Stereological Evidence of Non-Selective Hippocampal Neurodegeneration, Growth Factors Depletion, and Behavioral Deficit Following Short-Term Bilateral Adrenalectomy in Wistar Rats

Naserddine Hamadi

Department of Life and Environmental Sciences, College of Natural and Health Sciences, Zayed University, P.O. Box 144534, Abu Dhabi,

Ömür Deniz

Department of Histology and Embryology. Medical Faculty. Ondokuz Mayıs University, 55139. Samsun,

Ahlam Abi Issa

Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, United Arab Emirates University, P.O Box 17666, Al Ain,

Naheed Amir

Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, United Arab Emirates University, P.O Box 17666, Al Ain,

Saeed Minhas

Department of Anatomy, College of Medicine and Health Science, United Arab Emirates University, P.O Box 17666, Al Ain,

Nather Madjid

Department of Neuroscience, Karolinska Institutet, SE-171 77 Stockholm,

Fatima Khelifi-Touhami

Department of Animal Biology, Faculty of Life and Science, Constantine University-1, Ain Elbey street, 2500 Constantine,

Süleyman Kaplan

Department of Histology and Embryology. Medical Faculty. Ondokuz Mayıs University, 55139. Samsun,

Abdu Adem (✉ abdu.adem@ku.ac.ae)

Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Khalifa University, P.O Box 127788, Abu Dhabi,

Research Article

Keywords: neurodegeneration, adrenalectomy, passive avoidance, stereology

Posted Date: February 4th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-147752/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

In the current study we investigated the impact of short-term adrenalectomy on hippocampal neurons of Wistar rats. In addition, the underlying mechanism(s) of degeneration in these neurons was investigated by measuring the levels of insulin-like growth factor-1 and β -nerve growth factor. Moreover, we investigated the cognitive behavior in these rats.

The stereological counting in the hippocampus revealed a significant neuronal death in the dentate gyrus and CA3, but not in the CA2 and CA1, area 7 and 14 days post adrenalectomy. The ultrastructural examinations revealed degenerated and degenerating neurons only in the dentate as well as CA4, and CA3 areas over the course of 3, 7 and 14 days.

The levels of IGF-1 were significantly decreased in the hippocampus of ADX rats 12 h post adrenalectomy and lasted over the course of two weeks. However, β -NGF was significantly reduced in ADX rats only at 14 days postoperatively. Using passive avoidance test we found a cognitive deficit in the ADX compared to the sham operated rats over time.

In conclusion, both granule and pyramidal cells were degenerated in the hippocampus following short-term adrenalectomy. The early depletion of IGF-1 might play a role in the hippocampal neuronal degeneration. Consequently, the loss of the hippocampal neurons after adrenalectomy leads to cognitive deficits.

Introduction

There is a growing evidence that prolonged exposure to glucocorticoids induces pyramidal cells loss in the hippocampus of guinea pigs¹, rats² and vervet monkey³. Elevated concentration of glucocorticoids provide a clinical background to the pathogenesis of Parkinson and Alzheimer disease⁴. Furthermore, the administration of high levels of glucocorticoids believed to exacerbate pyramidal cells death in variety of neurodegeneration models^{2,5}. It is interesting to note that the only neuronal population that is vulnerable to such high levels of glucocorticoids is the pyramidal cells of the hippocampus.

In contrast, in the late eighties Sloviter et al.⁶ showed high dependence of granule cells in the hippocampus of Long Evans rats on glucocorticoids for their survival. They discovered that the withdrawal of glucocorticoids by adrenalectomy triggers a massive granule cells loss and spare the degeneration of pyramidal cells Sloviter et al.⁶. Several other groups replicated the findings⁷⁻¹⁰. However, Sapolsky et al.¹¹ were the first to show that in addition to the degeneration of hippocampal granule cell, a significant decrease in the number of CA4 pyramidal cells has taken place following the adrenalectomy. Our previous study¹² supported and extended the finding of Sapolsky et al.¹¹. We found, in our previous studies, using cell counting and electron microscopy, long-term adrenalectomy of Wistar rats caused pyramidal cells death not only in the CA4 but in the different areas of the Cornu Ammonis CA3, CA2, and CA1 beside a drastic and extensive degeneration in the granule cells of the dentate gyrus

^{12,13}. Furthermore, Martinez-Carlos et al. ¹⁴ demonstrated apical dendritic atrophy in CA3 region of the hippocampus following adrenalectomy.

The insulin-like growth factors (IGFs) are polypeptides with a sequence similar to insulin. IGF-I and IGF-II receptors are abundantly expressed in the brain where IGF signaling mediates neuronal growth, development, nervous system maintenance, myelination and synapse formation ¹⁵. These roles are attributed to their ability to cross the blood-brain barrier and their endocrine roles in the brain ¹⁶. A number of studies have observed the involvements of IGFs in the brain cognitive functions ¹⁷.

Beta-Nerve growth factor (β -NGF) is also a polypeptide found in the central nervous system (CNS) in which the hippocampus contains one of the highest levels of such hormone ¹⁸. β -NGF is an essential neurotrophic factor in the developing sympathetic and sensory nervous systems ¹⁹. It exerts a number of different effects on neurons, such as neurogenesis ²⁰, neuronal plasticity ²¹ development ²², and differentiation ²³. Neurons that fail to obtain sufficient β -NGF die by apoptosis ²⁴. It has been shown that adrenalectomy in young rats caused a drastic decrease of the β -NGF levels in the hippocampus ²⁵. Glucocorticoids and these growth factors play important roles in the brain, nonetheless, their interactions remain unclear although some studies have suggested a relation between them ²⁶. Hence, the hormonal activities of glucocorticoids were investigated whether they have an effect on the levels of β -NGF in the hippocampus. It was found that in developing rats, bilateral adrenalectomy considerably diminished β -NGF levels in the hippocampus and in the distribution of its receptor ²⁵.

The hippocampus is known for its major role in learning and memory, several investigations indicated a decline in memory performance tasks in ADX rats which highlights the possibility of the involvement of neuronal damage in the poor cognitive performance ^{12,27–29}. Therefore, we aimed in the current study to investigate cell death in the hippocampus after short-term adrenalectomy and explore whether both granule and pyramidal cells are vulnerable to the absence of glucocorticoids. In addition, we aimed to explore the impact of short-term adrenalectomy on the levels of two main growth factors IGF-1 and β -NGF. Moreover, we examined whether the neuronal death of different populations has an impact on the behavior of the animals.

