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ORIGINAL PAPER

Association Between Na^+,K^+-ATPase Activity and the Vulnerability/Resilience to Mood Disorders induced by Early Life Experience

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Abstract There is increasing evidence that early life events can influence neurodevelopment and later susceptibility to disease. Chronic variable stress (CVS) has been used as a model of depression. The objective of this study was to evaluate the interaction between early experience and vulnerability to chronic variable stress in adulthood, analyzing emotional, metabolic and neurochemical aspects related to depression. Pups were (1) handled (10 min/day) or (2) left undisturbed from day 1 to 10 after birth. When the animals reached adulthood, the groups were subdivided and the rats were submitted or not to CVS, which consisted of daily exposure to different stressors for 40 days, followed by a period of behavioral tasks, biochemical (plasma corticosterone and insulin sensitivity) and neurochemical (Na^+,K^+-ATPase activity in hippocampus, amygdala and parietal cortex) measurements. Neonatally-handled rats demonstrated shorter immobility times in the forced swimming test, independently of the stress condition. There was no difference concerning basal corticosterone or insulin sensitivity between the groups. Na^+,K^+-ATPase activity was decreased in hippocampus and increased in the amygdala of neonatally-handled rats. CVS decreased the enzyme activity in the three structures, mainly in the non-handled group. These findings suggest that early handling increases the ability to cope with chronic variable stress in adulthood, with animals showing less susceptibility to neurochemical features associated with depression, confirming the relevance of the precocious environment to vulnerability to psychiatric conditions in adulthood.

Keywords Neonatal handling · Depression · Chronic stress · Insulin sensitivity · Na^+,K^+-ATPase activity · Forced swimming test

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Introduction

During recent years, much research has been focused on early life events and their effects in adulthood. The association between the poor quality of the fetal environment and an increased risk for cardiovascular disease [1–3] and depression [4, 5] is well known. These findings are often suggested to be mediated by the programming of the hypothalamus-pituitary-adrenal (HPA) axis activity [4–7]. This programming is very important because it seems to generate many behavioral and physiological phenotypes and induce susceptibility or resilience to disease.

It is well known from animal research that the amount of care received in the first few days of life determines behavioral, hormonal and neurochemical aspects of the stress response, influencing especially the HPA axis activity [8–10]. Interventions that disrupt the mother-pup interaction can modify the SNC activation in response to chronic stress in adult life [11]. Studies in humans confirm that early adversity is associated with an increased prevalence of depressive symptoms and anxiety [12], as well as altered stress responses [13].

Neonatal handling is an interesting experimental approach [14] in which brief, repeated periods of separation from the mother are associated with an intensified maternal care when the pups return to the nest [8, 15, 16]. In adulthood, these animals show decreased stress responses when facing an acute stress situation [17], as well as chronic stress paradigms [18, 19].

In light of the suggestion that neonatal handling increases maternal care and this element is crucial to the susceptibility to mood disorders, we aimed at investigating the interaction between early handling and adult chronic variable stress using analyses that are indicative of depression states such as: forced swimming test [20], insulin sensitivity [21], basal plasma corticosterone [22–25], and Na^+,K^+-ATPase activity [26, 27] in different brain structures. Our hypothesis was that neonatally-handled animals would respond differently to chronic stress exposure, possibly being more resilient to the effects of this adversity in adult life.

Methods

Subjects

Pregnant Wistar rats bred at our animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 × 25 × 15 cm) with the floor covered with sawdust and maintained in a controlled environment until offspring (lights on between 07:00 and 19:00 h, temperature of 22 ± 2°C, cage cleaning once a week, food and water provided). All litters were culled within 24 h to eight pups and were maintained intact unless for handling procedures, which were carried out between 10:00 and 12:00 h. Included in this period were the time to set up the incubator, to bring the cages from the facility and briefly habituate the dams to the new room, to perform careful removal of the pups from the nest, the time of handling per se, the return of the pups to the dam and, again after a brief period, to return the cage to the facility room. The researcher also changed gloves for the manipulation of each litter to avoid the spread of any kind of odor from nest to nest.

