Immunohistochemical study to Localize the distribution of glucocorticoid receptors in the brain of frog *Rana Ridibunda*. (a)

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ABSTRACT

Glucocorticoids play essential role in, development, growth, behavior, neuroendocrine regulation and metabolic processes that are mediated by the glucocorticoid receptor (GR). During stress, activation of the hypothalamic-pituitary-adrenal (HPA) axis induces the release of high concentration of glucocorticoids which bind to GR through the central nervous system to maintain homeostasis. Although, the GR has been considerably studied in mammals, little is known about the distribution of GR in amphibians. Thus, the aim of the present study was to localize the GR-expressing cells in the diencephalon and telencephalon within the brain of frog *Rana ridibunda*. We demonstrate by Immunohistochemical process that GR-immunoreactive cells are widely distributed in the anterior preoptic area (Poa) of hypothalamus, a homolog of the mammalian supraoptic and paraventricular nucleus (PVN) and known to contain corticotrophin-releasing factor (CRF), and in the organum vasculosum known as the supraoptic crest. Also, in the telencephalon, several limbic system regions contain GR-immunoreactive neurons distributed notably in the medial pallium (mp, homolog of the mammalian hippocampus), lateral pallium, medial amygdala, lateral amygdala, and ventral amygdale, and Bed nucleus of the pallial commissure. Moreover, we observed GR-immunoreactivity in thalamic structures such as dorsal habenular nucleus, ventral habenular nucleus, thalamic eminence, and the ventromedial thalamic nucleus. In most GR-immunoreactive cells, the immunostaining was observed in both the cytoplasm and the nucleus. These results support the idea that the general patterns of glucocorticoid receptor distribution in the neuroendocrine nuclei and limbic system of the central nervous system are highly conserved among vertebrates. Thus GR is likely to play roles in mediating the effects of corticosteroids on frog brain similar to those in mammals, suggesting that the basic regulatory pathways for modulating the responsiveness of the stress axis may be an evolutionary conserved mechanism in vertebrates. Our data represent the first step to map the distribution of GR in the brain of the frog *Rana ridibunda*.

Key words: Glucocorticoid receptor, Immunohistochemistry, *Rana ridibunda*, Stress, HPA axis, Limbic system, Amygdala.

دراسة كيميائية نسيجية مناعية لتحديد تمركز مستقبلات القشرانيات السكرية في دماغ الضفدع رانا ريديبيوندا

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الملخص

تؤدي القشرانيات السكرية دوراً مهماً في التطور، والنمو، والسلوك، والتنظيم العصبي الغـدي، وتنظـيم السُبل الاستقلابية عبر ارتباطها بمستقبلات القشرانيات السكرية. أثنـاء الكـرب، يـؤدي تنـشيط المحـور الوطائي–النخامي–الكظري إلى إفراز تراكيز مرتفعة من القشرانيات السكرية التي ترتبط بمـستقبلاتها فـي الجملة العصبية المركزية لحفظ الاستتباب الداخلي. وبالرغم من أن مستقبلات القشرانيات الـسكرية حظيـت باهتمام واسع عند الثدييات، فإن المعلومات المتوفرة عنها في البرمائيات قليلة جداً. لـذلك هـدفت الدراسـة الحالية إلى تحديد تمركز الخلايا العصبية التي تحتوي على هذه المستقبلات في الدماغ الانتهـائي، والـدماغ البيني للضفدع رانا ريديبيوندا. نبين باستخدام الكيمياء النـسيجية المناعيـة أن الخلايـا المحتويـة لهـذه المستقبلات تتركز بكثرة في الباحة قبل البصرية الأمامية للوطاء، التي تقابل في الثدييات النواة فوق البصرية والنواة جانب البطينية، المعروفة باحتوائها على العامل المُطلق للحاثة الموجهة للكظر(CRF)، وفي العـضو الوعائي المعروف بالعرف فوق البصري. وفي الدماغ الانتهائي، تحتوي مناطق عدة مـن الجملـة الحافيـة (الطرفية) عصبونات ذات مستقبلات للقشرانيات السكرية تتركز بشكل خاص في الباليوم (الرداء) المتوسـط (الذي يقابل الحُصين عند الثدييات)، والباليوم الجانبي، وفي اللوزة المتوسطة، واللـوزة الجانبيـة، واللـوزة البطنية، ونواة ملتقى الباليوم. إضافة إلى ذلك، تتركز الخلايا المحتوية على مستقبلات القـشرانيات الـسكرية في أجزاء عدة من السرير البصري، مثل نواة العنان الظهرية، ونواة العنان البطنيـة، والبـارزة المهاديـة، والنواة المهادية البطنية الوسطى. وفي معظم الخلايا، لوحظ أن المستقبلات تتركز في كل مـن الـسيتوبلاسما والنواة. تؤكد نتائج هذه الدراسة أن النمط العام لتوزع مستقبلات القشرانيات السكرية فـي النـوى الخاصـة بالتنظيم العصبي–الغدي، وفي الجملة الحافية للجملة العصبية المركزية، قد حُفظ تطورياً عند الفقاريات. لـذا تؤدي القشرانيات السكرية عبر ارتباطها بمستقبلاتها عند الضفدع على الأغلـب دوراً ممـاثلاً لـدورها فـي الثدييات، مما يفترض أن آليات التنظيم لتعديل الاستجابة الناتجة عن تنشيط المحـور الوطـائي–النخـامي–الكظري خلال الكرب قد حُفظت خلال تطور الفقاريات. تمثل نتائج دراستنا الحالية خطوة أولى لوضع خارطـة لتوزع مستقبلات القشرانيات السكرية في دماغ الضفدع رانا ريديبيوندا.

