

Level of Vigilance Influences Licking Frequency in Rats

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Summary

In rats, the basic licking rhythm is generated by the central pattern generator located in the brainstem. Nevertheless, the licking frequency can be regulated between about 7.5 and 4 Hz by changing the drinking conditions. If these conditions are kept constant, the licking frequency can be influenced only to a minor degree by factors such as deprivation level, type of solution, and phase of the session. The aim of our study was to compare the licking frequency of rats at different levels of vigilance. We investigated spontaneous licking of rats by an electrical lick sensor; parallel behavior monitoring was also performed. Animals kept in a stable environment and showing a lower level of vigilance licked at a rate of 5.96 Hz, fully vigilant rats licked significantly more rapidly at a frequency 6.57 Hz. The fastest rate of licking (6.49 Hz and 6.82 Hz, respectively) was encountered in alert rats under a mild stress caused by the presence of a second animal in the experimental box. The vigilance level is thus another factor affecting the licking rate of rats that should be taken into account in behavioral licking experiments.

Key words

Central pattern generator • Drinking behavior • Licking frequency • Vigilance

Introduction

The stability of the licking frequency in rats has always been striking. For the first time the invariance of the rate of drinking in rats was noticed by Stellar and Hill (1952), who showed that rats either drink at a constant rate of 6 to 7 tongue licks per second or that they do not drink at all, regardless of the level of thirst. The volume of the water drunk increases after more severe deprivation (corresponding to the extent of water deprivation) but how much water a rat gets depends on how much time it spends in drinking. These observations were confirmed by others (Corbit and Luschei 1969). They admittedly described slowing down of the licking rate by improper placement of the drinking spout, reliable individual differences among subjects and the influence

of experience with the testing situation, but they concluded that the rate of licking in rats is highly stable.

Later, when previous results were examined by parametric analyses several factors were associated with statistically significant lick-rate variability (Cone 1974). In particular, it was the distance between the rat and the drinking tube that reduced the licking rate. Other authors confirmed this result (Weijnen 1977, Davis and Smith 1992). Diameter of the opening through which rat contacted the fluid is also an important factor of the drinking situation, because the lick rate is increasing significantly as a function of volume of fluid received per lick (Snyder and Hulse 1961). Thus the licking frequency could easily be manipulated between 7.5 and 4 Hz by changing access to the drinking spout (Weijnen 1998). If this configuration was kept constant, then the licking

frequency could only be influenced to a minor degree by factors such as deprivation level, type of solution and phase of the session (for review see Weijnen 1998). Individual differences also played some role (Keehn and Arnold 1960). These investigators did not exclude the influence of age of rats, as (7.03 licks per second in the first 5 days of the experiment and 7.52 licks per second a month later). Convincing relations of the licking rate and age was found in Mongolian gerbils in 26-day-old vs 200-day-old animals (Schaeffer and David 1973). On the other hand, no age-related shift in albino rats was described by Schaeffer and Premack (1961). No systematic relationship between sex and the licking rate was observed in infant rats (Schaeffer and Premack 1961), but a variability in licking frequency associated with sex was described in albino rats, females licking significantly more rapidly (6.41 Hz) than the males (5.75 Hz) (Cone 1974).

As far as we know, nobody has mentioned the influence of the stage of vigilance on licking. Three different factors could be listed which are closely related to the vigilance level: 1) The degree of experience with the testing situation (Corbit and Luschei 1969). Rats showed a relatively slow rate of drinking in initial tests but the rate gradually increased with continued testing. A similar observation accounts for the importance ascribed by others to testing in a novel or a familiar environment (Keehn and Arnold 1960). These authors found that the licking rate increases as a function of exposure to the test situation. 2) Circadian cycle. Licking rates in the *Virginia opossum* during their night high activity period and day low activity period were followed. Both sexes licked significantly faster at night than during the day. Similar results were obtained in albino rats, but the difference was statistically significant in females only (Cone 1974). 3) Drug administration. The basic licking rhythm is generated in the central pattern generator located in the pontobulbar reticular formation, specifically in the gigantocellular nucleus (Brožek *et al.* 1996). Drugs influencing nervous system had a major effect on licking frequency in rats. Chlorpromazine, pentobarbital, chlordiazepoxide and haloperidol always slowed down the licking rhythm (Knowler and Ukena 1973, Fowler and Wang 1998), whereas a different dose-dependent relation was seen with d-amphetamine (Knowler and Ukena 1973).