Weaning was on postnatal day 21. One or two male pups were used per litter per experiment. Rats were housed four per cage in home cages similar to those described above. Fifty-two experimental male rats were used in the different experiments, derived from 16 different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 13:00 and 16:00 h.

Neonatal Handling Model

In the non-handled group, pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at the same side by the principal researcher. In the handled group, pups were removed from their home cage and placed into a clean cage lined with clean paper towel, inside an incubator set to maintain an ambient temperature of 30–32°C (warm water at 34°C), being returned to their dams (which stayed in the home cage, next to the incubator) after 10 min. This procedure was carried out for the first 10 days of life, after which pups were left undisturbed until weaning.

Chronic Variable Stress Protocol

Chronic variable stress model was modified from other models of mild stress [26, 28]. At the age of 100 days, the animals were weighed and subdivided in four groups: non-handled control and chronically stressed, neonatally handled control and chronically stressed. A variate-stressor paradigm was used for the animals in the stressed groups. The following stressors were used: (i) 24 h of food deprivation; (ii) 24 h of water deprivation; (iii) 1 h of restraint, as described below; (iv) 1 to 3 h exposure to cold (4°C); (v) 10–15 min of noise; (vi) flashing light during 120–210 min as described below; (vii) inclination of the home cages at a 45° angle for 4–6 h, and (viii) isolation (2–3 days). Stress exposure started at different times every day, to minimize its predictability. Please refer to Table 1 to see the distribution of the stressors over the 40 days period of CVS exposure.

Restraint was carried out by placing the animal in a 25 × 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1-cm hole at the far end for breathing. Exposure to flashing light was made by placing the cage in a 50-cm-high, 40- × 60-cm open field made of brown plywood with a frontal glass wall. A 40-W lamp, flashing in a frequency of 60 flashes/min, was used.

Following this period, the forced swimming test was performed and since the test itself is stressful [29], the animals were not stressed on these 2 days (see details below).

Table 1 Schedule of stressor agents used during the chronic treatment

Day of treatment	Stressor used
1	Noise (15 min)
2	Flashing light (4 h)
3	Water deprivation (24 h)
4	Inclination of homecages (4 h)
5	Isolation
6	Isolation
7	Isolation
8	Food deprivation (24 h)
9	No stressor applied
10	Exposure to cold (1 h)
11	Noise (15 min)
12	Restraint (1 h)
13	Flashing light (4 h)
14	No stressor applied
15	Inclination of homecages (5 h)
16	Food deprivation (24 h)
17	Noise (15 min)
18	Restraint (1 h)
19	Isolation
20	Isolation
21	Isolation
22	Exposure to cold (1 h)
23	Flashing light (4 h)
24	Water deprivation (24 h)
25	No stressor applied
26	Isolation
27	Isolation
28	Isolation
29	Inclination of homecages (5 h)
30	Exposure to cold (1 h)
31	Food deprivation (24 h)
32	Noise (15 min)
33	Isolation
34	Isolation
35	Isolation
36	No stressor applied
37	Water deprivation (24 h)
38	Inclination of homecages (5 h)
39	Flashing light (3 h)
40	Restraint (1 h)

Forced Swimming Test

Two trials were given to the rats in which they were forced to swim in an inescapable polyvinyl carbonate cylinder aquarium, 60 cm in height and 30 cm in diameter filled with 30 cm tap water at 24°C. Rats were placed into the tank for 15 min on day 1 to induce a state of "helplessness." The rats were then dried off with a towel, and placed back into their home cage. Twenty-four hours after, a 5 min test was conducted [20]. After placing rats individually in the pool, they display vigorous activity and then adopt an immobile posture characterized by floating with the head just above the water surface, making very little movement with their body. This immobility behavior was scored using a chronometer on the test day.

