Stenosis of a portacaval anastomosis affects circadian locomotor activity in the rat: a multivariable analysis

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Córdoba, Juan, Joseé Dupuis, Jeanne Gottstein, and Andrés T. Blei. Stenosis of a portacaval anastomosis affects circadian locomotor activity in the rat: a multivariable analysis. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1218–G1225, 1997.—The study of hepatic encephalopathy is limited by the lack of standardized experimental models to assess behavior. We have shown that rats continuously monitored while running on a wheel show abnormalities of the circadian rhythm of locomotor activity after portacaval anastomosis (PCA), such that entrainment of running activity to the light-dark cycle is severely impaired. To identify factors that affect postoperative circadian behavior, we have performed a multivariable analysis of 69 sham-operated controls and 107 rats after PCA. Our results indicate that shunt stenosis, as determined by the pressure gradient from the splenic pulp to the inferior vena cava, ameliorates the postoperative deterioration of the circadian rhythm. In addition, postoperative behavior was affected by preoperative performance, diet, and gender. Postoperative body weight gain, spleen weight, and liver atrophy did not impact this model. Because shunt stenosis is known to ameliorate hepatic encephalopathy in humans, our findings support the validity of this behavioral end point as a correlate of hepatic encephalopathy. Measurement of the pressure gradient across the anastomosis and achievement of sufficient preoperative entrainment appear critical for the standardization of the model.

hepatic encephalopathy

THE STUDY OF THE pathogenesis of hepatic encephalopathy is limited by the lack of well-standardized animal models (4, 18). The rat after an end-to-side portacaval anastomosis (PCA) develops several behavioral abnormalities that may correspond to subclinical hepatic encephalopathy in humans (18). A better knowledge of the pathophysiology of subclinical encephalopathy, a frequent abnormality in cirrhosis (12), may help in understanding the mechanisms that result in hepatic coma and provide a basis for the development of new therapeutic approaches. However, inconsistent results in terms of behavioral, metabolic, and neurochemical features have been reported in this model. Factors that may influence the results include the surgical procedure used (9, 14), time after surgery (2, 10), diet composition (14), postoperative weight gain (10), liver atrophy (10), portacaval pressure gradient (9), and rat age (1) and gender differences (6). Ways to control for such variables have been proposed (18).

For the past several years we have been examining abnormalities in the circadian rhythm of locomotor activity that develop after PCA in rats entrained to run on a wheel (8, 28, 30, 34). In the experimental animal, studies of circadian rhythmicity can be carried out during cyclical changes of the external lighting conditions, in which case entrainment to the light-dark cycle can be measured. After adequate entrainment, the “free-running” period, an expression of the endogenous circadian function, can be assessed when the external cue is removed and constant light conditions are present. We have demonstrated a clear reduction in the capacity of PCA rats to entrain to the light-dark cycle and have shown a spectrum of abnormalities occurring during constant light, ranging from a decreased to an absent free-running period.

The validation of this paradigm as a correlate of hepatic encephalopathy requires that factors known to improve or worsen the human condition have a similar effect on the model. Our choice of this behavioral end point is supported by the observation of altered circadian rhythms in human cirrhosis (17, 29, 31). In a previous report, we showed that abnormal entrainment to the light-dark cycle is ameliorated by a low-protein diet (28), a factor known to improve human hepatic encephalopathy (19). In the current study, we have retrospectively analyzed the effect of shunt stenosis on circadian locomotor activity in a large group of rats. Stenosis or obstruction of a radiologically inserted stent or a surgically constructed portacaval shunt affords protection against the development of encephalopathy in humans (3). Because in the rat the degree of shunt stenosis influences other variables, such as postoperative body weight gain and the degree of liver atrophy (9, 14), factors that by themselves may have determined the postoperative behavior, the data were submitted to multivariable analysis.