The aim of our study was to compare licking frequency of rats showing different levels of vigilance.

Methods

Apparatus

During each experimental session, rats were placed in a drinking box equipped with a spout accessible through a small opening in the cage wall (Fig. 1). The opening could be closed manually by a sliding door. Our modification of former Weijnen's equipment (Weijnen 1989) enabled us to minimize the restraint of the animal's access to the drinking tube. Nevertheless, the modified position of the outer orifice of the spout allowed the rat to contact it only by the tongue during drinking. The inner diameter of the metal spout was 3 mm and provided free access to water from the container of total volume 12 ml. The animal was placed on the metal floor and our lick recording method involves making the animal a part of an electronic circuit so that contact of the tongue with the fluid closes the circuit (Fig. 2). Rats appear to be unable to detect direct current in the sub- μ A range (Weijnen 1998) so that this approach is suitable for behavioral experiments. The electrical lick sensor accurately follows each contact of the tongue with the drinking tube. Its output was fed to an IBM compatible PC, recorded and microanalysis of drinking behavior was performed. Each event (contact duration, CD; intercontact interval, ICI and lick-lick interval, LLI) was added to one of thirty 10 ms bins. CD corresponded to the contact duration of the rat's tongue with the spout, LLI was the time from the onset of one tongue-spout contact to the onset of the next one and ICI was the interval between the termination of one tongue-spout contact and the beginning of the next one. The median value of the bin was then taken as the duration of the event. Sum of all event duration was calculated and the mean of CD and LLI during at least one 24-s lasting record was computed. Values differing by more than three standard deviations were excluded from the processing. ICI was calculated as the difference between LLI and CD.

Animals and procedures

Seventeen male hooded rats of the Long Evans strain were housed individually in the animal room at constant temperature (22 °C) and natural lighting. Experiments were performed in summer time. Every animal was deprived of water for 24 h on the first experimental day to warrant sufficient motivation. This level of deprivation was well tolerated by rats and the Animal Protection Expert Commission of the Second

Faculty of Medicine, Charles University Prague, approved these experiments. On the second day the rats were allowed to drink *ad libitum* from freely accessible drinking tube in an experimental box to be accustomed with the environment. On the third day, individual rats were trained to drink from the spout in a position enabling recording of licking in undistorted form (Fig. 1). Tap water was used in a volume of 7 ml per rat. All rats were randomly assigned to one of two experimental groups on the third day and on the fourth day the experimental sessions were performed.

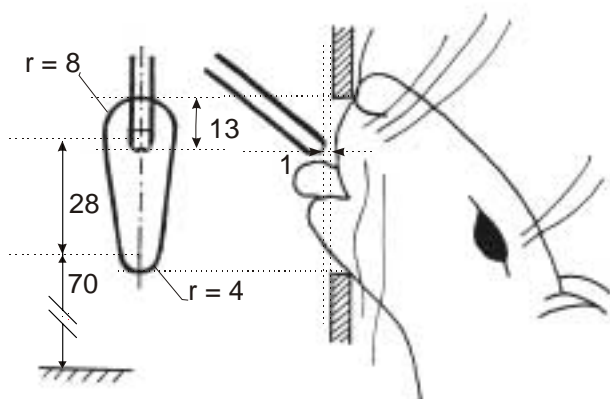


Fig. 1 Drinking setup. The position of the drinking tube enables a contact of the spout with the tongue only during drinking (modified after Weijnen 1989).