Plasma Collection and Biochemical Measurements

Animals were sacrificed by decapitation 24 h after the last stress session, being fasted in the previous 6 h. The trunk blood was collected into heparinized tubes for insulin, glucose and corticosterone determination. The tubes were centrifuged at 4°C and plasma was separated and frozen until the day of analysis. Hormonal measurements were performed with commercial rat ELISA kits: Cayman Chemical Co., Ann Arbor, MI, USA for corticosterone evaluation, and Alpco Diagnostics, Mercodia AB, Uppsala, Sweden to measure insulin. Plasma glucose was measured by the glucose oxidase method using a commercial kit, BioSystems, Barcelona, Spain. Insulin resistance was evaluated using the Quantitative Insulin Sensitivity Check Index (QUICKI), defined by 1/[log(fasting insulin) + log(fasting glucose)] [30, 31].

Neurochemical Studies

After decapitation, the brain was quickly removed and the hippocampus, amygdala and parietal cortex were dissected. For preparation of synaptic plasma membranes and determination of Na^+,K^+-ATPase activity, the structures were homogenized in 10 vol. 0.32 M sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared and the activity of Na^+,K^+-ATPase was determined. Synaptic plasma membranes were prepared according to a previously published method [32] with some modifications [33, 34]. They were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 × g for 110 min, the fraction between the 0.8 and 1.0 sucrose interface was taken as the membrane enzyme preparation. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 µl. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays [35]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [36]. Specific activity

of the enzyme was expressed as nmol Pi released per min per mg of protein. Protein was measured by the method of Lowry et al. [37] or Bradford [38] using bovine serum albumin as standard.

Statistical Analysis

Data were expressed as mean ± standard error of the mean, and were analyzed by Two Way ANOVA, followed by Student-Newmann-Keuls (SNK) post hoc when indicated [39]. The significance level was accepted as different when the P value was equal or less than 0.05. Sample size was calculated based on our previous studies, varying in each experiment, and is shown individually in the Results section.

Results

Forced Swimming Test

Handled rats were less prone to demonstrate immobility behavior in the forced swimming test (effect of the neonatal group seen by Two-Way ANOVA, [$F(1, 42) = 8.370$, $P = 0.006$, $n = 10$–12/group]. There was no effect of the chronic stress [$F(1, 42) = 0.8$, $P = 0.377$] and no interactions. Post hoc analysis revealed that the difference resides between the nonhandled_CVS (longer immobility time) and handled_CVS (shorter immobility time) groups. Please refer to Fig. 1.

Plasma glucose, Insulin and Corticosterone

Plasma glucose was not different between groups subjected to different neonatal invertentions [Two-Way ANOVA, $F(1, 31) = 0.491$, $P = 0.489$, $n = 7$–9/group] nor stress condition [$F(1, 31) = 0.140$, $P = 0.711$], and no interactions were seen. Insulin was not affected by the neonatal environment [$F(1, 31) = 1.005$, $P = 0.325$]. Although insulin was decreased in rats submitted to chronic stress [$F(1, 31) = 4.349$, $P = 0.046$], the insulin resistance index (QUICKI) showed no differences between neonatal groups [$F(1, 31) = 0.64$, $P = 0.803$] or stress condition [$F(1, 31) = 1.453$, $P = 0.238$] and no interactions. Basal plasma corticosterone was also not affected by the early life experience [Two-Way ANOVA, $F(1, 26) = 2.699$, $P = 0.114$, $n = 6$–8/group] nor chronic stress in adulthood [$F(1, 26) = 0.007$, $P = 0.933$], and no interactions were noted. See Table 2.