Materials And Methods

All animal handling procedures and experimental protocols conducted in accordance with relevant guidelines and regulations and approved by the regional ethical committee of the College of Medicine and Health Sciences, UAE University, United Arab Emirates (RECA/01/05). In addition, study was carried out in compliance with the ARRIVE guidelines.

2-1-Animals and adrenalectomy

Eight-weeks old males Wistar rats 170–220 g were obtained from College of Medicine and Health Sciences animal facility (Al Ain, United Arab Emirates) and used in the current study. Under pentobarbital

(35 mg/kg body weight, Ilium-Troy Laboratories, New South Wales, Australia) anesthesia, rats were subjected either to adrenalectomy or to sham operation (laparotomy) as described by Sloviter et al.⁶. Shaving the back of the animals was done by using an electric shaving machine (Wahl, Illinois, USA). The rat was placed on the surgical table ventrally and in order to perform an aseptic surgery, the back of the animal was cleaned with 70% ethanol (Sigma-Aldrich, Missouri, USA). Bilateral incisions were made into the skin and the dorsal muscle to access the peritoneal cavity. The size of the muscle incision was just big enough to expose the adrenal gland on the top of the kidney and some free space to remove it without leaving any intact residuals of the gland. The peri-adrenal fat was grasped using blunt forceps and the adrenal gland was exteriorized, with a tipped-blunt scissors a cut was made at the connective tissue between the kidney and the adrenal gland. The incision was sutured, and the animal was returned to the cage. The same procedure was applied to the sham operated animals except the removal of the adrenal gland.

All animals were placed in plexiglas cages, four rats per group, maintained in a temperature (22°C) and 70±5% of humidity. Rats were housed in a 12:12 h normal light–dark cycle and received ad libitum access to food and water throughout the experiment. ADX rats were provided with 0.9% saline instead of drinking water in order to maintain electrolyte balance and prevent the deleterious effects of sodium chloride insufficiency.

2-2-Determination of plasma corticosterone levels

The level of corticosterone (CORT) in the serum was used to assess the efficacy of adrenalectomy. Approximately 1 ml blood samples were taken directly from the heart at the time of sacrifice. The sera samples were stored at -80 °C until CORT levels were measured by Enzyme Immunoassay (EIA) Kit (Life sciences, Lausen, Switzerland) according to the manufacturer's protocol. The procedure uses a polyclonal antibody to CORT to bind in a competitive manner. A standard curve in the range from 32 to 20,000 pg/ml was constructed to calculate CORT concentrations in the samples.

2-3-Stereological analysis

Stereological quantification of pyramidal and granule cells was performed on the hippocampus as described earlier³⁰. The total number of animals used is 50 animals distributed as follows: 4 h (Sham = 5, ADX = 5), 24 h (Sham = 5, ADX = 5), 3 days (Sham = 5, ADX = 5), 7 days (Sham = 5, ADX = 5) and 14 days (Sham = 5, ADX = 5). Briefly, three series of tissue sections were processed for cresyl fast violet (Nissl) staining to label neuronal cells. Stereological analysis was performed with a stereology workstation (Computer Assisted Stereological Toolbox (CAST) -grid, Olympus, Denmark). Slides to count were determined in systematic random sampling as described earlier³⁰. The total number of pyramidal and granule cells was estimated using the optical dissector method. Counts included all neuronal cells distributed in a systematic-random fashion within an unbiased counting frame throughout the Hippocampus.

2-4-Transmission electron microscopy

At each designated time point (3,7 and 14 days), animals (n= 18) were deeply anesthetized with intra-peritoneal administration of sodium pentobarbital (35 mg/kg body weight). Transcardial perfusion fixation was performed through the ascending aorta, with 50 ml of 0.1 M phosphate-buffered saline followed by 300 ml of freshly prepared solution of Karnovsky's fixative, PH 7.2 at 3 days (n=6), 7 days (n=6) and 14 days (n=6) after the surgery. For electron microscopy samples were immersed in McDowell and Trump fixative for 3 h at 25 °C as described previously³¹. Tissues were rinsed with phosphate buffer saline and fixed with 1% osmium tetroxide for 1 h. Samples were washed with distilled water, dehydrated in graded ethanol and propylene oxide. Finally the tissue was embedded in Agar-100 epoxy resin at 65 °C for 24 h. Blocks were trimmed and semi thin and ultra-thin sections were cut with Reichert Ultracuts (ultramicrotome) approximately -3.7 mm relative to bregma according to Paxinos and Watson.³² The ultrathin sections (95 nm) mounted on 200 mesh Cu grids were contrasted with uranyl acetate followed by lead citrate double stain. The grids were examined and photographed under a Philips CM10 transmission electron microscope³¹.

2-5-Enzyme-linked immunosorbent assay (ELISA)

We used ELISA to evaluate the levels of two growth factors (IGF-1 and β -NGF) in the hippocampal homogenates of ADX and sham operated. Ninety two rats were used as follow: 30min (Sham = 6, ADX = 6), 2 h (Sham = 6, ADX = 6), 4 h (Sham = 6, ADX = 6), 12h (Sham = 6, ADX = 6), 24 h (Sham = 6, ADX = 6), 3 days (Sham = 6, ADX = 6), 7 days (Sham = 6, ADX = 6) and 14 days (Sham = 6, ADX = 6).

IGF-1, a trophic factor for neurons, has also been shown to be an important regulator of cell metabolism, differentiation, and survival. The Quantikine® ELISA from R&D Systems® assay was used for the quantitative determination of rat IGF-1 in tissue homogenates of the hippocampi as described in the manufacturer's protocol. A monoclonal antibody specific for rat IGF-I had been pre-coated onto a microplate. Any rat IGF-I present in the sample was bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for rat IGF-I was added to the wells. Following the formation of bound antibody-enzyme reagent, a substrate solution yielded a blue product that turned yellow with the addition of the Stop Solution. The intensity of the color measured at 450nm was in proportion to the amount of rat IGF-I bound in the initial step.