الكلمات المفتاحية: القشرانيات السكرية، الكيمياء النسيجية المناعية، رانا ريديبيونـدا، الكرب، المحور الوطائي–النخامي–الكظـري، الجملـة الحافيـة (الطرفية).

Introduction

Steroid hormones are produced by endocrine glands, including adrenal cortex, gonad and placenta. They exert a large array of biological effect in vertebrate. In particular, they play an important role in the development, growth, differentiation, proliferation, and maturation of the central and peripheral nervous system (1-5. for a review. see 6, 7). In addition, circulating steroid hormones play also a pivotal role in the control of a number of behavioral, neuroendocrine and metabolic processes such as regulation of food intake, locomotor activity, aggressiveness, anxiety, sexual activity, depression, stress , body temperature and blood pressure (8-10. for a review see 6,7). The production of glucocorticoids by adrenocortical cells (interrenal glands in amphibians) is largely controlled by pituitary adrenocorticotropic hormone (ACTH), whose synthesis and secretion is under hypothalamic regulation exerted by corticotropin releasing factor (CRF) and arginine vasopressin hormone (AVP) (for review. see 11-13).

During stress, activation of the hypothalamic-pituitary-adrenal (HPA) axis causes the release of high concentration of glucocorticoids which bind to glucocorticoid receptors through the central nervous system (CNS) to coordinate physiological and behavior adjustments to maintain homeostasis (for review. See 11-12). In fact, Elevated concentration of glucocorticoids exerts negative feed-back control at multiple levels of the Hypothalamic-pituitary-adrenal axis and suprahypothalamic limbic regions, such as the hippocampus and amygdala, to prevent continued activation (14-16). Feedback is mediated by the action of glucocorticoids at two types of steroid receptors, type I or mineralocorticoid receptors (MR) and type II or glucocorticoid receptor (GR). Both types of receptors have been extensively studied in the CNS of man (17-19), rat (20-21), dog (22) and mouse (23). These receptors belong to the nuclear receptor superfamily (24, 25) where its members act as ligand-dependent transcription factors. In the absence of ligand, the GR is located in the cytosol associated with heat shock proteins and immunophilins (26). Ligand binding causes disassociation of the protein complex and translocation of GR into the nucleus, where GR regulates transcription of its target genes (24-25, 27). In mammals, the MR is hypothesized to control the basal level and the circadian rhythm of circulating glucocorticoids, whereas glucocorticoid-depending physiological changes that occurs in response to stressors, and feedback regulation

by glucocorticoids is thought to be mediated by GR (28, for review. see 11, 29).

Moreover, the localization of GR receptor protein and mRNA was achieved in the brain of several vertebrate species such as Rhesus (30), mice (31), rat (32), chicken (1), Rainbow trout (33-34), and *Xenopus Laevis* (35). The highest Immunohistochemical staining of GR was found in the hippocampal region, hypothalamic paraventricular nucleus (PVN), preoptic nucleus, medial and central nucleus of the amygdala, Locus coeruleus, cerebellar cortex, olfactory pyramidal layers, and granule layer of the cerebellar cortex (31-36). The hippocampus expresses the highest level of GR in mammal brain, and glucocorticoid actions here evoke a tonic inhibition of neurosecretory neurons in the PVN via descending inhibitory projection to down-regulate HPA axis function (37). Also, the PVN neurons contain a high level of GR and thus they are a direct target for circulating glucocorticoids (15). Glucocorticoids affect also fear and cognition via their action in hippocampus and amygdala (38-40).

However, In spite of the good model of frog brain for the regulation of steroids biosynthesis and neuroendocrine regulation (41), the distribution of GR in the central nervous system of amphibian is relatively very little known. In the present study, we have attempted to demonstrate the distribution of glucocorticoid receptors (GR) in brain of a non-mammal vertebrate, the frog *Rana ridibunda*, especially in the diencephalon, and telencephalon nuclei, to localize the glucocorticoid targets in the central nervous system of frog, and to clarify the role of the different nuclei components of the limbic system in steroid regulation of HPA axis. Also our present study represents the first step to map the distribution of GR in the brain of frog *Rana ridibunda* .

Materials and methods

Animals

Adult male frogs (*Rana ridibunda*) of 50-60 g body weight were obtained from a commercial source. The animals were housed in a temperature-controlled room (8±1°C) under running water, on a 12 h light/dark schedule (lights on from 6:00 A.M. to 6:00 P.M.), for at least 1 week before use. To limit possible variations of steroid biosynthesis attributable to circadian rhythms, all animals were killed between 9:30 A.M. and 10:30 A.M.

Immunohistochemical Procedure

Animals were anesthetized by immersion in 0.1% of MS222 (3-aminobenzoic acid ethyl ester) and immediately perfused