Animals of the first „low level vigilance“ group L (7 rats, average weight 484 g) were kept in the laboratory in natural day lighting. Noise background during the actual experiment was about 60 dB. Each animal of the group L was separately and repeatedly placed into the experimental box in a stable laboratory environment and without any external disturbances. The sessions of the first animal of group L started at 09:45 h and other animals followed at 2-min intervals. Next sessions of the first animal followed at 10:00, 10:15, 10:30, 11:00 and at 12:00 h. Two separate records were taken at 13:00 h. At the beginning, the animal was kept alone in the licking box just as in all previous cases and the first recording was made. Then another unknown thirsty animal (visiting) was added into the box so that the animals came into physical contact and the second record of licking of the former (home) rat was taken. The access of the visiting rat to the drinking tube could be manually prevented by the sliding door. Animals

followed in 5-min intervals in this last session so that the exact time of the seventh rat session was 13:30 h.

The second, „high level vigilance“ group H consisted of ten animals (average weight 474 g) and was kept in a neighboring busy room from the third day of the experiments. There was no stable sound background there and unexpected noises occurred irregularly. First, experimental licking of each rat was preceded by 5-min contact with another previously encountered animal (a rat from the same original breeding group) to ensure a high level of vigilance in the experimental rat. Then the rat was allowed to drink alone in the licking box and the first record was taken. Consequently, another hitherto unknown thirsty animal was admitted into the drinking box and was present there during the second recording. The session of the first animal of group H started at 13:00 h and other animals followed in 5-min intervals as in the last session of the L group.

Short 5-min lasting observations of behavior were made in rats of both groups at 8:55, 10:55 and at 12:55 on the fourth experimental day. The position of each animal was observed and described, in case of locomotor activity its length was monitored. We observed how many times a rat crossed the cage and this number was multiplied by the length of the cage (39 cm). The individual breathing frequency of each animal was measured visually by observation of breathing movements during one minute. We also noted open or close eyes, whisking, grooming, exploration movements, sniffing etc.

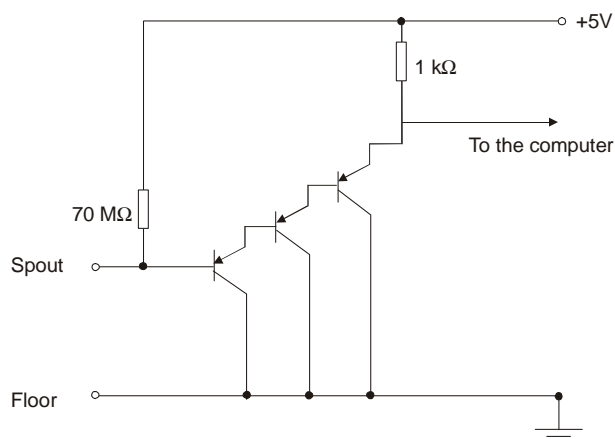


Fig. 2. A circuit of the lick sensor (modified after Weijnen 1977).

Statistical analysis of the data

One-way repeated measures ANOVA was used to compare the mean CD, LLI and ICI of all group

L sessions. All pairwise multiple comparison procedures were made by Newman-Keuls *post hoc* method. The paired t-test was used for statistical analysis of group H results and t-test for analysis between corresponding L and H group sessions. Comparison of the breathing frequency was done by paired the t-test. All evaluations were made by the Sigmastat program (Jansen).

Results

Five animals of group L did not show any locomotor activity at 8:55 h. They lay still but their eyes were open and their position obviously required retained muscle tonus. Two animals performed locomotion for 0.8 and 1.6 m, respectively. Mean breathing frequency of all animals was 104 ± 5.7 per minute (mean \pm S.E.M.). All animals lay in a position enabling muscle relaxation at 10:55 h. No locomotion was noted during 5-min observation, breathing frequency was 91 ± 8.7 per minute. Similar behavior was observed at 12:55 h with an even lower breathing frequency (75 ± 6.0 per minute; significantly different from the frequency at 8:55 h, $P=0.01$). Animals lay in a rolled up positions typical for rest and sleep. Some of them had closed eyes and no whisking was observed.