β -NGF is a trophic factor for neurons and is involved in the maintenance of the sympathetic and sensory nervous systems. A DuoSet® ELISA from R&D Systems® assay was used for the quantitative measurement of rat β -NGF in hippocampal tissue homogenate as described in the manufacturer's protocol. We used a specific antibody for rat β -NGF, goat anti-rat β -NGF, coated on a 96-well plate. The β -NGF present in a sample was bound to the wells by the immobilized antibody. Later, biotinylated goat anti-rat β -NGF antibody was added followed by HRP conjugated streptavidin. The addition of the substrate solution (1:1 tetramethylbenzidine and H_2O_2) allowed color development in proportion to the amount of β -NGF bound. Finally, the Stop Solution (2 N sulfuric acid; H_2SO_4) changed the color from blue to yellow. the intensity of the color was measured at 450 nm was in proportion to the concentration of β -NGF in the hippocampal homogenate.

2-6-Step-through passive avoidance test

In order to examine the impact of aforementioned biochemical and cellular alterations in the hippocampus on cognitive function the Passive Avoidance (PA) task was used as described earlier^{33,34}. Fifty five animals were used as follow: 3 days (Sham = 6, ADX = 9), 7 days (Sham = 8, ADX = 10) and 14 days (Sham = 7, ADX = 16). All animals were handled daily for at least 3 days before the experiments. The PA task was conducted using a two-compartment standard shuttle box (51x25x24 cm) (Harvard Apparatus, Massachusetts, USA). The two compartments were of equal size and had a stainless-steel bar floor connected by built in sliding door (7x7 cm). The right-hand compartment (shock compartment) was painted black to obtain a dark chamber, while the left-hand compartment was illuminated by a bulb (24V; 5W) installed on the top Plexiglas cover. PA training was conducted in a single session (day 1).

The rats were placed in the lighted compartment (with no access to the dark compartment) and were allowed to explore for 60s. During the exploration period in the PA apparatus, when 60s expired, the sliding door was automatically opened, and the rat was allowed to cross over into the dark compartment. Once the rat had entered the dark compartment with all four paws, the sliding door was automatically closed and a weak electrical current (constant current, scrambled, duration 3s, 0.3 mA) was delivered through the grid floor. Latency to cross into the dark compartment (training latency) was recorded. If a rat failed to move into the dark compartment within 300 s (cut off latency), the door was reopened and the rat was gently moved into the dark compartment, where it received the foot shock. After exposure to the foot shock, the rat was allowed to stay for 30s in the dark compartment before it was removed from the PA apparatus and returned to its home cage.

Retention was tested 24 h after training (day 2)^{33,34}. The animal was again placed in the lighted (safe) compartment with access to the dark compartment for a period of 300s. The latency to enter the dark compartment with all four paws was automatically measured (retention latency). If the rat failed to enter the dark compartment within 300 s, it was removed and assigned a maximum test latency score of 300s.

2-7-Statistical analysis:

All data are reported as the mean $\pm$ standard error of the mean and the analysis was considered significantly different if $P \leq 0.05$. Results were analyzed by two-tail Student t test SPSS version 25 (IBM, USA).

Results

3-1-Determination of plasma corticosterone levels

Adrenalectomy resulted in significantly lower CORT levels compared to sham operated rats (Fig.1). At 0.5h the CORT concentration of ADX rats ($1.11 \pm 0.59\text{ng/ml}$) was significantly ($P < 0.001$) lower compared to sham operated rats ($64.17 \pm 8.26\text{ng/ml}$). After adrenalectomy, at 2h rats also showed a significantly ($P < 0.001$) reduced CORT level ($0.03 \pm 0.01\text{ng/ml}$) compared to sham operated rats ($50.34 \pm 7.04\text{ng/ml}$).

The CORT levels were significantly ($P<0.01$) reduced in the serum of 4 hr ADX rats ($0.16 \pm 0.16\text{ng/ml}$) compared to sham operated rats ($17.07 \pm 4.81\text{ng/ml}$). While at later time-points, circulating CORT levels were undetectable by the kit at concentrations lower than 0.103 ng/ml . A significant effect of post-surgical time was present for sham operated rats. Mean CORT levels were 64.17 ± 8.26 , 50.34 ± 7.04 , 17.07 ± 4.81 , 18.02 ± 4.93 , 19.14 ± 7.52 , 13.95 ± 7.03 , 16.45 ± 4.60 , and $15.00 \pm 1.18\text{ng/ml}$ at the 0.5-hour, 2-hour, 4-hour, 12-hour, 1-day, 3-day, 7-day, and 14-day time-points respectively.

3-2-Stereological analysis

Our stereological results showed the effect of the removal of the adrenal gland is deleterious to the dentate gyrus of the hippocampus of the ADX compared to sham operated rats starting from the third day postoperatively. As shown in Fig.2 the first three days following adrenalectomy the number of granule cells in the DG was affected but it did not reach a statistical significance. However, after 7 days postoperatively the number of the granule cells decreased significantly ($P<0.001$), and such effect continued to be seen 14 days later in the ADX rats compared to the sham operated rats ($P<0.001$).

Our results showed the effect of adrenalectomy on the hippocampus is not selective. The removal of the adrenal glands leads to the reduction in the levels of glucocorticoids to undetectable levels that caused ultimately the death of different types of the neuronal cells throughout the hippocampus. The number of pyramidal cells in the CA1 was not affected significantly over the course of time (4h, 24h, 3 days, 7 days and 14 days) in both ADX and sham operated rats (Fig.3).

Similarly, to what we have seen with the pyramidal cells of CA1 the removal of the adrenal gland did not affect the neuronal population of the CA2 area (Fig.4).

Our stereology results showed that the removal of the adrenal gland affect directly the survival of the CA3 pyramidal cells in the hippocampus. We observed significant loss ($P<0.001$) of CA3 neurons 7 days and 14 days post adrenalectomy in the ADX rats compared to sham operated rats (Fig.5).

3-2-Transmission electron microscopy

In our previous ultrastructural study, we reported that the absence of glucocorticoids for long period of time (5 months) induces non selective degeneration in the hippocampus where different neuronal populations were affected through exhibiting signs of abnormalities and different cell death types were observed¹³. In the current study we aimed to examine the impact of the absence of such hormones on different cells of the hippocampus at an early stage and at different time points (3, 7 and 14 days).