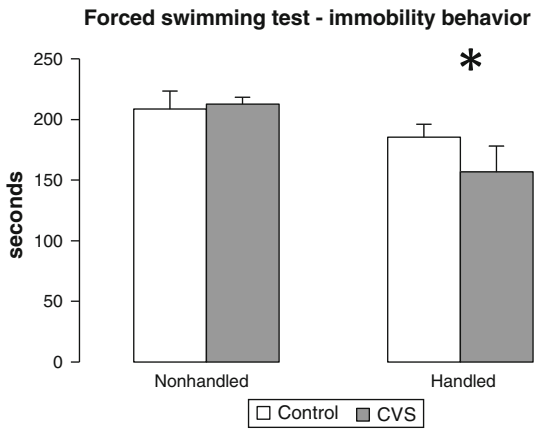

Fig. 1 Immobility time in the forced swimming test. Data are expressed as mean ± SEM of times in seconds. * A decreasing effect of neonatal handling on immobility time may be observed (Two-Way ANOVA, $P = 0.006$), but no effect of the chronic variable stress (CVS) nor interactions

Na^+,K^+-ATPase Activity in Different Brain Structures

Chronic stress induced a decrease in Na^+,K^+-ATPase activity in the three brain structures analyzed [Two Way ANOVA—hippocampus: $F(1, 19) = 18.150$, $P = 0.001$, n = 5–6/group; amygdala: $F(1, 22) = 4.552$, $P = 0.046$, $n = 5$–6/group; parietal cortex: $F(1, 18) = 5.848$, $P = 0.029$, $n = 4$–5/group]. Decreased Na^+,K^+-ATPase activity was observed in the hippocampus of neonatally-handled rats in general [$F(1, 19) = 6.277$, $P = 0.023$] without interaction between the two interventions (Fig. 2a). SNK post hoc test showed that nonhandled animals are statistically different depending on the CVS condition (decreased Na^+,K^+-ATPase activity in CVS exposed rats), but this does not happen among handled rats.

In the amygdala, there was an effect of the neonatal environment, increasing the enzyme activity in handled animals [$F(1, 22) = 5.182$, $P = 0.035$], without interactions (Fig. 2b). SNK analysis reveled that Na^+,K^+-ATPase activity was decreased after CVS exposure only in non-handled animals, but there is no such effect in handled ones.

In the parietal cortex, there was no effect of the early life experience [$F(1, 18) = 0.977$, $P = 0.339$]. However, an interaction between the early experience and chronic stress exposure in adulthood was observed [$F(1, 18) = 6.992$, $P = 0.018$], due to the fact that Na^+,K^+-ATPase activity was decreased by chronic stress in non-handled rats but not in the handled ones (Fig. 2c).

Discussion

In this study, we showed that neonatal handling induces a series of persistent alterations in adulthood, from behavioral to neurochemical parameters. Handled animals demonstrated shorter immobility time in the forced swimming test and a specific pattern of Na^+,K^+-ATPase activity in

Table 2 Plasma glucose, insulin, QUICKI and corticosterone measurements in nonhandled and neonatally handled rats with or without chronic stress exposure in adulthood. Data are expressed as mean ± S.E.M. for each measurement

Measurement	Nonhandled group		Neonatally handled group	
	Control	CVS	Control	CVS
Plasma glucose (mg/dl)	114.6 ± 4.3	120.5 ± 3.0	115.3 ± 5.9	113.0 ± 5.2
Plasma insulin (ng/ml)	2.1 ± 0.6	0.9 ± 0.2#	2.6 ± 0.6	1.5 ± 0.6#
QUICKI	0.46 ± 0.03	0.5 ± 0.02	0.47 ± 0.06	0.53 ± 0.04
Plasma corticosterone (ng/ml)	225.0 ± 52.8	248.6 ± 37.1	180.8 ± 30.6	163.8 ± 29.1

QUICKI Quantitative Insulin Sensitivity Check Index (see text for details). # Chronic variable stress (CVS) decreased the plasma insulin levels (Two-Way ANOVA, $P = 0.046$)

different brain regions (lower in the hippocampus and higher in the amygdala).