transcardially via the aortic bulb, first with 30 ml of 0.1M phosphate buffer solution (PBS, PH 7.4) containing 0.025g xylocaine, then with 50 ml of 4% paraformaldehyde in PBS supplemented with sodium metaperiod as previously described (42-44). The brain were quickly removed and postfixed overnight at 4 Cº in the same fixative solution. Then, the tissues were rinsed overnight in PBS containing 15% sucrose and then transferred into a 30% sucrose solution for at least 24 hours. Brain were placed in an embedding medium (O.C.T. Teck, Rrichert-Jung S.A. Wien, Austria) and frozen at -80Cº until use. Sections were cut at 8µm-thick on a cryostat ,taken on glass lames and processed for indirect Immunohistochemical procedure as previously mentioned (42-43). Briefly, consecutive tissue sections were incubated overnight at 4Cº in a humid atmosphere with the first antibody directed against glucocorticoid receptors (anti-GR 1:50 dilution) in PBS containing 0.3% Triton X-100 and 1% BSA. The antibody is a polyclonal antiserum raised in rabbit against *Xinopus laevis* glucocorticoid receptors, purified and tested for its efficiently as previously described (44). The sections were rinsed in three baths of PBS (10mM) and prepared for Immunoreactive reactions with a biotinylated secondary antibody using Vectastatin Elite ABC (rabbit) and Vector VIP substrate Kits, following the manufacturer's instructions (both Kits from Vector, Burlingame, CA; 0.5µg/ml affinity-purified polyclonal rabbit anti-xGR IgG). Finally, the sections were rinsed in PBS, mounted in PBS-glycerol (1/1), coverslipped, and examined on a Leitz orthoplan microscope equipped with 1300R fast digital camera and linked with a computer. Brightness, contrast, and evenness of illumination were adjusted uniformly for images shown in the figures using Adobe Photoshop. The specificity of the immunoreaction was controlled either by substituting the primary antisera with PBS or by preabsorption with the antigenic xGR peptide as previously published (44).

Results

The distribution of GR in the brain of *Rana ridibunda* was analyzed by indirect immunohistochemical procedure. The analysis showed that the GR- immunoreactive cells are widely distributed in the diencephalon and telencephalon of the frog *Rana ridibunda.* In most GR-immunoreactive cells, the immunoreactivity was localized in both the cytoplasm and the nucleus, and in some regions the immunostaining was higher in the cytoplasm than in the nucleus. The anatomical drawings of frog brain regions schematized in figure-1 are from Yao et al 2008 and Do-rego et al (44, 57).

The distribution of GR-immunoreactive cells in the studied regions of the sections of frog brain is described as below:

1- Diencephalon: In particular, the GR-expressing cells are located in high density in the neuroendocrine component of the brain, notably the anterior preoptic area (Poa), homologues of the supraoptic and paraventricular nuclei (PVN) of mammal's brain, as shown in figure 2. In more caudal regions of the Poa, GR-immunoreactive cells were observed in the ventral part of magnocellular preoptic nucleus (Mgv) and the dorsal part of the magnocellular preoptic nucleus (Mgd) as illustrated in figure 3. The GR-immunoreactivity in these cells was found in both the nucleus and cytoplasm. In addition, GR-immunoreactivity was also seen in several structures of the thalamus of frog brain, including the dorsal habenular nucleus (Hd), the ventral habenular nucleus (Hv) as shown in figure 4, and in the thalamic eminence (TE), and in the ventromedial thalamic nucleus (VM) as shown in figure 5. We also observed a strong immunoreactivity in the organum vasculosum (OV) which represents one of the circumventricular of the third ventricle of the brain (see figure 5).

2- Telencephalon: The sites showing GR-immunoreactivity in telencephalon were localized in neurons of the medial pallium (MP), a homolog of the mammalian hippocampus, as shown in figure 6, in the lateral pallium (LP), and to a lesser extent but identifiable immunostaining cells were seen in the dorsal pallium (DP) as illustrated in figures 6. Also, a strong GR-immunoreactivity was localized in the Bed nucleus of the pallial commissure (BN), in the medial amygdale (MA), and in the lateral amygdale (LA) as shown in figures 7 and 8. In most cases, the immunoreactivity was distributed both in the nucleus and in the cytoplasm. We observed in several cases that some epithelial cells of the third and lateral ventricles demonstrated also a strong GR-immunoreactivity.