The examination of thin sections of the hippocampi of sham operated and ADX rats under the electron microscope three days postoperatively showed granule cells degeneration on the tip of the upper blade of the dentate gyrus where the cells exhibited condensed chromatin and irregular cell membrane and the beginning of vacuolation in the cytoplasm only in ADX rats (Fig.6 A,B,C,D). Interestingly we have not noticed cell abnormalities in the rest of the dentate gyrus of the ADX rats (Fig.6 B,D).

Concerning the different areas of the Cornu Ammonis (CA areas) three days post adrenalectomy, we found solely CA4 pyramidal cells displayed signs of cell damage following the removal of the adrenal gland of ADX rats (Fig.7 B,D) compared to sham operated rats (Fig.7 A,C). Nevertheless, pyramidal cells of CA1, CA2 and CA3 cells did not show any signs of neurodegeneration after three days of surgery in both groups (Results are not shown).

Seven days following adrenalectomy the ultrastructural examination of the hippocampus revealed a progression in neurodegeneration compared to the third day of adrenalectomy where cell death was seen all over in both blades of the dentate gyrus and more extensively on the tip of the upper blade where we observed more exacerbation of the neurodegenerative process (Fig.8A'). In addition to what we have seen on the third day after adrenalectomy more degenerative cells were observed in the CA4 seven days postoperatively (Fig.8B'). Electron microscopy examination of the neuropil in the CA3 area of the hippocampus of ADX rats revealed cell death of the pyramidal cells for the first time seven days following adrenalectomy (Fig.8C'). No sign of degeneration was seen in the CA1 and CA2 of the adrenalectomized rats. The hippocampus of the sham operated rats did not show any abnormalities in the different neuronal populations (Fig. 8A, B, C)

We observed 14 days post adrenalectomy, an extensive cell death on the tip of the upper blade of the dentate gyrus followed by less degree of cell death in lower blade. Through the examination of granule layer of the dentate gyrus, we observed differences in the gross appearance of the neuropil between the ADX and sham operated rats. In the ADX animals, the granule layer showed empty areas with multiple "blank" spaces that indicated already degenerated cells. In addition to the changes in the neuropil, we observed ultrastructural changes in the upper and lower blade of the dentate gyrus (Fig.9). We have observed 14 days following adrenalectomy the condensation of chromatin beside the shrinkage of the cell body revealing both apoptotic and necrotic process occurring in the dentate gyrus of the hippocampus. These observations indicate the major vulnerability of the granule cells of the hippocampus to the absence of glucocorticoids achieved by the removal of the adrenal gland (Fig.10). In contrast, in the sham operated rats the neuropil was continuous with abundant, tightly packed granule cells. As the granule cells are small neurons, the nucleus occupied most of the cell body with only a thin rim of cytoplasm surrounding it. The examination of the Cornu Ammonis of the ADX rats revealed an extensive cell death in the CA4 area where chromatin condensation and vacuolation were seen (Fig.11).

Electron microscopy examination of the CA3 area of the ADX rats, 14 days post adrenalectomy, revealed the occurrence of cell death of the pyramidal cells along the principal layer of this area where extensive chromatin condensation, and reduction of the soma volume and the wavy shrinkage of the nuclear membrane and the vacuolization of mitochondria occurs (Fig.12). Sham operated rats exhibited healthy clustered pyramidal cells with regular plasma membrane, and well defined nucleus with consistent dispersed chromatin. More importantly, our results showed no ultrastructural abnormalities of the CA1 CA2 pyramidal cells of both groups (Results not shown).

3-5-Growth Factors Analysis

Using ELISA, our results showed differences in IGF-1 protein levels. In the hippocampus, the IGF-1 protein levels were significantly decreased in the ADX rats at 12h ($P<0.01$) compared to the sham operated rats. The results remained consistently low at 24h ($P<0.01$), 3 days ($P<0.05$), 7 days ($P<0.01$), and 14 days ($P<0.05$) in the hippocampus of ADX rats compared to the sham operated (Fig. 13).

The evaluation of the levels of β -NGF in the hippocampus homogenates of ADX rats exhibited a significant decrease ($P<0.05$) in β -NGF levels only at 14 days postoperatively compared to sham operated rats. It is worth noting that there was no statistical difference between the groups prior to 14 days (Fig. 14).

3-6-Animal behavior test

Based on our histological and biochemical findings we expected a negative impact on the behavior of the ADX rats and hence Passive Avoidance Test was performed. The evaluation of the latency time for both ADX and sham operated rats over the course of time (3, 7 and 14 days) indicated a significant increase ($P<0.05$) on the third day following surgery (Fig.15).

Furthermore, at day seven a significant increase ($P<0.05$) in the latency time was observed in ADX rats compared to the sham (Fig.16).

Our results showed 14 days following adrenalectomy a significant increase in the latency time persisted between the two groups ($P<0.05$) (Fig.17).

Discussion

In the current study using stereological techniques and ultrastructural examination we investigated the impact of short-term adrenalectomy on different hippocampal neuronal populations in Wistar rats. In addition, the underlying mechanism(s) of degeneration in these neurons was investigated by measuring the levels of two important growth factors in the hippocampus, Insulin-like growth factor-1 (IGF-1) and β -nerve growth factor (β -NGF). Moreover, we examined whether the biochemical and histological changes in the hippocampus, after short-term adrenalectomy, have an impact on the behavior of Wistar rats.

Our results showed, half an hour following the bilateral removal of the adrenal gland, a drastic depletion in the levels of corticosterone in the ADX compared to the sham operated rats. Such decrease persists over the course of time of the experiment (0.5, 2, 4, 12 hours, and 1, 3, 7, 14 days). The adrenal gland is known as the major producer of corticosterone in the body and the significant depletion that was observed in the ADX rats is attributed to its removal^{6,35-38}.

The impact of long-term removal of the adrenal gland on neurodegeneration of hippocampal neurons has been a subject of extensive research. However, there is a controversy as to whether long-term removal selectively affects the granule cells or not. Long-term withdrawal of the glucocorticoids by adrenalectomy was wrongly suggested as a selective model of granule cells' degeneration^{6,28,39,40}. However, Sapolsky et

al.¹¹ and Adem et al.¹² showed indeed the pyramidal cells are also vulnerable to such hormonal manipulation. We therefore aimed to investigate the effect of short-term adrenalectomy on different neuronal populations of the hippocampus.