Multivariable analysis examines interactions between variables, identifying elements that independently affect the dependent variable. In this study, we analyzed whether shunt stenosis maintained its effect on behavior (dependent variable) when other variables were considered. The results are supported by the inclusion of a large number of rats (n = 176), which confers a high statistical power to the analysis. Our analysis also aimed at the identification of elements that should be controlled in future experiments. Altogether, this study validates and standardizes abnormalities of the circadian rhythm of locomotor activity after PCA as a correlate of hepatic encephalopathy.

MATERIALS AND METHODS

Animals and Maintenance

Albino rats of the Sprague-Dawley strain (Charles River, North Bloomington, MA) weighing ~250–350 g were used for all studies. On receipt and until the end of the experiment, the animals were housed in individual cages with wire mesh floors and standard bedding. Lighting conditions were controlled for a 12:12-h light-dark cycle (light-dark cycle). Each...
cage was equipped with a running wheel, the revolutions of which were continuously inscribed in a computerized recording device. All studies were performed with a protocol approved by the Animal Care Committee at the Lakeside Veterans Affairs Medical Center. Approximately half of the animals were included in previous reports from our laboratory (8, 28, 30, 35).

Surgery

PCA. Using the technique of Lee and Fisher (15), an end-to-side anastomosis between the portal vein and the inferior vena cava was constructed with continuous suture under methoxyflurane anesthesia and clean technique. The inferior vena cava and portal vein were clamped for not more than 15 min; after unclamping, the bowel was evaluated for cyanosis. If cyanosis persisted, the animal was killed with an overdose of Nembutal (Abbott Laboratories, Chicago, IL). After satisfactory surgery, the abdomen was sutured in two layers. All interventions were performed by the same operator (J. Gottstein). The postoperative mortality after more than 100 interventions was <10%.

Sham operation. The procedures were identical to those for PCA with clamping, but with no transection of the portal vein, and took place over a period of time similar to that of the PCA surgery.

Experimental Conditions

Design. During an entrainment period of 3–4 wk, the animals became acclimated to the new environment and ran on the wheel. After this period, surgery was performed. Animals were immediately returned to their activity cages and maintained under the same housing conditions for another period of 6 wk.

Diet. The majority of animals received a diet of standard chow and water ad libitum. A group of rats were fed with a purified diet (AIN-76A, Milwaukee, WI). The diet was liquid and was delivered by custom-designed ball valve sipper tubes attached by rubber stopper to plastic bottles. No other access to water was provided. In this group, daily feeding was performed in total darkness during the last 3 h of the dark cycle to avoid the masking effects of anticipatory behavior on activity onset (32).

Behavioral Variables

Data collection. Locomotor activity (wheel revolutions) was continuously recorded during the entire experiment (Fig. 1). However, to maximize the effect of training and minimize the influence of potential deleterious metabolic changes during the immediate postoperative course, only the data of the week before surgery (preoperative) and the third postoperative week were used for the analysis. Computer analysis was performed using a software program designed to continuously analyze activity data (Chronobiology kit, Stanford Software Systems, PaloAlto, CA).

Total activity. Total activity was calculated as the number of wheel revolutions per 7 days.

Day-to-total activity ratio. This ratio was calculated by dividing the activity during the 12-h light period (in rodents, which are nocturnal animals, the day is a period of inactivity) by total activity for the same 7-day period.

Amplitude of entrained rhythm to light-dark cycle. This variable is a measure of the degree of entrainment of the
locomotor activity to the light-dark cycle. The amplitude of the entrained rhythm to the light-dark cycle \((Q_p)\) was calculated by the \(x^2\) periodogram method (26) as the integral of the difference between mean activity and values above the mean when modeled as a sinusoidal curve and measured every minute. Its value reflects the intensity with which the change in the lighting condition is translated into a change in locomotor activity.

Terminal Studies

Rats were killed 6 wk after surgery. At this time, animals were weighed and the pressure gradient from the splenic pulp to the inferior vena cava was measured. Animals were anesthetized and killed by decapitation; the liver and spleen were resected and weighed.