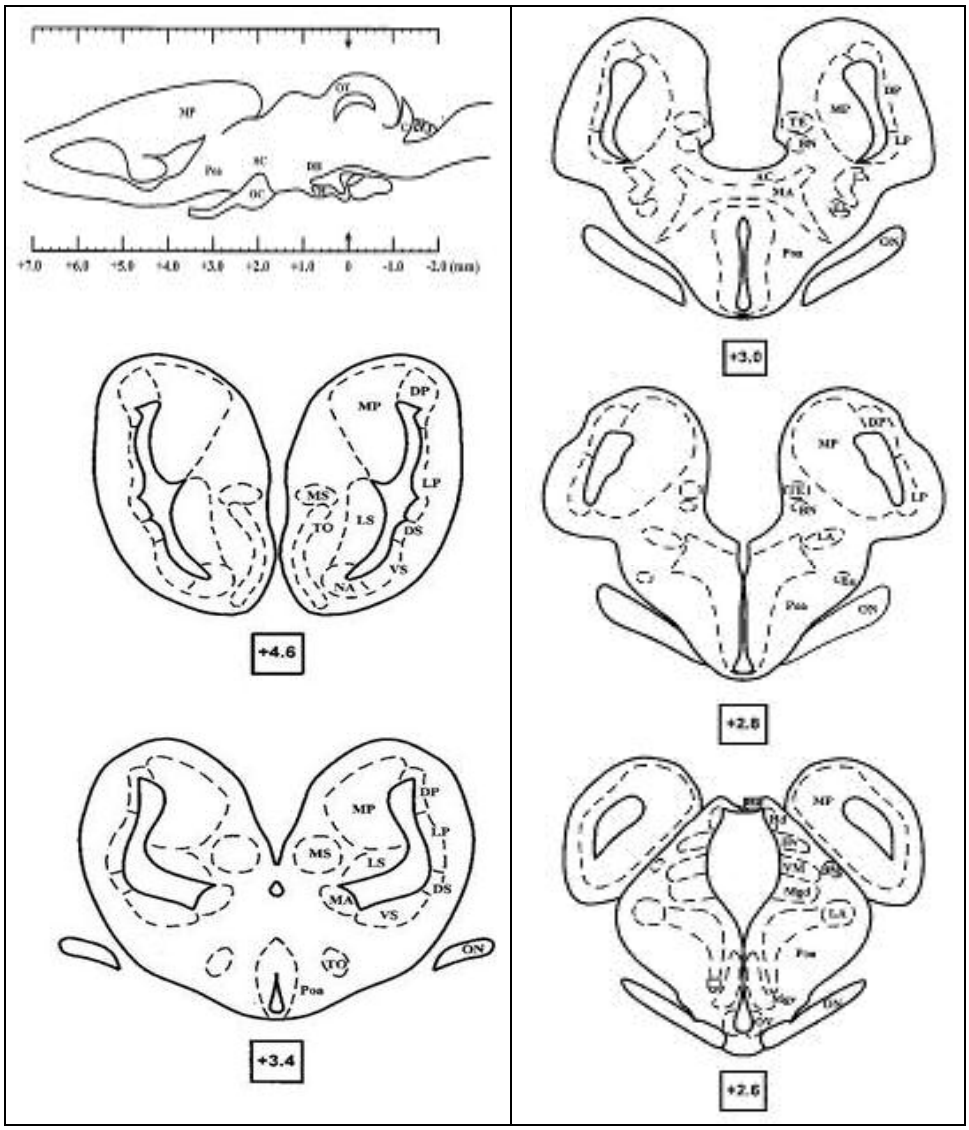


Fig.1. Anatomical illustration for the regions studied in the brain of frog *Rana ridibunda* to localize the distribution of GR-immunoreactive cells. The drawings are fromYao *et al* 2008 (44), and Do Rego et al 2007(57). The drawing at the left top of the figure shows a lateral view section of the frog brain.

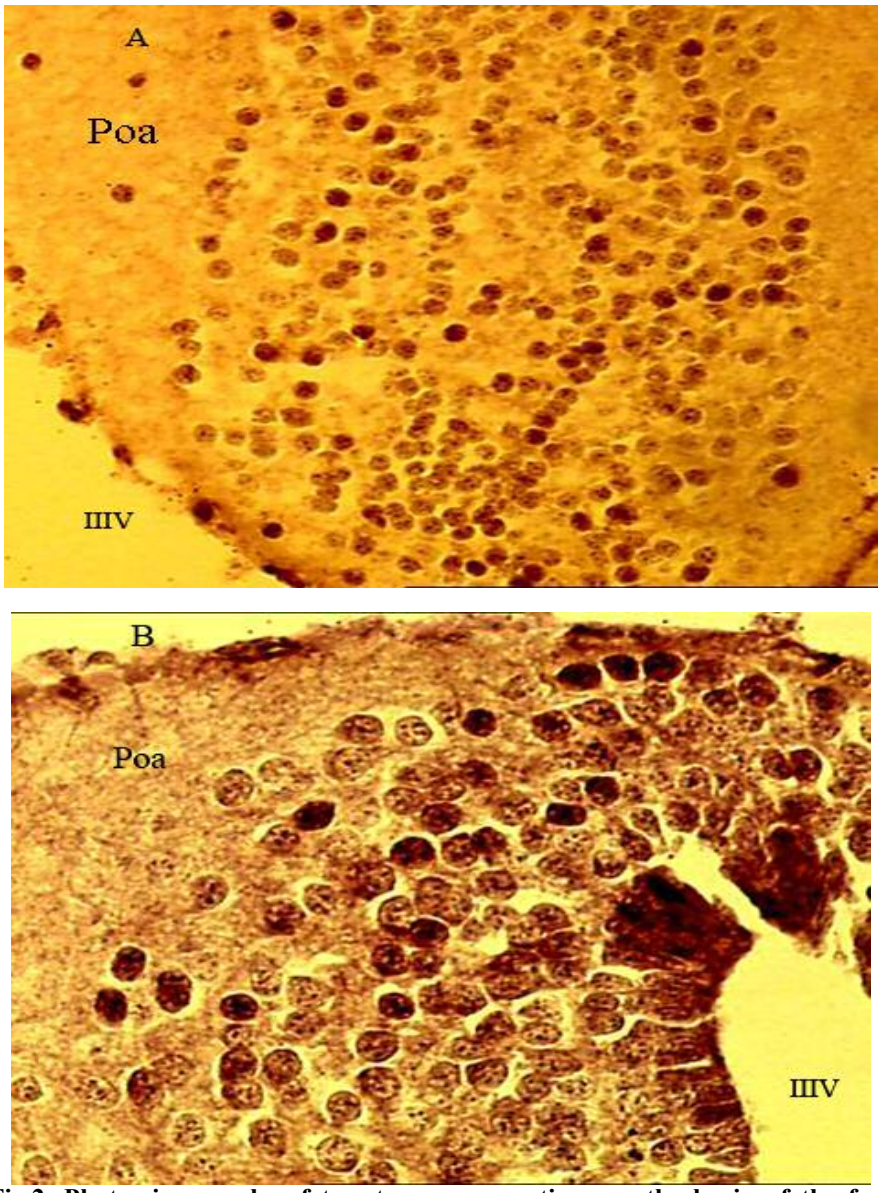


Fig.2. Photomicrographs of two transverse sections on the brain of the frog *Rana ridibunda* through the diencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells in the anterior preoptic area. (A: X10, B: X40). Also, the third ventricle (IIIV) appeared in the two sections.