We have previously shown, using Fluoro-Jade B (FJB) staining, few dentate granule cells death on day three which increased progressively on day 7 and 14 post adrenalectomy⁴¹. In line with our previous findings in the present study, using stereological counting technique, we observed a decrease in the number of granule cells at day three, albeit not significant, which decreased significantly at day 7 and 14 post adrenalectomy. In addition, the stereological counting revealed a significant decrease in the number of CA3 pyramidal cells at day 7 and 14 post adrenalectomy.

Our ultrastructural observation of the hippocampus using transmission electron microscopy showed that granule cells of the dorsal blade appeared to be the first cells that get affected by the absence of glucocorticoids which occurred on the third day following adrenalectomy. Our results showed cell abnormalities appeared in the form of condensed chromatin and irregular cell membrane. These observations are in line with the findings of Sloviter et al.^{39,40} where they examined the granule cells' degeneration after four days following adrenalectomy. In contrast to the granule cells, no significant signs of damage have been seen along the pyramidal cells of the Cornu Ammonis.

Interestingly, seven days post adrenalectomy we have observed cell death of CA4 and CA3 pyramidal cells in addition to significant granule cell degeneration in both blades of the dentate gyrus. Moreover, 14 days post adrenalectomy a substantial cells loss all over the dentate gyrus where the morphological abnormalities of such cells appeared in cell membrane disintegration and chromatin condensation beside the invasion of the damaged area by glia. In addition, we observed a progression in pyramidal cells degeneration of CA4 and CA3 where more damaged and affected cells were seen. Similar to the granule cells, the pyramidal cells showed morphological abnormalities such as cell membrane disintegration, chromatin condensation and invasion of the damaged area by glia.

Few studies have investigated the relationship between glucocorticoids and IGF-1. It has been demonstrated that postnatal injection of dexamethasone causes a significant decrease in the levels of IGF-1 accompanied by cell death in the hippocampus⁴². In line with the later, it has been shown that in Alzheimer's disease the cortisol levels are significantly elevated with low levels of IGF-1⁴³. In contrast, in the current study after short-term bilateral adrenalectomy we observed a significant reduction in IGF-1 levels in the hippocampus at 12 hours, 1 day, 3 days, 7 days, and 14 days. Our present results support our previous findings⁴⁴ where we observed an alteration of IGF-1 receptor and its messenger ribonucleic acid (mRNA) in the hippocampus after long-term adrenalectomy using *in vitro* receptor autoradiography and *in situ* hybridization immunohistochemistry, respectively. Significantly decreased levels of IGF-1 receptor and its mRNA were noted in the DG and CA1–CA4 regions of the hippocampus after long-term adrenalectomy suggesting that the level and expression of IGF-1 receptors in the hippocampus is influenced by adrenal hormones. In the present study the significant reduction of IGF-1 seen at 12 hrs after adrenalectomy takes place long before the death of the neurons which is observed on day three post

adrenalectomy. Our results suggest that bilateral adrenalectomy increases susceptibility of hippocampal neurons to degeneration through early decrease in IGF-1 levels. These findings indicate that IGF-1 exerts potent neurotrophic and neuroprotective/ antiapoptotic activities in the hippocampus as suggested previously⁴⁵⁻⁴⁷.

In contrast to IGF-1, the levels of β -NGF were significantly reduced in the hippocampus only 14 days post adrenalectomy. Our findings are in line with those of Aloe²⁵ who found a significant decrease in the levels of β -NGF in young adult rat hippocampus 12 days after adrenalectomy. Taken together these findings indicate that β -NGF might not be involved in the early neurodegeneration seen in the hippocampus after bilateral adrenalectomy. In addition to IGF-1 other trophic factors, like β -NGF and others, which have impact on hippocampal neurons are affected later after bilateral adrenalectomy and may exacerbate the neurodegenerative process in the hippocampus.

The hippocampus is well known for its role in cognition^{48,49}, most importantly in learning and memory⁵⁰. As it was aforementioned due to the high concentration of glucocorticoid receptors in the hippocampus, it is considered the most targeted area of the brain by adrenal hormones^{51,52}. Several studies have shown the withdrawal of these hormones causes degeneration of different neuronal cell populations in the hippocampus. The impact of the neuronal damage caused by long-term absence of adrenal hormones on the behavior of rats was investigated by different investigators. We showed 19 weeks post adrenalectomy, a significant increase in the latency in the Morris maze task and a significant lower rearing scores by the ADX rats compared to the sham operated⁵³. A significant decrease in ambulatory and rearing activities was observed following chronic adrenalectomy, while grooming and defecation scores were not altered⁵⁴. Moreover, Conrad and Roy⁵⁵ showed that after long-term adrenalectomy, the ADX rats exhibited difficulties in acquiring a new spatial memory. Furthermore, Spanswick et al.²⁹ showed the inability to reverse such deficit in the spatial memory of long-term ADX rats by chronic treatment (6 weeks) with corticosterone or by an alternative neurogenic compound, Fluoxetine. The later finding indicates the crucial role of hippocampal formation in building memories and acquiring new information. In the current study, using the passive avoidance test we investigated the effect of short-term adrenalectomy on cognitive functions. Our results of the passive avoidance test showed that the ADX rats failed to retain their cognitive capacity compared to the sham operated where we have seen in days 3, 7 and 14 a significant increase in the latency of the ADX rats revealing a cognitive decline in these rats. These findings are in line with those of Oitzl and de Kloet⁵⁶ who found that adrenalectomy impaired the performance of rats in the maze 3 days after the surgery. Moreover, ADX rats were also significantly less active than controls on days 3 and 4 of testing as a result of a greater drop in activity over the four days of testing⁵⁷. Taken together our present results and those of others^{53,55,56} are in line with our previous results of long-term adrenalectomy and suggest that cognitive behavior impairment might start as early as day 3 following glucocorticoid withdrawal.

In conclusion, our results showed hippocampal granule and pyramidal neurons degeneration after short-term adrenalectomy. The significant reduction of IGF-1 in the hippocampus seen at 12 hrs after

adrenalectomy takes place long before the death of the neurons which is observed on day three post adrenalectomy. Our results suggest that bilateral adrenalectomy increases susceptibility of hippocampal neurons to degeneration through early decrease in IGF-1 levels. These findings indicate that IGF-1 exerts potent neurotrophic and neuroprotective/antiapoptotic activities in the hippocampus. Consequently, the loss of the hippocampal neurons after adrenalectomy leads to cognitive behavioral deficits.