Pressure gradient from the splenic pulp to the inferior vena cava was evaluated using the \(x^2\) periodogram method (26) as the integral of the difference between mean activity and values above the mean when modeled as a sinusoidal curve and measured every minute. Its value reflects the intensity with which the change in the lighting condition is translated into a change in locomotor activity.

Statistical Analysis

Results are expressed as means ± SE. Statistical significance in contingency tables was evaluated using \(x^2\). Comparisons of continuous variables between groups were performed with unpaired Student's t-test or Mann-Whitney rank-sum test when appropriate. Changes in behavioral variables between preoperative and postoperative values were assessed using paired Student's t-test or the nonparametric Wilcoxon test for variables that did not follow a normal distribution. Comparisons among multiple groups were assessed using one-way analysis of variance (ANOVA) or the Kruskall-Wallis nonparametric ANOVA on ranks. Post hoc multiple pairwise comparisons were calculated with Dunn's method. Correlations were assessed with Pearson's coefficient.

A multivariable analysis was designed to study the effect on postoperative behavior (total activity, day-to-total activity ratio, \(Q_p\)) of the following variables: 1) preoperative performance (preoperative values of total activity, day-to-total activity ratio, and \(Q_p\)), 2) rat characteristics (gender, diet, and initial weight), and 3) postoperative course (weight gain, liver weight, spleen weight, and pressure gradient).

For this reason, multiple correlations were computed analyzing PCA and Sham separately. Variables that did not follow a normal distribution were normalized using arithmetic transformations (e.g., logarithmic). The variables that showed a significant correlation in the univariate analysis were included in a stepwise multiple regression analysis. Because diet was unevenly distributed between gender (no

RESULTS

General Aspects

A total of 176 rats were included in the study. Table 1 shows the characteristics of Sham and PCA rats. Both groups did not differ in terms of diet, gender, and initial body weight. However, as described in detail previously (8, 23), PCA rats experienced a blunted weight gain and developed liver atrophy. In spite of the decrease in body and liver weight, spleen weight was slightly increased.

Behavior

The behavioral characteristics of Sham and PCA rats running on a wheel were similar to those reported previously (8, 28, 30, 35). After the initial training period, Sham and PCA rats did not show differences in their preoperative performance (Fig. 2). However, the postoperative course was markedly different. After surgery, PCA rats presented an important decrease in total activity (−63%) and a disruption in the entrainment to the light-dark cycle (Fig. 1), as shown by a decrease in \(Q_p\) (−25%). In Sham rats, the level of total activity was not modified by surgery. Furthermore, the degree of entrainment was sharpened during the postoperative course with an increase in \(Q_p\) (10%). Changes in day-to-total activity ratio were opposite those seen in \(Q_p\). Day-to-total activity ratio was increased for PCA rats (235%) and decreased for Sham rats (−43%).

Table 1. Characteristics of the rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>69</td>
<td>107</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>48:21</td>
<td>66:41</td>
</tr>
<tr>
<td>Diet, chow/purified</td>
<td>42:27</td>
<td>63:44</td>
</tr>
<tr>
<td>Initial weight, g</td>
<td>308 ± 6</td>
<td>304 ± 5</td>
</tr>
<tr>
<td>Liver/body weight, %</td>
<td>29 ± 2</td>
<td>10 ± 2†</td>
</tr>
<tr>
<td>Spleen/body weight, %</td>
<td>3.42 ± 0.07</td>
<td>2.14 ± 0.03†</td>
</tr>
<tr>
<td>Pressure gradient, mmHg</td>
<td>5.5 ± 0.3</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sham, sham operated; PCA, portal-caval anastomosis. Weight gain, (weight at death – preoperative weight)/preoperative weight. Liver weight-to-body weight and spleen weight-to-body weight ratios are at time of death. Pressure gradient, pressure gradient of the splenic pulp to inferior vena cava. *P < 0.05, †P < 0.001 vs. Sham. There were no significant differences between both groups in terms of sex, diet, and initial weight. Due to the different significance of pressure gradient in Sham (pressure gradient across the liver) and PCA rats (pressure gradient across PCA), comparisons between both groups were not performed.
Factors that Affect Behavior

The multivariable analysis (Tables 2 and 3) showed that 40% to 62% of the variance of the postoperative behavior could be explained by the following factors.