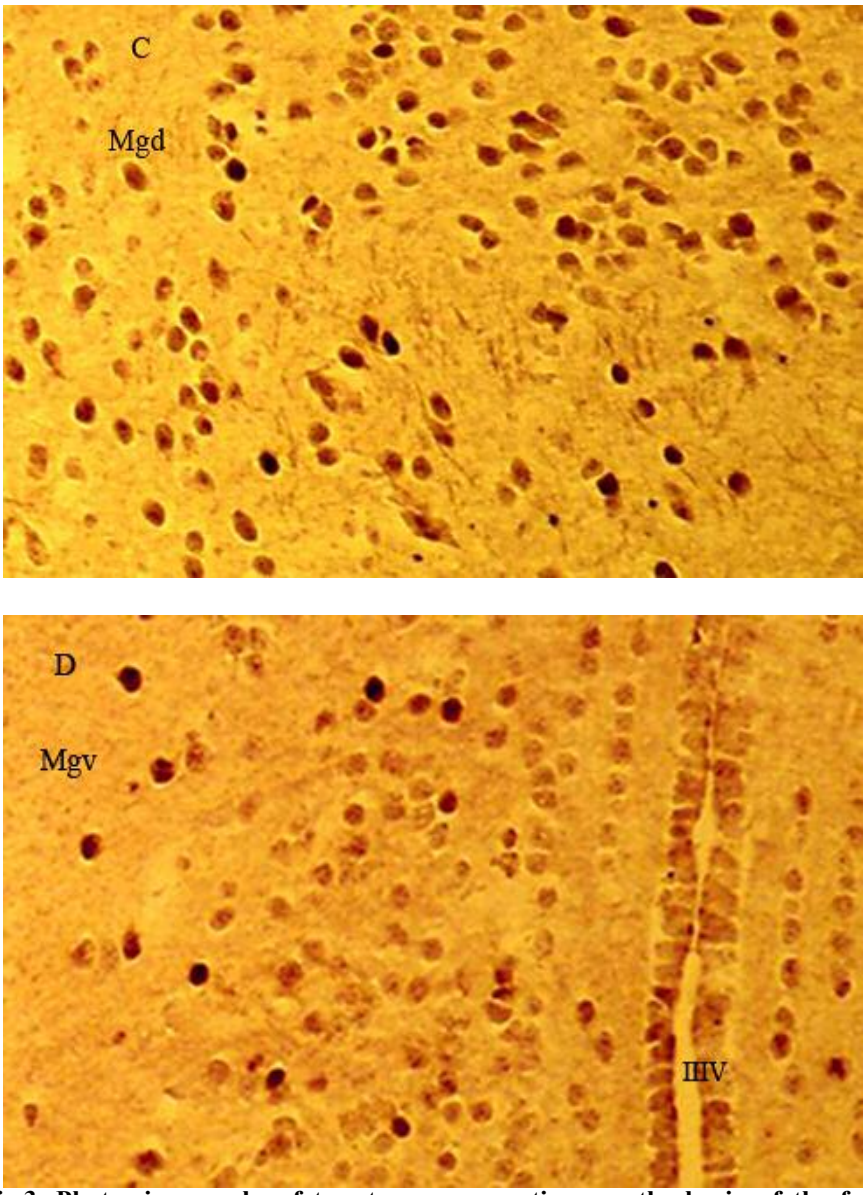
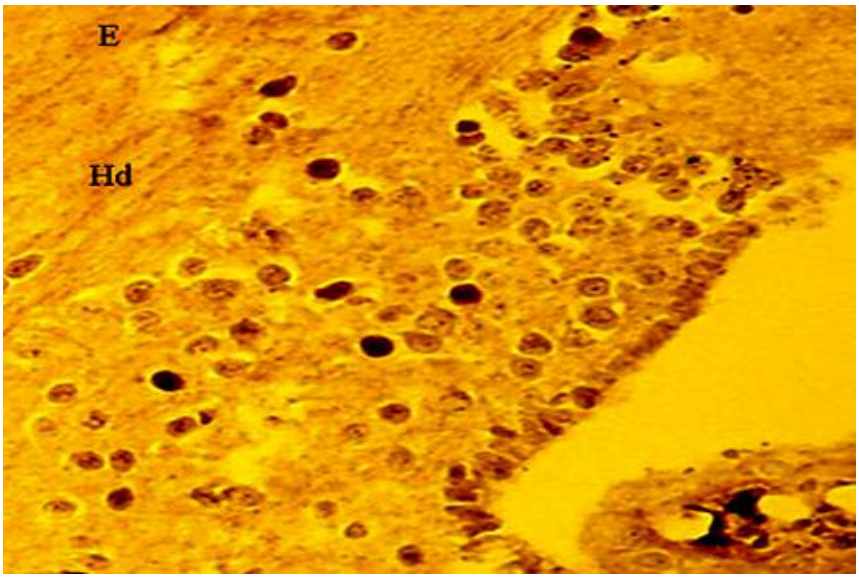


Fig.3. Photomicrographs of two transverse sections on the brain of the frog *Rana ridibunda* through the diencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells. C: in the dorsal part of magnocellular preoptic nuleus (Mgd), and D: in the ventral part of magnocellular preoptic nuleus (Mgv). X10.



