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Does behavioral response to novelty influence paw withdrawal latencies in repeated Hargreaves test?

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Abstract

Background and Purpose: *Only recently, we have reported that single retesting session significantly decreases rat paw withdrawal latencies (PWL) in Hargreaves tests. We wondered, whether decrease in PWL values obtained during reexposure to Hargreaves test might be associated with reaction to the new environment. Therefore, we investigated PWL together with the open field behavior in an enclosure of the Hargreaves test device during the period of 3 subsequent days.*

Materials and Methods: *Ten male Wistar rats were tested once a day. Each rat was first exposed to the open field behavior test for five minutes and then to the Hargreaves test. Rearing (vertical movements), grooming (animal cleaning its face or body), defecation, the distance travelled, time mobile and time immobile were recorded. Data were analyzed using one way ANOVA and Bonferroni test (PWL) or Kruskal Wallis and Mann Whitney U tests (open field behavior).*

Results: *PWL significantly declined from the first to the second and third exposure to Plantar test device (ANOVA, $P< 0,01$; Bonferroni $P< 0,05$). Decline in PWL values was accompanied with concomitant increase in grooming (Kruskal Wallis $P< 0.01$; Mann-Whitney U test $P< 0.05$), decrease in rearing behavior (Kruskal Wallis $P< 0.001$; Mann-Whitney U test $P< 0.001$) and defecation during retesting sessions. Conclusion. Decrease in the PWL values obtained during reexposure to Hargreaves test device was accompanied by alterations in behavioral reactions to the new environment. Time course and direction of these changes suggest that reaction to novelty might be related to decrease in PWL values observed during retesting sessions of the Hargreaves test.*

INTRODUCTION

By definition, »…open field test is an enclosed open arena where an animal is placed and some form of behavior, usually activity, is measured *(1)*.« The open field test was originally developed by comparative psychologists *(2, 3)*. Its use has gradually spread out from psychology to neuroscience and psychopharmacology. Nowadays it is one of the most widely used tests in behavioral research. Basically, in the open field test small rodents were subjected to naturally aversive situation by placing an individual animal in the center of an unknown open arena for a fixed amount of time. There is no chance for animal to escape, since open arena is surrounded by walls. Complex behavioral reactions produced by exposure to this aversive environment could be interpreted as indicators for exploration, locomotion, emotionality and fear *(4)*. Hargreaves test is widely used for assessing tolerance to thermally induced pain in rats. Pharmacologists successfully use this test for revealing analgesic drug action and for predicting their analgesic effect in humans. Another popular pain tests, Randall Sellito test *(5),* the tail flick test *(6),* and the hot plate test *(7)* are all sensitive to the training phenomenon. In these tests the pain response decreases with repeated exposure of animals to experimental conditions *(8)*. Only recently, we reported that Hargreaves test is also susceptible to the training phenomenon *(9)*. On the second exposure to Hargreaves test, we noticed almost 30% decrease in paw withdrawal latencies (PWL). As far as we know, this was the first report suggesting the effect of training on Hargreaves test results. During these experiments, we became aware of the possibility that reaction to novelty might be of importance for elucidation of these findings. As first, in this study we used naive rats, unfamiliar to Hagreaves test device. Besides, transparent enclosure of Hargreaves test device could be considered as a small, enclosed open arena. Finally, it was obvious that a decrease in PWL on the 2nd and 3rd exposure to Hargreaves test was accompanied with decrease in the movements of animals along Hargreaves test device. We had strong impression that vertical movements were especially reduced. These simple experimental observations encouraged us to suggest that decrease in rat PWL values obtained during repeated exposures to Hargreaves test might be associated with aversion to open and unfamiliar environment of the Hargreaves test device. To test this hypothesis, we decided to study paw withdrawal latencies together with the open field behavior in enclosure of the Hargreaves test device throughout the period of 3 subsequent days.