Preoperative performance. Preoperative values influenced all three postoperative behavior parameters in Sham and PCA rats. There was a positive correlation (positive coefficient) for all variables, except for $Q_p$ in PCA rats. In the latter group, a higher preoperative $Q_p$ was associated with a more marked postoperative decrease in $Q_p$, suggesting that the post-PCA deterioration of $Q_p$ was better detected in animals with “good entrainment” (see below).

Rat characteristics. The effect of rat characteristics on postoperative performance differed between Sham and PCA rats. Diet and gender affected total activity and $Q_p$ after PCA but not after sham surgery. In PCA rats, a purified diet and female gender protected from the postoperative decrease in total running activity and entrainment to the light-dark cycle. The lack of influence of diet and gender on day-to-total activity ratio in PCA rats was possibly due to a masking effect, because diet and gender influenced day-to-total activity ratio in Sham rats in an opposite direction than would be expected in PCA rats. Preoperative weight did not influence postoperative behavior.

Table 2. Stepwise multiple regression analysis in Sham rats

<table>
<thead>
<tr>
<th>Postoperative Performance</th>
<th>$R^2$</th>
<th>F</th>
<th>P</th>
<th>Variable</th>
<th>Coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity</td>
<td>0.62</td>
<td>13.82</td>
<td>0.0001</td>
<td>Preop total activity</td>
<td>0.60</td>
<td>0.0001</td>
</tr>
<tr>
<td>Day/total activity</td>
<td>0.42</td>
<td>6.11</td>
<td>0.0001</td>
<td>Preop day/total activity</td>
<td>0.50</td>
<td>0.0001</td>
</tr>
<tr>
<td>$Q_p$</td>
<td>0.40</td>
<td>5.39</td>
<td>0.0001</td>
<td>Preop $Q_p$</td>
<td>0.54</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Fig. 2. Preoperative and postoperative values of total activity (A), day-to-total activity ratio (B), and $Q_p$ (C) in Sham ($n = 69$) and PCA rats ($n = 107$). Data are expressed as means ± SE. Pre- and postoperative comparison: *P < 0.05, **P < 0.01, ***P < 0.001. There were nonsignificant preoperative differences between Sham and PCA rats.

See Table 2 for an explanation of the multiple regression function and the variables shown.
Postoperative course. Weight gain after surgery influenced the day-to-total activity ratio in Sham and PCA rats but did not have any influence on total activity and $Q_p$. In PCA rats, the increase of the pressure gradient across the PCA (a sign of shunt stenosis) protected against the postoperative decrease in total activity and $Q_p$. After adjustment for the other variables, neither liver nor spleen weight affected postoperative behavior.

Preoperative Entrainment

According to multivariable analysis, postoperative entrainment to the light-dark cycle in Sham rats was only influenced by preoperative values. Thus this group was adequate for the study of the effect of preoperative performance on postoperative values. Rats with high preoperative $Q_p$ showed homogeneous behavior after sham surgery (Fig. 3). However, low preoperative performance was associated with heterogeneous postoperative behavior. Using the method of comparison of variances, we found the maximal differences between the group above and the group below predetermined cut-off values at preoperative $Q_p = 900$ (P < 0.0001). In contrast to the whole group of sham rats (Fig. 2), a selection of sham rats with preoperative $Q_p > 900$ (n = 30) would have led to nonsignificant differences between preoperative (1,142 ± 27) and postoperative $Q_p$ (1,082 ± 44). The same criteria applied to PCA rats (n = 58, 54% of total) would have led to a more marked postoperative deterioration of $Q_p$ (−31 ± 4%).