References

1. Mühlen K aus der, null & Ockenfels, H. [Morphological alterations in the diencephalon and telencephalon following disturbances to the feedback mechanism adenohipophysis-adrenal cortex. 3. Studies on the guinea pig after administration of cortisone and hydrocortisone]. *Z Zellforsch Mikrosk Anat* **93**, 126–141 (1969).
2. Sapolsky, R. M. & Pulsinelli, W. A. Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. *Science* **229**, 1397–1400 (1985).
3. Sapolsky, R. M., Uno, H., Rebert, C. S. & Finch, C. E. Hippocampal damage associated with prolonged glucocorticoid exposure in primates. *J. Neurosci.* **10**, 2897–2902 (1990).
4. Ábrahám, I. M., Meerlo, P. & Luiten, P. G. Concentration Dependent Actions of Glucocorticoids on Neuronal Viability and Survival. *Dose Response* **4**, 38–54 (2006).
5. Morse, J. K. & Davis, J. N. Regulation of ischemic hippocampal damage in the gerbil: Adrenalectomy alters the rate of CA1 cell disappearance. *Experimental Neurology* **110**, 86–92 (1990).
6. Sloviter, R. S. *et al.* Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy. *Science* **243**, 535–538 (1989).
7. Conrad, C. & Roy, E. Selective loss of hippocampal granule cells following adrenalectomy: implications for spatial memory. *J Neurosci* **13**, 2582–2590 (1993).
8. Sloviter, R. S., Dean, E. & Neubort, S. Electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat: apoptosis in the adult central nervous system. *J. Comp. Neurol.* **330**, 337–351 (1993).
9. Sloviter, R. S., Sollas, A. L., Dean, E. & Neubort, S. Adrenalectomy-induced granule cell degeneration in the rat hippocampal dentate gyrus: characterization of an in vivo model of controlled neuronal death. *J. Comp. Neurol.* **330**, 324–336 (1993).
10. Sousa, N., Madeira, M. D. & Paula-Barbosa, M. M. Structural alterations of the hippocampal formation of adrenalectomized rats: an unbiased stereological study. *J Neurocytol* **26**, 423–438 (1997).
11. Sapolsky, R. M., Stein-Behrens, B. A. & Armanini, M. P. Long-term adrenalectomy causes loss of dentate gyrus and pyramidal neurons in the adult hippocampus. *Experimental Neurology* **114**, 246–249 (1991).
12. Adem, A., A, I., N, B., K, C. & B, W. Loss of neurones after long-term adrenalectomy in the adult rat hippocampal formation. *Neuroreport* **5**, 2285–2288 (1994).

13. Islam, A. *et al.* Ultrastructural analysis of the hippocampus of adult rats after long-term adrenalectomy. *Brain Research* **849**, 226–230 (1999).
14. Martínez-Claros, M. *et al.* Adrenalectomy and corticosterone replacement differentially alter CA3 dendritic morphology and new cell survival in the adult rat hippocampus. *Journal of Chemical Neuroanatomy* **48–49**, 23–28 (2013).
15. Labandeira-Garcia, J. L., Costa-Besada, M. A., Labandeira, C. M., Villar-Cheda, B. & Rodríguez-Perez, A. I. Insulin-Like Growth Factor-1 and Neuroinflammation. *Front Aging Neurosci* **9**, (2017).
16. Lewitt, M. S. & Boyd, G. W. The Role of Insulin-Like Growth Factors and Insulin-Like Growth Factor–Binding Proteins in the Nervous System. *Biochem Insights* **12**, 1178626419842176 (2019).
17. Stern, S. A., Chen, D. Y. & Alberini, C. M. The effect of insulin and insulin-like growth factors on hippocampus- and amygdala-dependent long-term memory formation. *Learn Mem* **21**, 556–563 (2014).
18. Conner, J. M. *et al.* NGF Is Essential for Hippocampal Plasticity and Learning. *J Neurosci* **29**, 10883–10889 (2009).
19. Lockhart, S. T., Turrigiano, G. G. & Birren, S. J. Nerve Growth Factor Modulates Synaptic Transmission between Sympathetic Neurons and Cardiac Myocytes. *J Neurosci* **17**, 9573–9582 (1997).
20. Chen, W.-H., Mao, C.-Q., Zhuo, L.-L. & Ong, J. L. Beta-nerve growth factor promotes neurogenesis and angiogenesis during the repair of bone defects. *Neural Regen Res* **10**, 1159–1165 (2015).
21. Cho, H.-K. *et al.* Beta-nerve growth factor gene therapy alleviates pyridoxine-induced neuropathic damage by increasing doublecortin and tyrosine kinase A in the dorsal root ganglion. *Neural Regen Res* **15**, 162–168 (2020).
22. Petruska, J. C. & Mendell, L. M. Nerve Growth Factor. in *Encyclopedia of Neuroscience* (ed. Squire, L. R.) 71–78 (Academic Press, 2009). doi:10.1016/B978-008045046-9.00672-0.
23. Martorana, F. *et al.* Differentiation by nerve growth factor (NGF) involves mechanisms of crosstalk between energy homeostasis and mitochondrial remodeling. *Cell Death Dis* **9**, (2018).
24. Kristiansen, M. & Ham, J. Programmed cell death during neuronal development: the sympathetic neuron model. *Cell Death & Differentiation* **21**, 1025–1035 (2014).
25. Aloe, L. Adrenalectomy decreases nerve growth factor in young adult rat hippocampus. *PNAS* **86**, 5636–5640 (1989).
26. Cheung, P. T., Wu, J., Banach, W. & Chernausk, S. D. Glucocorticoid regulation of an insulin-like growth factor-binding protein-4 protease produced by a rat neuronal cell line. *Endocrinology* **135**, 1328–1335 (1994).
27. Armstrong, J. N., McIntyre, D. C., Neubort, S. & Sloviter, R. S. Learning and memory after adrenalectomy-induced hippocampal dentate granule cell degeneration in the rat. *Hippocampus* **3**, 359–371 (1993).
28. Conrad, C. D. & Roy, E. J. Selective loss of hippocampal granule cells following adrenalectomy: implications for spatial memory. *J. Neurosci.* **13**, 2582–2590 (1993).