MATERIAL AND METHODS

Animals

Ten male Wistar rats obtained from the Charles River Laboratory, Italy, were used to collect the data. The rat weight was 0.41–0.53 kg at the time of testing. Tap water and standard rodent chow were available *ad libitum*. Rats were handled on the daily base during acclimatization and testing period. The experiments reported here were approved by the Croatian Ministry of Agriculture, Forestry and Water Management and by the institutional Animal Care and Ethics Committee. In addition, international guidelines on ethical standards for investigations of experimental pain in animals were followed *(10)*. All efforts were made to reduce the number of animals used and to minimize animal pain and distress. The heat stimuli emitted by device were tested on the author's hands. Author's experienced only brief sense of pain followed with mild discomfort.

Hargreaves test

Paw withdrawal latencies to radiant heat are measured by using the method originally described by Hargreaves *(11)*. The method used here was slightly modified, as we described recently *(9)*. All measurements were done using commercially available Hargreaves test (Plantar test, Ugo Basile, Italy). Paw withdrawal latencies were defined as the time in seconds from the activation of radiant heat source to the withdrawal of rat hind paw as the result of pain. A simple protective flexion movement of irradiated hind limb was used as behavioral end point. Other behavioral responses related or not related to pain stimuli were not counted. Two PWL values were obtained alternatively from each hind paw within a three minute interval. Individual PWL values were determined as the mean of four measurements on both paws.

Open field behavior

The open field arena used in our study was a transparent, plastic cage of the Hargreaves test device (22 cm x 17cm with 14-cm-high walls). The Web camera (Logitech, Quick cam Pro 5000, USA) used for video capturing of behavior was positioned 59 cm above the center of the open arena floor. From this position, the entire arena was in the zone of camera's view. The camera equipped with Carl Zeiss lens and wide angle view technology was able to produce 640x480 VGA video. Video tracking software (ANY-maze, Stoelting, USA) was used for detection and tracking of animal behavior inside the open field arena. Before the experiment was actually performed, the software had been used for setting up a tracking protocol. Basic elements of tracking protocol used in the experiments presented here were as follows:

Apparatus – Dimensions of the open field arena were automatically calculated from manual drawing of apparatus map. In our case the dimensions were 22 x 17 cm (the floor of a Hargreaves test cage).

Zones – Apparatus map can be split into discrete zones. Considering the size of the cage and animal size, as well as the objective of the study, we did not divide apparatus map into zones. Therefore, behavioral measures were scored for the apparatus as a whole.

Beginning, end and duration of test – Individual animal was placed in the center of open arena. The test started manually by pressing S key on the computer keyboard and ended automatically after five minutes. The rat was removed from the open field arena and the arena was cleaned with 70% ethanol solution before the next rat was tested.

Detection of movement – In our study animal movements were detected by tracking the animal's center point.

Definition of immobility – Sensitivity was set at 70% (70% of animal body had to remain in place to be considered immobile). The animal had to remain immobile for 2 seconds in order for the Any maze software consider it as immobile.

Keys – By pressing keys G, R or B on the computer keyboard, the number of grooming, rearing and fecal boli were scored.

Any maze software was also used for analysis and report of results, as well as for saving and reloading of both the results and the tracking protocol. The following behavioral parameters were obtained:

Rearing – The number of times the animal started to rear (moved vertically with hind limbs on the floor) during the test.

Grooming – The number of times the animal started to groom (cleaned its face with forepaws, or cleaned, licked and scratched the various parts of own body) during the test.

Fecal boli – The number of times the animal excreted feces during the test.

Distance traveled – Total distance (in meters) that the animal traveled during the test.

Time immobile – Total amount of time (in seconds) the animal was immobile during the test.

Time mobile – Total amount of time (in seconds) the animal was mobile during test.