Pressure Gradient Across PCA

As shown by the multivariable analysis, the pressure gradient across the anastomosis had an independent effect on total activity and $Q_p$ that persisted after adjusting for preoperative performance. Stenosis of the shunt (high pressure gradient) was associated with an amelioration of postoperative deterioration on total activity and $Q_p$ (Fig. 4). In addition, a high pressure gradient across PCA ($> 5$ mmHg) was associated with a lower rise in brain glutamine (Fig. 5). In a previous study (9), we observed that a pressure gradient cut-off value of 2 mmHg separated rats with a different postoperative course and different levels of glutamine in the cerebrospinal fluid. In the current study, PCA rats with a pressure gradient $> 2$ mmHg (n = 33) had a less marked postoperative deterioration on behavior (total activity, $-51 \pm 4\%$ vs. $-77 \pm 4\%$, P < 0.0001; $Q_p$, $-16 \pm 3\%$ vs. $-39 \pm 4\%$, P < 0.0001). Furthermore, brain glutamine was lower in PCA rats with a pressure gradient $> 2$ mmHg (227 ± 19%, n = 34, $P = 0.011$; multiple comparisons, *$P < 0.05$).
vs. 270 ± 19%, n = 26), a difference that approached statistical significance (P = 0.09).

**DISCUSSION**

This study demonstrates that a factor known to improve hepatic encephalopathy in humans, stenosis of a PCA, ameliorates the disruption of entrainment of the circadian rhythm of locomotor activity seen in rats after PCA. In addition, we have identified preoperative entrainment as a critical variable affecting postsurgical behavior.

The rat after PCA has been extensively used for the study of hepatic encephalopathy (4, 18). A main limitation of this model is the observation of variable results both between and within institutions. Some of the differences may be explained by differences in surgical technique (14). Minimal stenosis, not detectable by visual exam, can cause a different pattern of portal-systemic shunting, a critical factor in the development of hepatic encephalopathy (3). In a previous study (9), we demonstrated that an increase in the portal venous pressure gradient as a result of a stenosed shunt results in lower portal-systemic shunting (due to hepatic collateral), lower ammonia delivery to the systemic circulation, and a lower concentration of glutamine in the cerebrospinal fluid (9). We also showed that rats with an "open" anastomosis had a significantly smaller weight gain and a greater degree of liver atrophy (9). Neurobehavioral abnormalities in rats after PCA have been attributed not only to portal-systemic shunting but also to the loss of hepatic mass and liver function (5, 13). We proposed that assessment of the pressure gradient across PCA may be needed to standardize this experimental model. A behavioral correlate was needed to support this claim.

For the last five years we have been performing experiments in a similar fashion, allowing us to carry out this retrospective analysis. We have been systematically measuring the splenic pulp to inferior vena cava pressure gradient in rats after PCA to assess a potential stenosis of the anastomosis. The splenic pulp pressure is simple to measure and provides a good estimate of portal pressure (21). Our results in over 100 PCA rats demonstrate that this gradient influences the development of a well-described behavioral abnormality (8, 28, 30, 35). A high pressure gradient across PCA ameliorates the disruption of the entrainment of the circadian rhythm of locomotor activity, as revealed by changes in Qp. Furthermore, because the data were submitted to multivariable analysis, it was possible to exclude an effect of other confounding factors. Neither body weight gain nor liver atrophy influenced the postoperative Qp in accordance with our previous results in pair-fed rats (28). Thus the pressure gradient across the anastomosis affects Qp independently of other factors, suggesting that the abnormalities of circadian rhythmicity may be secondary to the toxic effects on the brain of substances arising from the gut. This interpretation is supported by the observation of a lower increase in brain glutamine, the product of ammonia detoxification, in rats with a higher pressure gradient.