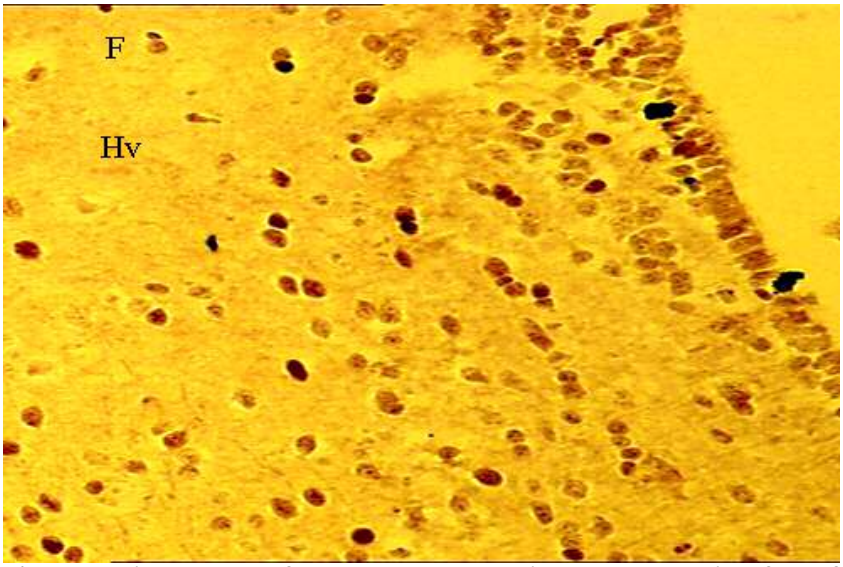
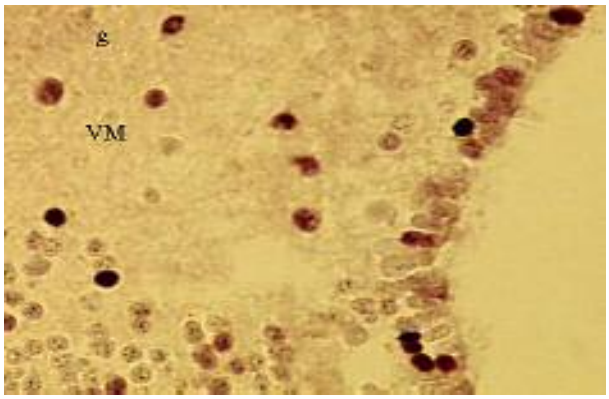
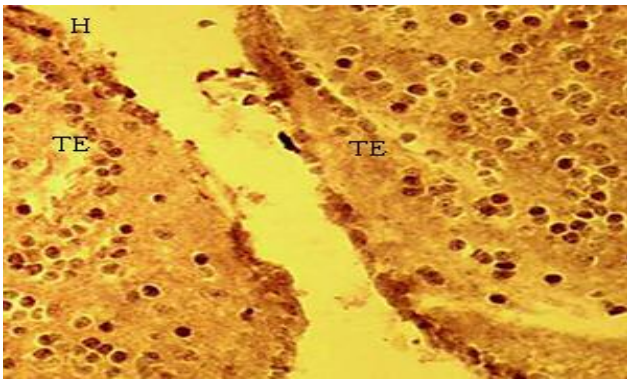


Fig.4. Photomicrographs of two transverse sections on the brain of the frog *Rana ridibunda* through the diencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells in: E, the dorsal habenular nucleus (Hd), and F, the ventral habenular nucleus (Hv). X10.





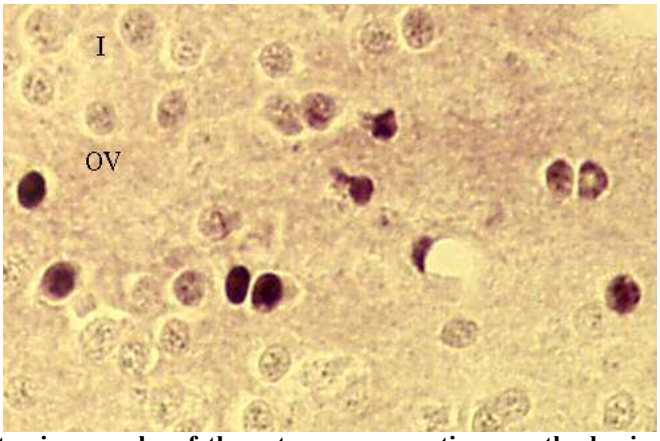


Fig.5. Photomicrographs of three transverse sections on the brain of the frog *Rana ridibunda* through the diencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells: g (X40): in the ventromedial thalamic nucleus (VM), and H(X10), in the thalamic eminence (TE), and I, in the organum vasculosum (OV). X40.