Experimental design

Two weeks before the start of the experiment rats were transferred from the animal room into the experimental room for acclimatization to conditions in the experimental room (constant temperature and humidity, 12/12 hours light/dark cycle starting at 07:00). All behavioral measurements were conducted inside the experimental room, during summer season. Only one person handled animals and operated the Hargreaves test instrument throughout the experiment. Another person always operated the Any maze video tracking system. The experimental room was not entered by other persons during testing sessions. The entire room was illuminated with blue, fluorescent light. Naive rats were used, unfamiliar with the Hargreaves test device and testing procedure. Animals were first exposed to the open field test and five minutes later to the Hargreaves test. Both tests were performed once a day, during 3 subsequent days, in the light phase of the day, always between 09 a.m. and 01 p.m.

Statistical analysis

Mean values of paw withdrawal latencies for 3 consecutive testing sessions analyzed using one way ANOVA and Bonferroni post hoc multiple comparison test. Unlike the paw withdrawal response data, which followed a normal Gaussian distribution, the data obtained for open field behavior differed from the Gaussian distribution based on the Kolmogorov-Smirnov test. The open field data were therefore analyzed with the nonparametric Kruskal Wallis and Mann Whitney U tests. Although the open field data were statistically analyzed in a manner described before, the same data in figures were presented in their untransformed form, directly obtained from the Any maze software. Comparisons between the amount of time the animals spent mobile and the amount of time they spent immobile during the test were determined for 3 subsequent days, by using one-sample t-test. In all cases $P < 0.05$ was used as the criterion for significance.

RESULTS

Hargreaves test

Paw withdrawal latencies to radiant heat were shortened from 12.2±0.9 seconds (mean ± SEM) on the first day, to 9.4±0.5 seconds on the 2nd day and 9.6±0.2 seconds on the 3rd day of testing period (Figure 1). As indicated by one way Analysis of variance (ANOVA), a decline in paw withdrawal latencies observed on day 2 and day 3, when compared to day 1, reached the statistical significance ($p< 0.01$ ANOVA, $F (2.27) = 6.31$, $p< 0.05$ Bonferroni). There was no significant change in PWL from the second to the third exposure to the Hargreaves test device.

Open field behavior – rearing and grooming

As shown in Figure 2. The number of rearing episodes recorded in testing cage of the Hargreaves test device

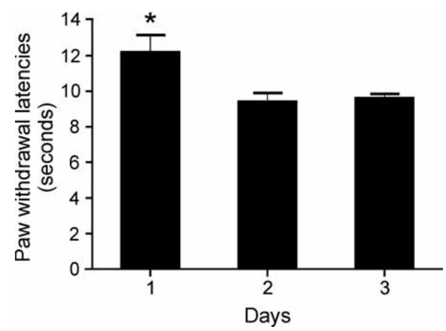

Figure 1. *Pain response to radiant heat measured throughout the period of 3 subsequent days. Male Wistar rats were used. Data were collected using Hargreaves test. Each column represents the mean ± SEM. N = 10 for each daily session. *P< 0.05 day 1 versus days 2 and 3.* Paw withdrawal latencies*: time in seconds from the activation of radiant heat source to the withdrawal of rat hind paw as a consequence of pain.*

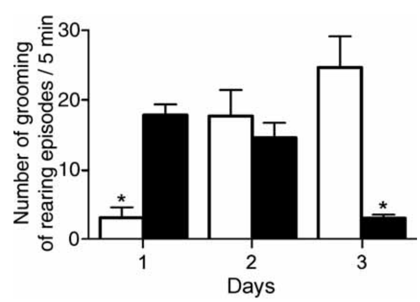

Figure 2. *Grooming and rearing behavior recorded in the testing cage of the Hargreaves test device throughout the period of 3 subsequent days. Male Wistar rats were used. Data were collected and analyzed using a web camera and Any maze tracking software. Each column represents the mean ± SEM of total the number of rearing (■) or grooming (□) episodes recorded during a 5 minute test. N = 7 (day 1); N = 10 (day 2 and 3). (■) *P< 0.01 day 3 versus days 1 and 2. (□) *P< 0.05 day 1 versus days 2 and 3.* Rearing episode: *Animals moved vertically with hind limbs on the floor of the Hargreaves test device.* Grooming episode: *Animal cleaned its face with forepaws or washed, licked and scratched the various parts of own body.*

TABLE 1

Locomotion and defecation recorded in the testing cage of the Hargreaves test device throughout the period of 3 subsequent days.