Animals of group H were not allowed to rest by frequent disturbances. Their behavior activities were observed at 08:55 h: grooming, exploration movements with sniffing and feeding. Rats were either lying or showing locomotion or motor activity at the place. Their breathing rate was 130 per minute or more. Somewhat lower activity was observed at 10:55 h. Five rats were lying still, four moved at the lace, and only one rat changed its location in the cage. Another animal was added into each cage immediately before the session at 13:00 h. The visiting animal always exhibited exploration activity with sniffing in the cage and sniffing the air from their neighborhood through the perforated parts of the cage. Locomotion, sometimes even over the other animal, and digging the bedding were also observed. The experimental (home) animals sniffed the visiting rat every time, especially its anal region as well as genital and abdominal regions, face and tail. Home animals always contacted the back, head and shoulders of the visiting animal with their forelimbs. In home animals, we observed face washing and grooming of the hips,

forelimbs and belly, as well as sniffing of the air coming from their neighborhood through the perforated parts of the cage. Rarely, digging the bedding, stretching and watching the investigator were observed. Home animals moved within a mean length of 3.9 ± 0.2 m per animal per 5 min. Their breathing rate could not be counted – more than 130 per min. They were put into the licking box when fully vigilant.

Figure 3 gives an overview of main results. Five lick records per animal of group L were made between 09:45 and 11:00 h (L1-L5). The mean duration of LLI was 158 ± 3 ms. Frequency of licking varied in the range 156-159 ms and was very stable. Longer part of this time – ICI – was 89 ms on the average (range 86-90 ms). CD was shorter, being 69 ms on the average (range 68-70 ms).

ANOVA of the LLI mean values of group L showed a significant main effect of sessions ($F(6, 42) = 8.57, P < 0.001$). LLI in rats of group L was significantly longer at 12:00 h (165 ms) in comparison with the first record at 09:45 h (159 ms, $P=0.009$). This difference additionally increased at 13:00 (LLI 168 ms, $P=0.001$). This prolongation coincided in time with more conspicuous behavioral signs of somnolence. LLI of group H was 152 ms at 13:00 h and was not significantly different from the control record of group L at 09:45 h. However, we found a significant difference between both groups at 13:00 h ($P=0.011$). Animals of both groups had an unknown partner in the experimental box in the last session. Although it was only a few minutes after the previous session, the licking frequency in the group L was substantially more rapid (paired t test, $P < 0.001$) than the previous one. It was even the fastest of all records of group L, but did not differ significantly from the first record at 09:45 h. A smaller but still significant difference was found in the group H ($P=0.017$). Groups L and H did not differ from each other under the conditions when two animals were present in the licking box.

No difference in CD was found within the group H and L, respectively, in individual sessions (H7 and H8, L1-L8), as well as between groups H and L in the same sessions (L7 vs H7, L8 vs H8). ICIs in both groups copied the course of LLIs, and except the result of group L at 12:00 h the statistical significance was also the same as in LLI (Fig. 3B).

