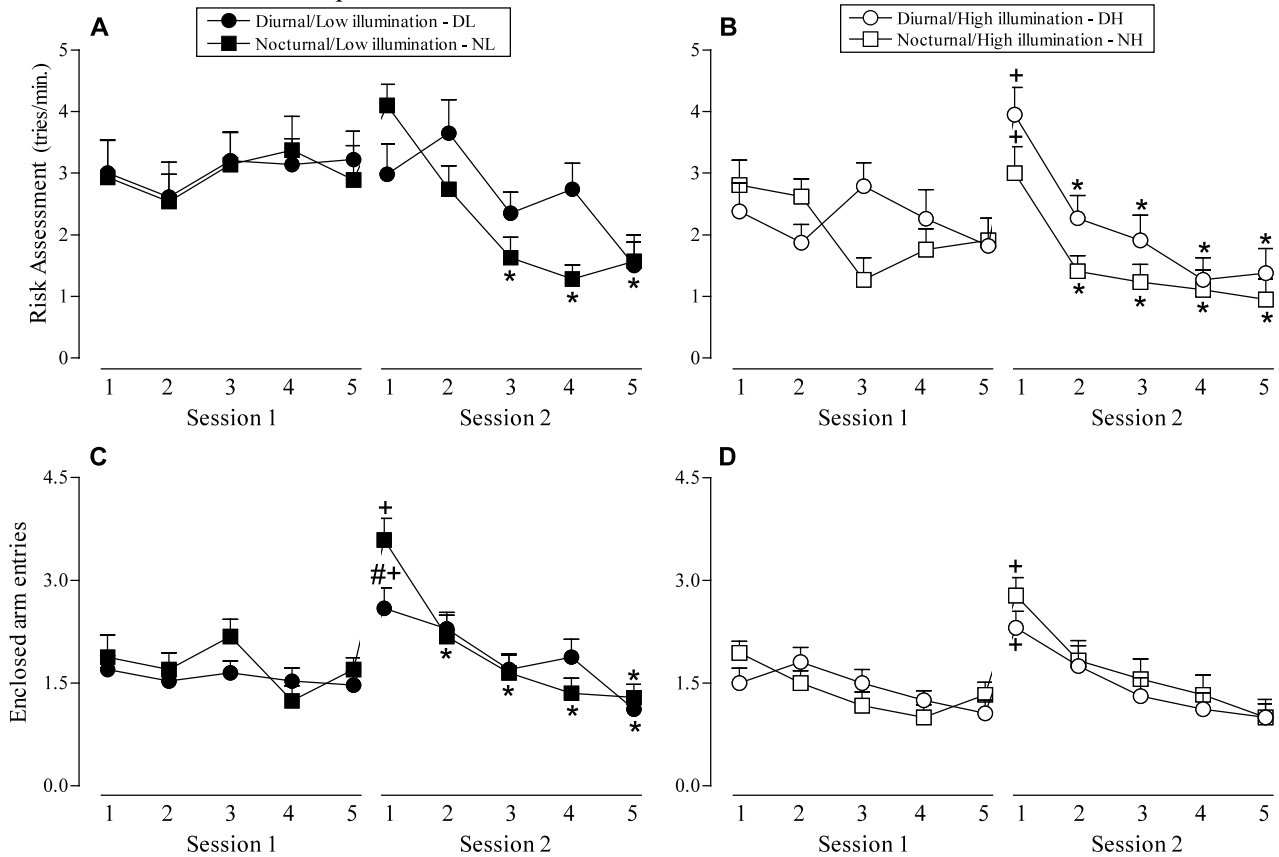

Fig. 4. Minute by minute analysis of the circadian phase and illumination condition effects on risk assessment behavior (A) and enclosed arm entries (B) in rats submitted to the elevated plus-maze (EPM) in both session 1 (S1) and session 2 (S2), revealed by three-factor (group × session × time bin) repeated measures ANOVA followed by post-hoc Newman–Keul's test ($P < 0.05$). Data are presented as mean ± S.E.M. *Statistical difference from first minute in same group and session; # statistical difference between circadian phases; + statistical difference from last minute of S1.