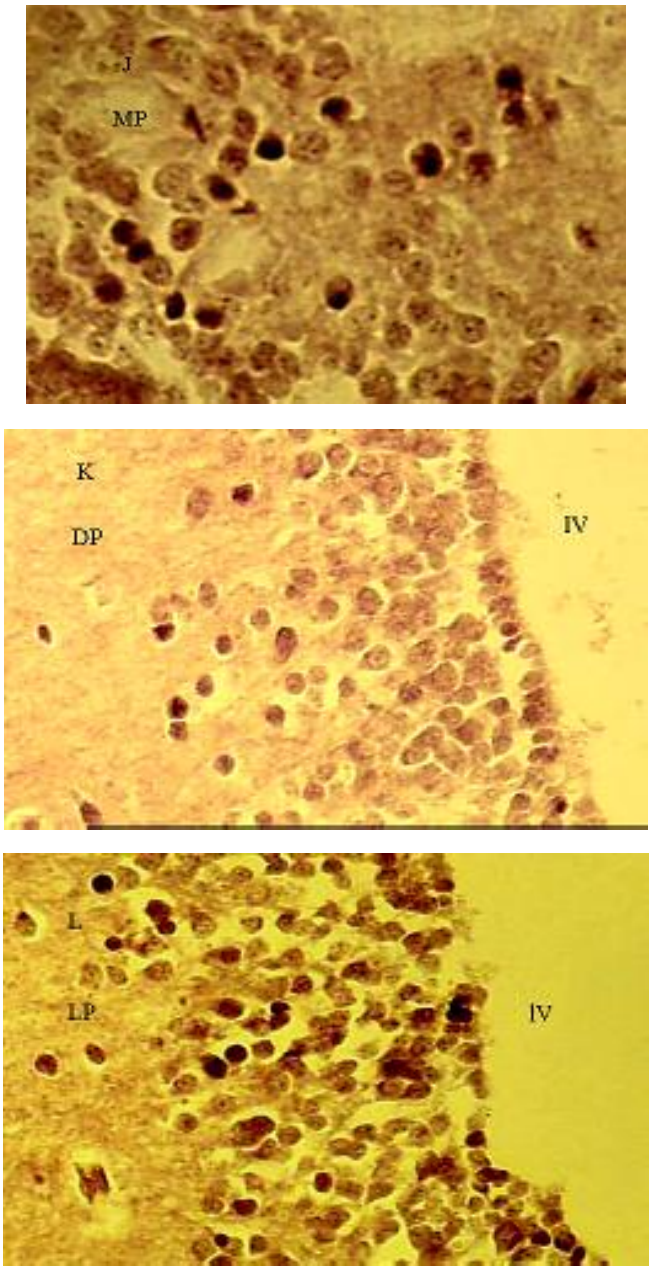


Fig.6. Photomicrographs of three transverse sections on the brain of the frog *Rana ridibunda* through the telencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells. J (X40) in the medial pallium (MP), K(X40); in the dorsal pallium (DP), and L(X40): in the lateral pallium (LP).

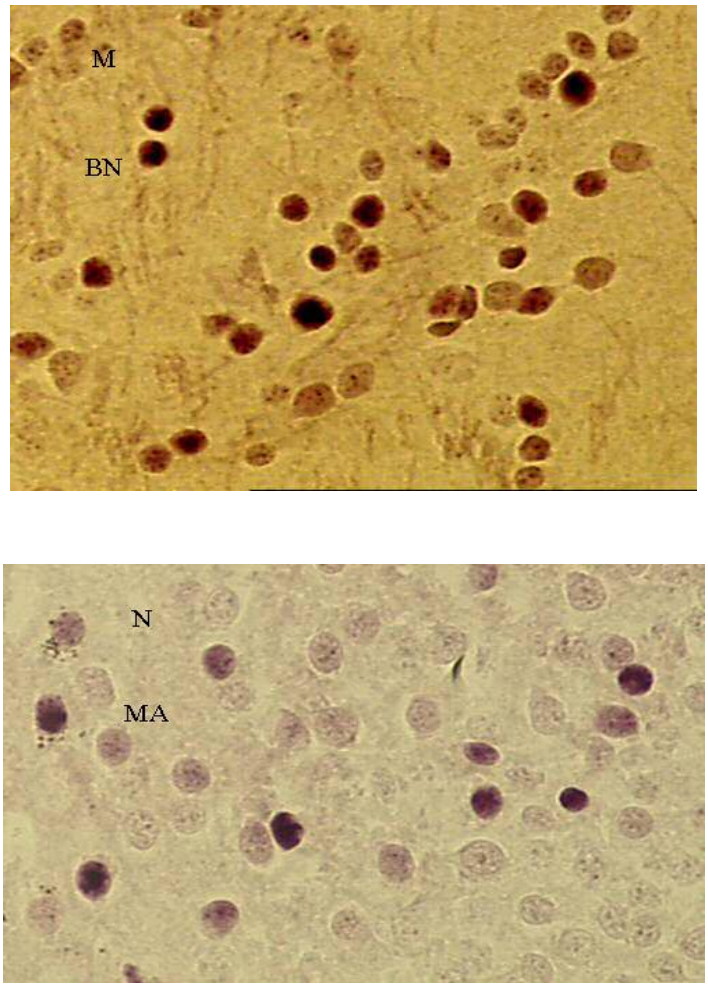


Fig.7. Photomicrographs of two transverse sections on the brain of the frog *Rana ridibunda* through the telencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells. M; in the Bed nucleus of the pallial commissure (BN), and N, in the medial amygdale (MA).X40