	Open field behavior			
	Distance (m)	Time mobile (s)	Time immobile (s)	Defecation*
Day 1	2.11 ± 0.50	61 ± 15	**239 ± 15	9
Day 2	1.90 ± 0.58	51 ± 15	**249 ± 15	8
Day 3	1.46 ± 0.38	45 ± 16	**255 ± 16	0

Male Wistar rats were used. Data were collected and analyzed using a web camera and Any maze tracking software. *Results were expressed as the total number of times the animals excreted feces during the test. Other results are presented as the mean ± SEM. N = 7 (day 1); N = 10 (day 2 and 3). **P< 0.001 immobile versus mobile time. *Distance* – Total distance (in meters) that the animals traveled during the test. *Time immobile* – Total amount of time (in seconds) the animals were immobile during the test. *Time mobile* – Total amount of time (in seconds) the animals were mobile during the test.

decreased from 18±1.5 (mean ± SEM) and 15±2.1 on the first and second day, to 3.1±0.48 on the 3rd day of the testing period. Kruskal Wallis test indicated a significant decline in the number of rearing responses during the third testing session, when compared to the first and the second testing session (P< 0,001 Kruskal Wallis, Chi-Square = 17.52; P< 0,001 Mann-Whitney U test). At the same time, the number of grooming episodes increased (Figure 2) from 3.1±1.5 (mean ± SEM) on the first day, to 18±3.8 on the 2nd day and 25±4.5 on the 3rd day of the testing period. The animals displayed significantly less grooming behavior during the first exposure to the testing cage of the Hargreaves test device as compared to the second and third retraining sessions (P< 0.005 Kruskal Wallis, Chi-Square = 10.85; P< 0.05 Mann-Whitney U test).

Open field behavior – ambulatory locomotion and defecation

As shown in Table 1, the animals spent more than 80% of testing time in motionless position throughout the testing period. One sample t-test indicated a significant difference between mobile and immobile time for the first (P<0.001 t-test; t = 5.57), second (P<0.001 t-test; t = 6.02) and third (P<0.001 t-test; t = 5.65) exposure to the Hargreaves test device. Moreover, on the day by day basis, mobility time measured in the open arena showed a tendency to decrease (from 61±15 seconds on the first day, to 51 ±15 seconds on the 2nd day and 45 ±16 seconds on the 3rd day), and immobility time to increase (from 239 ± 15 seconds on the first day, to 249 ± 15 seconds on the 2nd day and 255 ± 16 seconds on the 3rd day of the testing period). However, these tendencies failed to reach statistical significance. The distance traveled recorded in the testing cage of the Hargreaves test device was slightly shortened from 2.1±0.5 and 1.9±0.6 meters on the first and 2nd day to 1.5±0.4 meters on the 3rd day of the testing period (Table 1). Again, this difference did not reach the statistical significance. Finally, as shown in Table 1, pronounced decrease in defecation was observed on the last testing session (the number of fecal boli = 0; N=10), as compared to the first (the number of fecal boli = 9; N=7) and the second testing session (the number of fecal boli = 8; N=10). Practically, defecation ceased at the end of the testing period.