The current results agree with other studies, which have suggested that neonatal handling is associated with a greater ability to cope with a repeated chronic forced swimming stress in males [40]. Neonatal handling is also associated with a reduced helplessness behavior using inescapable shock [41], possibly through noradrenergic mediation [42]. It is interesting to note that despite the fact that the procedure for early-life intervention used in this study (10 min/day and returning to the cage) is a very mild disturbance, it has the ability to induce long-lasting effects. Other recent studies have reported that even a more subtle intervention (1 min handling) has anatomical, functional and behavioral effects that persist to adulthood [43–46]. On the other hand, it has been reported that the neonatal handling paradigm has the ability to induce robust rises in corticosterone secretion that are delayed for many hours after the procedure [47]. In addition, there are studies reporting that returning to the cage increases the maternal care towards the pups [8, 15, 16], which could be involved in the long term resilience to the effects of chronic stress described here [8].

Metabolic syndrome [48] and, more especifically, insulin resistance [21] have recently been associated with depression in humans. Hypercortisolemia has been suggested as the main factor leading to disturbed glucose utilization in depressed patients [49]. In our study, although CVS decreased plasma insulin levels, we did not find a difference regarding the insulin resistance index QUICKI between the neonatal groups.

Evidence show that neonatally-handled rats have a decreased response to acute stress, demonstrating plasma corticosterone levels that return to baseline faster after an acute insult, with a more efficient glucocorticoid negative feedback due to an increased level of glucocorticoid receptors in the hippocampus [17] and decreased in the amygdala [50]. Interestingly, Na^+,K^+-ATPase has subunits that are responsive to glucocorticoids [4, 51]. In this study, we showed that neonatally handled rats had a decreased enzyme activity in the hippocampus and increased in the amygdala. The differential pattern of central glucocorticoid distribution in neonatally handled rats could be accounting for the differential pattern of Na^+,K^+-ATPase activity seen at baseline in these animals. It is important to mention that while it seems to us that neonatal handling is associated with a stable hippocampal Na^+,K^+-ATPase activity independent of the CVS exposure, as opposed to the nonhandled group, we cannot exclude the possibility that there was no further decrease in the handled animals exposed to CVS because the Na^+,K^+-ATPase activity is already downregulated.

The brain Na^+,K^+-ATPase activity decreases with age, and it seems to contribute to the age-related brain deterioration [52]. Accumulating evidence proposes that the activity of this enzyme may be involved in the etiology of mood disorders in animal models [26] and peripheral tissues in humans [53, 54]. There is a report of a decreased brain activity in the Na^+,K^+-ATPase in the parietal cortex in depressed patients [27]. Interestingly, in our study, CVS induced a decrease in the enzyme activity in this brain region, and neonatal handling was able to protect against this effect. In the hippocampus and amygdala, similarly to what was observed in the parietal cortex, CVS also decreased the Na^+,K^+-ATPase activity as previously demonstrated [26], but this effect was less evident in neonatally handled rats, which may account for their baseline characteristics described above.

Na^+,K^+-ATPase is an integral membrane protein complex responsible for establishing the electrochemical gradients of Na^+ and K^+ ions across the plasma membranes of mammalian cells. This complex is present in high concentrations in brain cellular membranes, consuming about 40–50% of the ATP generated in this tissue [55]. Besides its essential role in maintaining the cellular membrane electrochemical gradient, it works as an oubain receptor, mediating intracellular signaling and activating nuclear transcriptional factors [56]. Intracerebroventricular injection of ouabain induces behavioral changes in rats, which has been suggested as an animal model for bipolar disorder [57]. In addition, an interesting study shows that mice