29. Spanswick, S. C., Epp, J. R., Keith, J. R. & Sutherland, R. J. Adrenalectomy-induced granule cell degeneration in the hippocampus causes spatial memory deficits that are not reversed by chronic treatment with corticosterone or fluoxetine. *Hippocampus* **17**, 137–146 (2007).
30. Odaci, Dr. E., Bas, O. & Kaplan, S. Effects of prenatal exposure to a 900 MHz electromagnetic field on the dentate gyrus of rats: A stereological and histopathological study. *Brain research* **1238**, 224–9 (2008).
31. Bastaki, S. M. A., Amir, N., Saeed, T. & Adeghate, E. Effect of diabetes mellitus on vitamin B12, pepsinogen and gastric intrinsic factor levels in rats. *Hamdan Medical Journal* **13**, 93 (2020).
32. Paxinos, G. & Watson, C. The Rat Brain in Stereotaxic Coordinates - 6th Edition. <https://www.elsevier.com/books/the-rat-brain-in-stereotaxic-coordinates/paxinos/978-0-12-374121-9?aaref=https%3A%2F%2Fwww.google.com%2F> (2007).
33. Ahlander-Lüttgen, M., Madjid, N., Schött, P. A., Sandin, J. & Ogren, S. O. Analysis of the role of the 5-HT1B receptor in spatial and aversive learning in the rat. *Neuropsychopharmacology* **28**, 1642–1655 (2003).
34. Misane, I. & Ögren, S. O. Selective 5-HT 1A Antagonists WAY 100635 and NAD-299 Attenuate the Impairment of Passive Avoidance Caused by Scopolamine in the Rat. *Neuropsychopharmacology* **28**, 253–264 (2003).
35. Gould, E., Woolley, C. S. & McEwen, B. S. Short-term glucocorticoid manipulations affect neuronal morphology and survival in the adult dentate gyrus. *Neuroscience* **37**, 367–375 (1990).
36. Jaarsma, Postema, F. & Korf, J. Time course and distribution of neuronal degeneration in the dentate gyrus of rat after adrenalectomy: A silver impregnation study. *Hippocampus* **2**, 143–150 (1992).
37. Sloviter, R. S., Dean, E. & Neubort, S. Electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat: Apoptosis in the adult central nervous system. *Journal of Comparative Neurology* **330**, 337–351 (1993).
38. Spanswick, S. C., Lehmann, H. & Sutherland, R. J. A Novel Animal Model of Hippocampal Cognitive Deficits, Slow Neurodegeneration, and Neuroregeneration. *Journal of Biomedicine and Biotechnology* vol. 2011 e527201 <https://www.hindawi.com/journals/bmri/2011/527201/> (2011).
39. Sloviter, R. S., Dean, E. & Neubort, S. Electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat: apoptosis in the adult central nervous system. *The Journal of comparative neurology* (1993) doi:10.1002/cne.903300305.
40. Sloviter, R. S., Sollas, A. L., Dean, E. & Neubort, S. Adrenalectomy-induced granule cell degeneration in the rat hippocampal dentate gyrus: Characterization of an in vivo model of controlled neuronal death. *Journal of Comparative Neurology* **330**, 324–336 (1993).
41. Hamadi, N. *et al.* Increased pro-inflammatory cytokines, glial activation and oxidative stress in the hippocampus after short-term bilateral adrenalectomy. *BMC Neuroscience* **17**, (2016).
42. Feng, Y., Famuyide, M. & Bhatt, A. J. Dexamethasone decreases insulin-like growth factor-I and -II via a glucocorticoid receptor dependent mechanism in developing rat brain. *Neuro Endocrinol Lett* **34**, 624–634 (2013).

43. Murialdo, G. *et al.* Relationships between cortisol, dehydroepiandrosterone sulphate and insulin-like growth factor-I system in dementia. *J Endocrinol Invest* **24**, 139–146 (2001).
44. Islam, A. *et al.* Changes in IGF-1 receptors in the hippocampus of adult rats after long-term adrenalectomy: receptor autoradiography and in situ hybridization histochemistry. *Brain Res* **797**, 342–346 (1998).
45. Beilharz, E. J., Bassett, N. S., Sirimanne, E. S., Williams, C. E. & Gluckman, P. D. Insulin-like growth factor II is induced during wound repair following hypoxic-ischemic injury in the developing rat brain. *Molecular Brain Research* **29**, 81–91 (1995).
46. Knusel, B., Michel, P. P., Schwaber, J. S. & Hefti, F. Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J Neurosci* **10**, 558–570 (1990).
47. Russo, V. C., Gluckman, P. D., Feldman, E. L. & Werther, G. A. The Insulin-Like Growth Factor System and Its Pleiotropic Functions in Brain. *Endocrine Reviews* **26**, 916–943 (2005).
48. Strange, B. A., Witter, M. P., Lein, E. S. & Moser, E. I. Functional organization of the hippocampal longitudinal axis. *Nat. Rev. Neurosci.* **15**, 655–669 (2014).
49. Zeidman, P. & Maguire, E. A. Anterior hippocampus: the anatomy of perception, imagination and episodic memory. *Nat. Rev. Neurosci.* **17**, 173–182 (2016).
50. Morgado-Bernal, I. Learning and memory consolidation: linking molecular and behavioral data. *Neuroscience* **176**, 12–19 (2011).
51. Conrad, C. D. Chronic Stress-Induced Hippocampal Vulnerability: The Glucocorticoid Vulnerability Hypothesis. *Rev Neurosci* **19**, 395–411 (2008).
52. Rogalska, J. Mineralocorticoid and glucocorticoid receptors in hippocampus: their impact on neurons survival and behavioral impairment after neonatal brain injury. *Vitam. Horm.* **82**, 391–419 (2010).
53. Islam, A., Henriksson, B., Mohammed, A., Winblad, B. & Adem, A. Behavioural deficits in adult rats following long-term adrenalectomy. *Neuroscience Letters* **194**, 49–52 (1995).
54. Veldhuis, H. D., De Kloet, E. R., Van Zoest, I. & Bohus, B. Adrenalectomy reduces exploratory activity in the rat: A specific role of corticosterone. *Hormones and Behavior* **16**, 191–198 (1982).
55. Conrad, C. D. & Roy, E. J. Dentate gyrus destruction and spatial learning impairment after corticosteroid removal in young and middle-aged rats. *Hippocampus* **5**, 1–15 (1995).
56. Oitzl, M. S. & de Kloet, E. R. Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. *Behav. Neurosci.* **106**, 62–71 (1992).
57. Joffe, J. M., Mulick, J. A. & Rawson, R. A. Effects of adrenalectomy on open-field behavior in rats. *Hormones and Behavior* **3**, 87–96 (1972).