DISCUSSION

Pain response to radiant heat decreased on the first retraining session and remained stable on the second retraining session (Figure 1). These results are in agreement with our own results published recently *(9)*. Time course, direction and intensity of changes in paw withdrawal latencies were very similar in those two studies. Basically, we confirmed our previous findings in an independent experiment done with another 10 animals. The results presented here are in agreement with those published by Anseloni and coworkers *(12)* and Taiwo *et al. (13)*. A decrease in paw withdrawal latencies was accompanied with pronounced changes in open field behavior recorded inside the Hargreaves test device. Elevated defecation rate, high frequency of rearing and low frequency of grooming were obtained on the first exposure of animals to the aversive environment of open arena. Paw withdrawal latencies, frequency of rearing and defecation rate decreased, while grooming increased with repeated exposure to open arena (Figure 2, Table 1). Those were the main findings of the present study. As mentioned before, exposure of small rodents to an unknown open arena produces a very complex pattern of behavioral reactions: ambulatory locomotion (distance traveled, mobile/immobile time), defecation, urination, freezing (resting immobile), rearing, grooming, sniffing (air and floor of the open arena), tigmotaxis (wall seeking behavior), time in the center, latency to leave the center, jumping, attempts to escape, vocalization, etc. *(14)*. Expression of a particular type of open field behavior in small rodents depends on many factors: motivation (food and water presence inside the open arena or food and water deprivation), level of illumination and noise (in experimental room and open arena), intensity of aversion (presence of shelters, electric shock, odor of a predator), familiarity with the open arena (single exposure, repeated testing), housing conditions before open field test, sex of animals, strain, etc. *(15)*. Moreover, literature review reveals a great variability in the shape (circular, rectangular, square) and size (from 20 cm to an entire room) of the open arena used by different authors. This should be taken into account when comparing results from different laboratories and interpreting results of open field behavior in terms of emotionality, fear/anxiety, exploration and novelty stress. From the original description of open field method, high defecation rate and low level of ambulatory locomotion were commonly used and accepted to indicate fear/emotionality response of the rat to a new, potentially dangerous environment *(14)*. Defecation rate should decrease and ambulatory locomotion increase with repeated exposure to the open field test *(16)* In our study, pronounced decrease in defecation rate was observed in the last testing session, as compared to the first and second testing session (Table 1). It seems that our rats were going through biphasic emotional response. At the beginning of the testing period, the animals manifested fear/high emotional response to the Hargreaves test device. But, at the end of testing period, rats became »fearless« and clearly expressed low emotional response to the Hargreaves test device. As evident by the total amount of immobile/mobile time (Table 1), continuously low level of ambulatory locomotion was recorded throughout the testing period. Locomotion did not increase with repeated exposure of rats to the Hargreaves test device in our study, as it could be expected. Since we used large animals (400 – 500 g) in a relatively small open arena (22 x 17 cm), horizontal movements of animals were limited. This lack of space for horizontal movements probably makes ambulatory locomotion inadequate as an indicator of emotionality in our study. Rearing behavior is considered to be a measure of exploratory activity in the open field test *(17)* It is positively correlated with defecation rate. With repeated exposure to the open arena, rearing behavior should decrease *(14)*. In our study, rearing behavior gradually decreased throughout the testing period. These results clearly showed that rats expressed high level of exploratory activity in unfamiliar environment. During retraining sessions, rats lost interest for exploration of the open arena as the consequence of habituation to the Hargeaves test device. It should be emphasized that exploratory behavior showed the same response as emotional activity (defecation). On the contrary, grooming behavior gradually increased, while defecation rate and rearing decreased from the start to the end of the testing period (Figure 2). Increased grooming behavior can be viewed as indication of stress to environment *(18)* and restrain *(19)* in rodents. If we accept this view, gradual increase in grooming behavior throughout the testing period implies gradual increase in the amount of stress. In this case, defecation rate, as an indicator of fear/emotionality should have increased, not decreased. Yet, we observed a decrease in defecation rate at the end of testing period (Table 1). Therefore, we assumed that grooming behavior, in our testing condition, was not a measure of stress. We believe that grooming behavior, in our testing conditions, might be a measure of locomotion. Vertical movements of animals (grooming and rearing) were not limited in the Hargreaves test enclosure because of 14 cm high walls. As we mentioned before, locomotion and defecation should be negatively correlated. This means, with repeated exposure to open arena defecation decreases, and locomotion (expressed as grooming behavior) increases.