Nowadays, the EPM analysis has included, along with the traditional parameters, a variety of ethological measures related to RA behavior which allows a detailed behavioral description of the rodent performance, increasing sensitivity of the EPM in detecting the anxioselective effects of a wide range of drugs [13,25,43]. Full session data showed that RA behavior was decreased under high illumination for both circadian phases in S1, when compared to low illumination conditions. Subjects under high illumination also showed decreased open arm exploration, suggesting that the illumination condition account for the mainly aversive promoting factor for the EPM performance. Rodgers et al. [47] showed that the corticosterone response was correlated with measures of RA behavior, but not with measures of open arm or general locomotor activity, in rodents submitted to the EPM. It is also interesting to note that minute by minute analysis revealed an increased RA behavior, mainly under high illumination, in the first minute of S2 when compared to the last minute of S1. Thus, an increased RA, described as information-gathering behavior in potentially threatening situations [7], at the beginning of S2 may be optimizing the most adaptive behavioral strategy (open arm avoidance). The reduced RA throughout S2 suggests a decrease in the approach while increasing avoidance behavior, therefore confirming the aversion to the open arms. Similar findings have also been observed for the murine EPM [25] model and for the elevated T-maze model of anxiety in rats [49].

Our results during the nocturnal phase agree with previous studies [9,27] showing an increased general locomotor activity (enclosed arm entries) in rats submitted to the EPM under low illumination, when compared to high illumination in S1. Minute by minute analysis also revealed an increased enclosed arm entries, for all groups, in the first minute of S2 when compared to the last minute of S1, a feature similar to RA behavior, thus reinforcing the suggestion of the subject's initial risk assessment to acquire prior knowledge of the situation.

As pointed out by Morin [37], 5-HT may be modulating the sensitivity of the circadian rhythm to light, with a clear rhythm in its extracellular levels (the maximum occurring during the onset of the nocturnal phase-darkness). It is thought that increased 5-HT levels in limbic forebrain areas may produce an enhanced anxiety state because 5-HT_{1A} agonists that reduce 5-HT release in these areas concomitantly decrease fear-potentiated behavior in the EPM [31]. In line with this evidence, Rodgers et al. [44] found in mice that LY297996, a 5-HT_{1A} antagonist, was anxiolytic in the mid-dark phase but not in the mid-light phase and Handley and McBlane [22] showed that the same dose of the 5-HT_{1A} agonist 8-OH-DPAT produced anxiolytic or anxiogenic effects under high or low illumination conditions, respectively. Analyzing the data in the literature and the results obtained in this study, the reduced open arm activity observed in the nocturnal phase, intensified under high illumination conditions, could represent increased 5-HT neurotransmission in various areas of the brain, including the forebrain, where 5-HT activation is related to fear-potentiated behavior in the EPM.

In summary, the results of the present study confirm that prior exposure to the EPM decreased open arm exploration for all groups in S2, regardless of the circadian phase or illumination condition. However, subjects tested during the nocturnal phase decreased open arm exploration when compared to the diurnal phase in both illumination conditions. Further minute-by-minute analysis also showed the presence of a progressive avoidance to the open arm, starting around the second or third minute of S1, which persisted throughout S2. RA behavior was decreased in rats tested under high illumination, for both circadian phases in S1. Further analysis also revealed that it was increased in the first minute of S2 when compared to the last minute of S1, thus reinforcing the most adaptive behavioral strategy (open arm avoidance). Although open arm exploration and RA behavior were decreased in rats tested under high illumination in both circadian phases, when compared to low illumination conditions, general locomotor activity was only decreased during the nocturnal phase (active period), suggesting that general EPM exploration is circadian phase dependent. Based on this fact, investigators should be aware of the potential influence arising from the rat's active versus resting period. Taken together, the results support the idea that rodent circadian variation could be a possible source of variability in the EPM. Further experiments are currently assessing whether these changes in behavior profile also reflect circadian variation in drug effects.

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