Figures

Figure 1

Bar graph showing levels of serum corticosterone. Levels of corticosterone in the serum of ADX compared to the sham operated rats over the course of time (0.5, 2, 4, 12 hours, and 1, 3, 7, 14 days). **P<0.01; ***P<0.001. Data are expressed as mean ($\pm$ SEM).

Figure 2

Bar graphs showing the number of DG granule cells in the hippocampus of ADX and sham operated rats. No difference in the number of neurons was observed 3 days postoperatively. However, a significant decrease in the number of granule cells was observed 7 and 14 days following adrenalectomy in ADX rats compared to sham operated. *** $P < 0.001$. Data are expressed as mean ($\pm$ SEM).

Figure 3

Bar graphs showing the number of CA1 neurons in the hippocampus of ADX and sham operated rats. No difference in the number of neurons was revealed over the course of time (4h, 24h, 3 days, 7 days and 14 days) in both ADX and sham operated rats. Data are expressed as mean ($\pm$ SEM).

Figure 4

Bar graphs showing the number of CA2 neurons in the hippocampus of ADX and sham operated rats over the course (4h, 24h, 3 days, 7 days and 14 days). No difference in the numbers of neurons following adrenalectomy. Data are expressed as mean ($\pm$ SEM).

Figure 5

Bar graphs showing the number of CA3 neurons in the hippocampus of adrenalectomized and sham operated rats over the course (4h, 24h, 3 days, 7 days and 14 days). No difference in the number of CA3 neurons up to 3 days but a significant decrease was observed 7 and 14 days following adrenalectomy. ***P<0.001. Data are expressed as mean ($\pm$ SEM).

Figure 6

Electron micrograph of the upper blade of the dentate gyrus three days postoperatively of ADX rats compared to sham operated. (A) Usually the granule cells of the dentate gyrus of the hippocampus have a round shape and a big nucleus occupies the whole cell body, in such cells the nucleus surrounded by thin layer of cytoplasm. (B) in the ADX rats a few degenerated granule cells were seen on the tip of the upper blade where deformation of the cell morphology was seen, in addition to the shrinkage of the cell body. Scale bar = 5 μ m. (C) and (D) higher magnification of granule layer of dentate gyrus of sham operated and ADX rats respectively. Scale bar = 2 μ m.

Figure 7

Electron micrograph of CA4 pyramidal cells three days postoperatively of ADX rats compared to sham operated. (A) Healthy pyramidal cells showing well-defined nucleolus and nucleus and homogenous karyoplasm. (B) multiple degenerated pyramidal neurons of the CA4 with irregular nucleus membrane and signs of chromatin condensation, the arrow showing already degenerated neuron where reduction of the nucleus volume is apparent and dark compacted chromatin. Scale bar = 5 μ m. (C) and (D) higher magnification of pyramidal layer of CA4 of sham operated compared to ADX rats respectively. Scale bar = 2 μ m.

Figure 8

Electron micrograph showing degeneration in different areas of the hippocampus of ADX rats compared to the sham operated were taken seven days postoperatively. (A) intact granule cell with well-defined nucleus and cell membrane of sham rats. (A') degenerated granule cell of the dentate gyrus with condensed chromatin and irregular cell and nuclear membrane. (B) and (C) healthy pyramidal cells of CA4 and CA3 respectively. (B') showing ultrastructure of CA4 dead neurons with disintegration of the cell body, condensation of the chromatin and reduction of the nucleus volume. (C') degenerated pyramidal neurons of the CA3 showing irregular nucleus membrane and chromatin condensation. Scale bar = 1 μm.

Figure 9

Electron micrograph taken from the upper blade of the dentate gyrus 14 days following adrenalectomy showing granule cell engulfed by microglia (white arrows), in addition, it shows other granule cells undergoing degeneration where fragmentation of the nucleus and vacuolation in the cytoplasm is very apparent (black arrow) Scale bar = 5μm.

Figure 10

High power microscopy of degenerated granule in the hippocampus of adrenalectomized rats 14 days following adrenalectomy. Appearance of vacuolation in the cytoplasm with condensed chromatin (thin arrow) and adjacent granule cell in the process to be engulfed by microglia (big arrow) Scale bar = 1 μm.

Figure 11

High power microscopy taken from CA4 area of the hippocampus following 14 days of adrenalectomy showing many empty spaces in the pyramidal layer. In addition, many neurons with compacted chromatin surrounded by irregular nuclear membrane black arrow. A glial cell in the process to phagocyte pyramidal cell of the CA4 white arrow Scale bar = 5μm.

Figure 12

Electron micrograph showing CA3 pyramidal cells at different stage of degeneration 14 days following adrenalectomy confirming that withdrawal of glucocorticoids by adrenalectomy is not selective where different neuronal populations in the hippocampus are subjected to substantial degeneration Scale bar = 2 μ m.

Figure 13

Bar graph showing IGF-1 level in the hippocampus of ADX rats and sham operated rats. IGF-1 level was measured by ELISA over the course of time (0.5, 2, 4, 12 hours, and 1, 3, 7, 14 days). *P<0.05; **P<0.01. Data are expressed as mean ($\pm$ SEM)

Figure 14

Bar graph showing β -NGF level in the hippocampus of ADX rats and sham operated rats. β -NGF level was measured by ELISA over the course of time (0.5, 2, 4, 12 hours, and 1, 3, 7, 14 days). * $P < 0.05$. Data are expressed as mean ($\pm$ SEM)

Figure 15

Bar graphs showing latency time of ADX and Sham operated rats following three days of bilateral adrenalectomy. * $P < 0.05$. Data are expressed as mean $\pm$ SEM.

Figure 16

Bar graphs showing latency time of ADX and Sham operated rats following 7 days of bilateral adrenalectomy. * $P < 0.05$. Data are expressed as mean $\pm$ SEM.

Figure 17

Bar graphs showing latency time of ADX and Sham operated rats following 14 days of bilateral adrenalectomy.*P<0.05. Data are expressed as mean $\pm$ SEM.