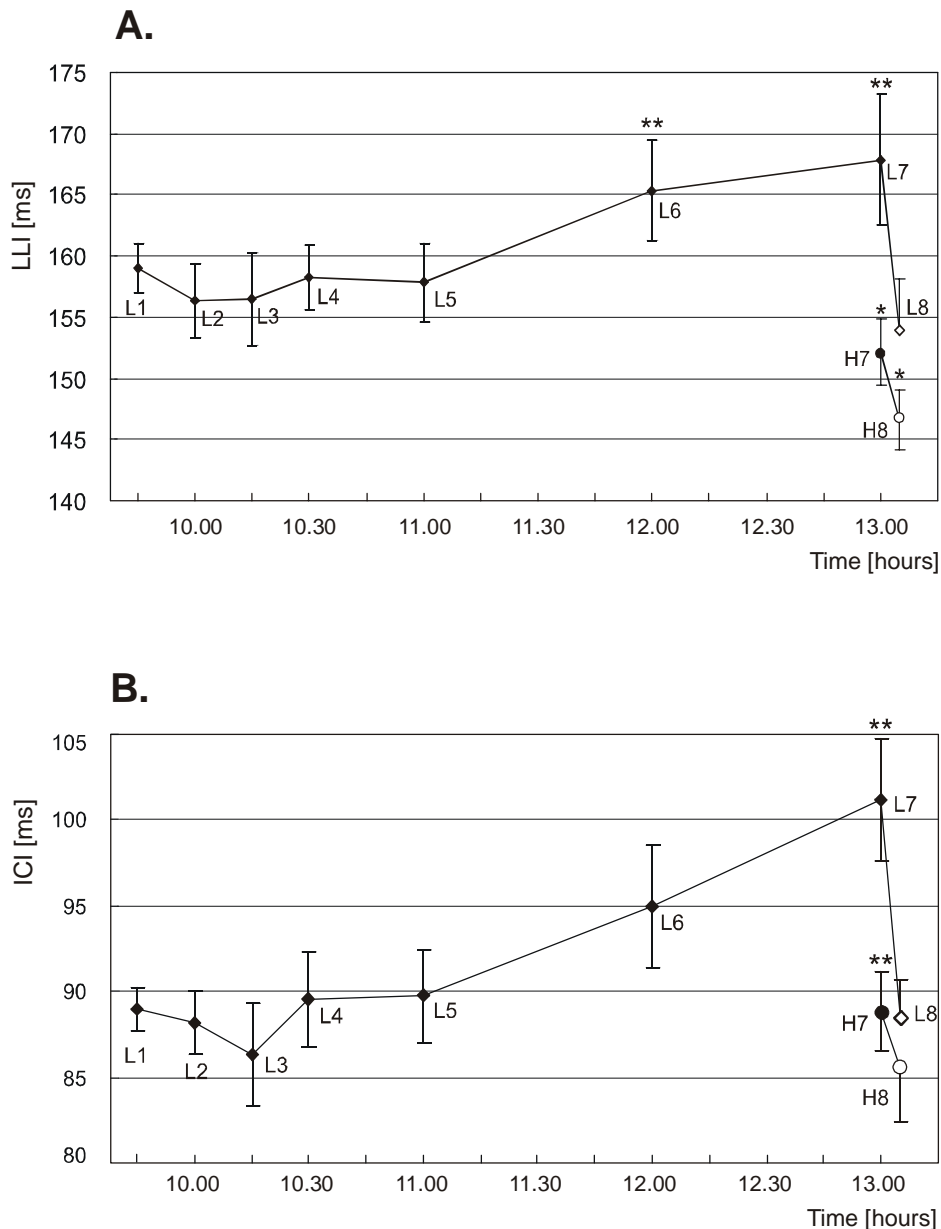


Fig. 3. Time course of lick-lick interval duration (A) and intercontact interval duration (B) in separate sessions of group L (diamonds) and group H (circles). Open symbols are used in 2-animal sessions. All data are given as arithmetic means \pm S.E.M., asterisks above the L group denote values significantly different from the control L1 session; asterisks above the H group values denote significant differences between sessions of H and L groups at the same time. (** $P < 0.01$, * $P < 0.05$).

Discussion

As we used a weight- and age-homogeneous batch of animals, we assumed that the age was not involved in the differences either of vigilance level or of licking performance between the groups. Both groups were composed of males only so that sex-related differences were also excluded.

A low level of vigilance in animals of group L was observed and corresponded to descriptions of the low behavioral activity in laboratory rats during the light period of the day compared with the darkness (van den Buuse 1999). It was also in good agreement with the finding that both paradoxical and slow wave sleep occupy more time of the light phase than of the dark period (12.8 % vs 3.9 % and 54.4 % vs 36.2 %, respectively) in rats

housed in standard laboratory plexiglass cages (Nicolaidis *et al.* 1979).