In conclusion, results presented here suggest that exploratory behavior and fear/emotionality are positively correlated with paw withdrawal latencies on repeated exposure to the Hargreaves test device. Further experiments are needed to elucidate the neurochemical background of this phenomenon.

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REFERENCES

1. CHOLERIS E, THOMAS A W, KAVALIERS M, PRATO F S 2001 A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. *Neurosci Biobehav Rev 25*: 235–260
2. YOSHIOKA J G 1932 Learning versus skill in rats. *Jour Genet Psychol 41*: 406–416
3. HALL C S J 1936 Emotional behavior in the rat III. The relationship between emotionality and ambulatory activity. *J Comp Psychol 22*: 345–352
4. ANTONIOU K, PAPATHANASIOU G, PANAGIS G, NOMIKOS G G, HYPHANTIS T, PAPADOPOULOU-DAIFOTI Z 2004 Individual responses to novelty predict qualitative differences in *d*-amphetamine-induced open field but not reward-related behaviors in rats. *Neurosci 1230*: 613–623
5. RANDALL L O, SELITTO J J 1957 A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther 111*: 409–419
6. D'AMOUR F E, SMITH D L 1941 A method for determination of pain sensation. *J Pharmacol Exp Ther 72*: 74–79
7. WOOLFE G, MCDONALD A D 1944 The evaluation of analgesic action of pethidine hydrochloride (Demerol). *J Pharmacol Exp Ther 80*: 300–307
8. LE BARS D, GOZARIU M, CADDEN S W 2001 Animal models of nociception. *Pharmacol Rev 53*: 597–652
9. KOCEVSKI D, TVRDEIĆ 2007 The effect of repeated daily measurements on paw withdrawal latencies in Hargreaves test. *Coll Antropol 3 (Suppl 3)*: 515–519
10. COVINO B J, DUBNER R, GYBELS J, LIEBESKIND J C, STERNBACH R A, VYKLICKY L, YAMAMURA H, ZIMMER-MANN M 1980 Ethical standards for investigations of experimental pain in animals. *Pain 9*: 141–143
11. HARGREAVES K, DUBNER R, BROWN F, FLORES C, JORIS J 1988 A sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain 32*: 77–88
12. ANSELONI V C Z, ENNIS M, LIDOW M S 2003 Optimization of the mechanical nociceptive threshold testing with the Randall-Selitto assay. *J Neurosci Meth 131*: 93–97
13. TAIWO Y O, CODERRE T J, LEVINE J D 1989 The contribution of training to sensitivity in the nociceptive paw-withdrawal test. *Brain Res 487*: 148–151
14. RAMOS A, MORMEDE P 1998 Stress and emotionality: a multidimensional and genetic approach. *Neurosci Biobehav Rev 22:* 33–57
15. WALSH R N, CUMMINS R A 1976 The open-field test: a critical review. *Psychol Bull 83*: 482–504
16. HALL C S 1936 Emotional behavior in the rat I. Defecation and urination as measures of individual differences in emotionality. *J Comp Psychol 22*: 385–403

17. ERDOĞAN F, GÖLGELI A, ARMAN F, ERSOY A Ö 2004 The effects of pentylenetetrazole-induced status epilepticus on behavior, emotional memory and learning in rats. *Epilepsy Behav 5*: 388–393

18. MIDZYANOVSKAYA I S, SHATSKOVA A B, SARKISOVA K Y, VAN LUIJTELAAR G, TUOMISTO L, KUZNETSOVA G D 2005 Convulsive and nonconvulsive epilepsy in rats: effects on behavioral response to novelty stress. *Epilepsy Behav 6*: 543–551

19. D'AQUILA P S, PEANA A T, CARBONI V, SERRA G 2000 Exploratory behaviour and grooming after repeated restraint and chronic mild stress: effect of desipramine. *Eur J Pharmacol 399*: 43–47