In addition to the pressure gradient, diet conditioned the entrainment of circadian locomotor activity. Rats fed a purified diet (casein based) showed a less pronounced postoperative decrease in Qp than rats fed a chow diet (cereal based). A better postoperative recovery (higher weight gain) has been described in PCA rats fed a purified diet (14). In our series, this is not the mechanism by which purified diet improves postoperative Qp, as the multivariable analysis indicated independence of diet from body weight. Casein-based diets have favorable effects in experimental models (14, 20) and in humans with hepatic encephalopathy (11). The mechanisms by which casein diet improves encephalopathy are poorly understood (19) but may include interfering with the generation of neurotoxic substances (36). Accordingly, diet composition by itself may account for improvements in the entrainment of the circadian rhythm of locomotor activity, as it does for hepatic encephalopathy. A "training effect" on the circadian rhythm as a result of administering the purified diet in a liquid form cannot be excluded, although the experimental procedure we followed makes this unlikely (29).

Furthermore, diet also conditioned total locomotor activity, another behavioral variable that is determined by pressure gradient and that is not subjected to anticipatory behavior.

Our results show the limitations of an experimental model to detect factors with slight effects on outcome before the model has been fully standardized. In a previous study, we reported that there were no striking gender differences in the disruption of locomotor activity in rats after PCA (30). However, the current results indicate that female rats have a less disrupted circadian rhythm after PCA. These discrepancies can be explained by an insufficient number of rats in the former study. We have estimated that at least 37 rats are necessary to detect the 15% improvement in Qp present in female rats. Because this effect was independent of weight gain and pressure gradient, other factors, such as hormonal differences, may be involved. After PCA, dramatic and gender-dependent changes in estradiol and testosterone occur (25), which may modulate circadian rhythms (16). In spite of some controversial observations (22, 27), clinical experience does not suggest that females are protected from the development of hepatic encephalopathy.

Several requirements have been proposed to validate an experimental model as a correlate of the human condition (34). These include 1) predictive validity (the model correctly identifies factors that improve or worsen human behavior) and 2) construct validity (the behavior in the model reproduces the symptomatology of the condition being modeled). The PCA model shows several features that allow it to be considered a correlate of hepatic encephalopathy. Entrainment to the light-dark cycle improves with dietary measures and shunt stenosis (predictive validity) and may correspond to the spectrum of circadian abnormalities that develop in patients with encephalopathy (24), such as dampening of the circadian rhythm of motor activity (31) (construct validity). The day-to-total activity ratio has been widely
used in other paradigms as a behavioral correlate of hepatic encephalopathy (33). In the current model, the day-to-total activity ratio appears to be a less-sensitive variable than $Q_p$, because the day-to-total activity ratio was not influenced by shunt stenosis.

In terms of standardization of the model, two factors appear critical: 1) achievement of good preoperative entrainment and 2) avoidance of even a minimal degree of shunt stenosis. As with other behavioral paradigms, baseline entrainment can be improved by extending the preoperative period. This study indicates an adequate level of baseline $Q_p$ to ensure a postoperative behavior not influenced by an insufficient preoperative entrainment. In addition, our current and previous results (9) suggest that to obtain homogeneous effects of PCA on behavior and on neurochemical abnormalities in the brain, rats with a pressure gradient $\geq 2$ mmHg should be excluded from the final analysis. The avoidance of such minimal levels of shunt stenosis will require surgical refinements, because in our hands only 35% of PCA rats had a pressure gradient $< 2$ mmHg. For laboratories not well equipped for hemodynamic monitoring, a water manometer can be used for pressure measurements.

In conclusion, our results show that assessment of entrainment of the circadian rhythm of locomotor activity is a sensitive and objective tool to assess behavioral abnormalities after PCA. In addition, this study supports the measurement of the pressure gradient across the PCA as a way to standardize experiments in this model. Such an approach may allow the comparison of results from different laboratories and will result in a better understanding of the pathophysiology of hepatic encephalopathy.

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