Licking frequency 6.35 Hz (LLI 158 ms) of group L between 09:45 and 11:00 h was in the faster part of the range found by other authors (e.g. Weijnen 1998). The duration of CD 69 ms was almost in the middle of the interval from 65 to 75 ms (Halpern and Tapper 1971), duration of ICI 89 ms was 10 ms shorter than that given by the above authors. The relatively faster licking rate and shorter ICI was probably due to the low-restriction licking environment that we succeeded to create by the adaptation of the experimental set up (Fig. 1). We did not find any significant difference among mean LLI of five sessions of group L recorded at separate times between 09:45 and 11:00 h and this result supported the stability of the rate of licking under a constant arrangement (Weijnen 1998). There were also no significant differences among mean ICI or CD, respectively. It could therefore be concluded that no internal variability was hidden in the stable LLI.

The group L rats slowed down the licking frequency with continuing light phase of the day. Several possible factors could explain this observation.

- 1) The animals gradually gained higher degree of experience with the testing situation. But the direction of the change in our experiments was opposite to that found by other authors (Keehn and Arnold 1960, Corbit and Luschei 1969). This factor thus acted against the change observed.
- 2) Lower deprivation could be the reason for the decreasing licking rate (Weijnen 1998). Nevertheless, there are two objections against taking this factor into account for sufficient explaining the slower licking frequency in our experiments. First, the licking rate increased again in group L at 13:00 h (2 animals in the session), in spite of the fact that the animals were less deprived than in the previous session; second, the more deprived group H licked significantly faster at the same time of the day.
- 3) The phase of the circadian cycle could be regarded as the reason of the gradually slowed licking rate in group L. Decreased licking frequency during light, low activity period (Cone 1974) corresponds to our results. However, this possibility cannot give complete explanation due to the evident reason. Fast licking frequencies in L8 session and in both H7 and H8 sessions were recorded in the midday low activity phase of the day time.
- 4) Our results indicate that the change of licking frequency responded to the differences in vigilance

level of animals just before the session. Animals of the low level vigilance group L at 13:00 h licked at the rate 5.96 Hz (LLI 168 ms), fully vigilant rats of group H at 13:00 h licked significantly faster with a frequency 6.57 Hz (LLI 152 ms). The fastest rate of licking in animals of both groups was encountered in alert rats under mild stress caused by the presence of the second animal – a rival – in the experimental box (6.49 Hz, LLI 154 ms and 6.82 Hz, LLI 147 ms). These frequencies were significantly different from sessions of the same group held only a few minutes earlier. All observed shifts were due to change of the ICI, while CD did not change significantly.

Principles of the effect of vigilance level on the licking rate could be found in analogous experiments with CNS-influencing drugs (Knowler and Ukena 1973, Fowler and Wang 1998). The frequency of licking could be both accelerated or slowed down by direct influence on the brainstem central pattern generator, for example by the local administration of drugs (Vajnerová and Brožek 2000). The level of vigilance also changes muscle tonus especially *via* the gamma system. Under natural conditions, the rhythmical motor output can be influenced from higher brain centers and/or through the feedback from sensory afferents (Fowler and Mortell 1992, Weijnen 1998). Thus, the slowing down of the licking frequency in less vigilant animals can be mediated through the more synchronous activity of upper levels of the central nervous system that influences the central pattern generator for licking. Alternatively it can act *via* lower muscle tonus during a state of low level vigilance, which could reduce the force of tongue protrusion or interfere with the postural adjustment of the rat, causing a change of licking frequency by means of a feedback mechanism.

For the objectivity and confirmation of our behavioral observations, EEG and EMG records or perhaps a lick force sensor reading should be used simultaneously with the licking records to indicate exactly the stage of synchronization of brain activity and to determine the tonus especially of postural and tongue muscles. Lick sensors that made the animal a part of an electrical circuit could introduce artifacts in electrophysiological recordings (Weijnen 1989). Mechanical or optical principles could be used (Weijnen 1998) with high-restriction and thus with less advantageous licking configuration. The best chance now is probably offered by a new method using fiber optic technology that is fully compatible with behavioral

electrophysiology without a restrictive lick recording (Schoenbaum *et al.* 2001).

The level of vigilance should to be taken into account in behavioral licking experiments. The influence of its level on other central pattern generators can also be expected.

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Reprint requests

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