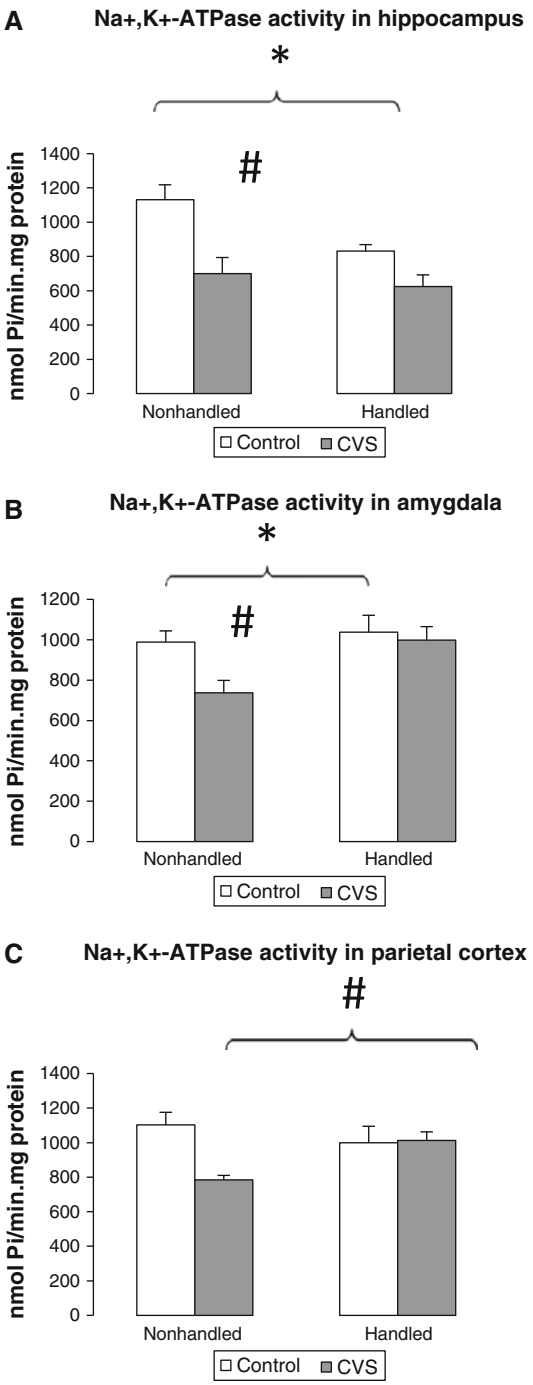

Fig. 2 Na^+, K^+-ATPase activity (nmol inorganic phosphate released per min per mg protein) in different brain structures. Data are shown as mean ± SEM **a** Hippocampus—both * neonatal handling (Two Way ANOVA, $P = 0.023$) and # Chronic variable stress (CVS) ($P = 0.001$), decreased the enzyme activity. No interactions were seen. **b** Amygdala—* neonatal handling increased the enzyme activity ($P = 0.035$), while # CVS decreased it ($P = 0.046$), without interactions. **c** Parietal cortex—# CVS decreased the enzyme activity ($P = 0.029$), and there was an interaction between CVS and the neonatal experience ($P = 0.018$)

having a genetic mutations resulting in 15% reduced neuronal Na^+,K^+-ATPase activity are vulnerable to develop increased depression-like endophenotypes in a chronic variable stress (CVS) paradigm compared to their wild-type littermates [58]. Therefore, alterations in the activity of Na^+,K^+-ATPase could well be involved in the vulnerability/resilience to mood disorders induced by early life experience as we propose in this study.

It is common for patients with depression to demonstrate a hyperactive HPA axis [59], and treatment with antidepressants is usually associated with normalization of HPA axis activity [60]. Although we did not find a difference in basal corticosterone between the groups in our study, we could propose that neonatal handling leads to a persistently differential functioning of several systems, resulting in a characteristic pattern of activity in behavioral and neurochemical outcomes. Our results suggest that, when exposed to a rat model of depression, handled animals have a lower tendency to have their baseline status affected by the chronic adversity.

In summary, we propose that early life experience influences the ability to cope with chronic stress in adulthood. In the case of neonatal handling, it seems that this brief, repeated separation from the mother in the postnatal period was able to modulate the way that these animals deal with some effects induced by the exposure to a rat model of depression in adult life; it is possible that this susceptibility is associated with altered Na^+,K^+-ATPase activity in different brain structures. This experimental paradigm is, therefore, an important model to study the pathophysiology of the mood disorders and to contribute to the enlightenment of future therapeutic approaches.

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