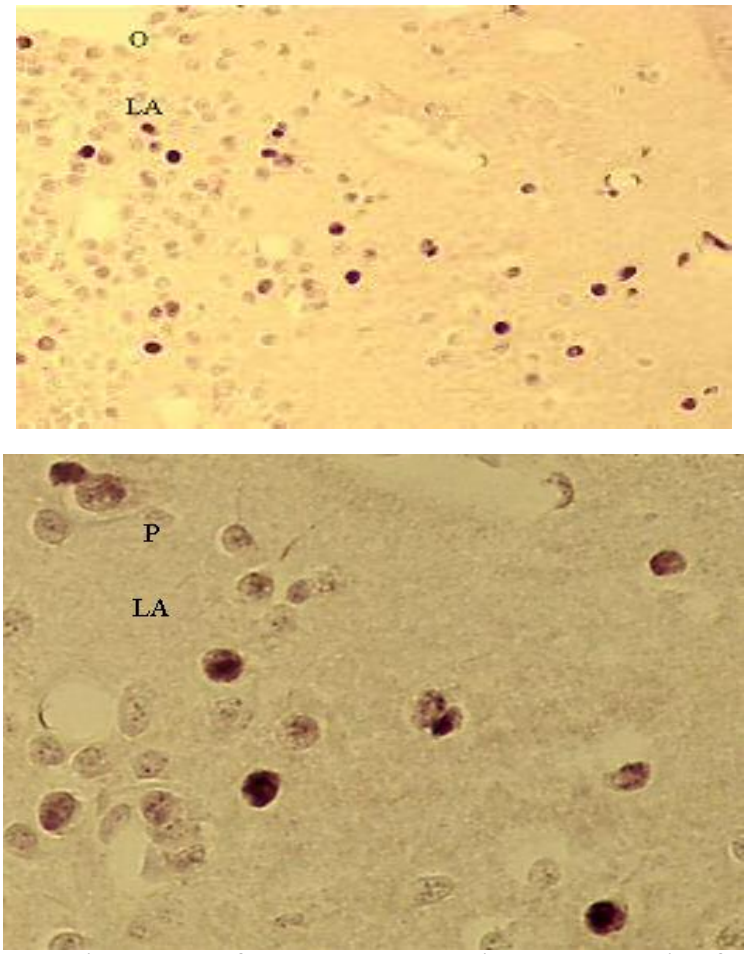


Fig.8. Photomicrographs of two transverse sections on the brain of the frog *Rana ridibunda* through the telencephalon showing the distribution of glucocorticoid receptor-immunoreactive cells in lateral amygdala (LA). O: X10, P: X40.

Discussion

Stress activates hypothalamic-pituitary-adrenal (HPA) axis which initiates a series of neuronal responses, and causes the release of high concentration of glucocorticoids that bind to glucocorticoid receptors (GR) through the central nervous system (CNS) to coordinate physiological and behavior adjustments and to prepare the organism to adapt to new environmental challenges. In the present study, we found that the glucocorticoid receptor-expressing cells are located in high

density in the neuroendocrine component of the brain of the frog *Rana ridibunda*, notably in the anterior preoptic area (Poa), homologues of the supraoptic and paraventricular nuclei (PVN) of the brain of mammals. The GR-immunoreactivity in these cells was found in both the nucleus and cytoplasm. These data are in accordance with previous studies conducted on fish (33-34) and *Xenopus laevis* (35, 44-46) which showed that the glucocorticoid receptor (GR) is widely distributed in these regions of the brain. Neurons in these regions contain and secrete a number of neuropeptides including CRF and vasotocin (46-48). The high expression of GR in preoptic neurons suggests a direct effect of circulating glucocorticoids on these neurons. This is further supported by the finding that CRF and GR are colocalized in the Poa of amphibian *Xenopus laevis* (48). Thus, glucocorticoids induce negative feedback control on neurosecretory CRF neurons in the frog similar to that in mammals and fish (for a review see 49).

A significant difference between the distributions of GR in the brain of the frog *Rana ridibunda* and that of birds and mammals is that we found a high density of GR-immunoreactivity in the dorsal part of magnocellular preoptic nucleus (Mgd) and less, but albeit identifiable GR-expressing cells in the ventral part of the magnocellular preoptic nucleus (Mgv), whereas no GR-immunoreactivity was detected in the magnocellular division of the PVN in intact rat or Japanease quail (50). Also, high GR-immunoreacitivity was detected in rainbow trout and kokanee salmon (33,51). These findings of high GR in the magnocellular of Poa in the frog and in fish and their absence from the homologous regions of the bird or mammals could be related to the aquatic vs. terrestrial life histories of these species. In addition, we observed a strong immunoreactivity in the organium vasculousum (supraoptic crest) which is known as one of the circumventricular organs of the brain and is strongly interconnected with the median preoptic nucleus of the hypothalamus (the nucleus medianus). This structure form together with the subfornical organ and area postrema the anterior and ventral region of the third ventricle known as AV3V. The AV3V region is very important in the regulation of fluid and electrolyte balance, by controlling thirst, sodium excretion, blood volume regulation, and vasopressin secretion. Thus, glucocorticoid receptors of frog brain, as in mammals, regulate a number of behavioral, neuroendocrine and metabolic processes. Furthermore, we have observed a positive immunoreactivity in many regions of the limbic system of frog brain, notably in the medial pallium (mp), which

is the amphibian and fish homologous of the mammalian hippocampus. Also, we found GR-immunoreactivity in the medial and lateral amygdala (MA, LA), and in the Bed nucleus of the pallial commissure (BN). In support of these data, the presence of GR-immunoreactivity in the limbic structure of mammals, such as hippocampus, dentate gyrus, and amygdala, is well documented (31,32). In addition, similar distribution of GR-Immunoreactivity detected in frog medial pallium was observed in fish brain (33-34, 51).

Moreover, many studies conducted in mammals have demonstrated that the amygdala is involved in stress-related reactions and in the regulation of the HPA axis (52-54). The amygdala contains high levels of CRF, and CRF-containing fibers have been traced from the amygdala to the lateral hypothalamus and may directly innervate CRF-containing neurons within the PVN (53-56). Thus, glucocorticoids can negatively regulate the activity of PVN neuron secreting CRF in mammals via a descending inhibitory pathway originating from hippocampus and amygdala (55-56). Thus, our present findings showing that glucocorticoid receptors (GR) are distributed in the homologous regions of the brain of the frog *Rana ridibunda* , medial pallium, and amygdala, and the study demonstrated that CRF and GR are colocalized in the Poa of amphibian (48) suggest that these structures could play a similar function in frog as they do in mammals.

In fact, autoregulation of glucocorticoid receptor in the central nervous system by circulating glucocorticoids is considered as the more important mechanism to regulate the hypothalamic-pituitary-adrenal (HPA) axis during stress in man and all mammals studied until now.

In conclusion, our results support the idea that the general patterns of glucocorticoid receptor distribution in the neuroendocrine nuclei and limbic system of the central nervous system are highly conserved among vertebrates. Thus glucocorticoid receptor is likely to play roles in mediating the effects of corticosteroids on frog brain similar to those in mammals, suggesting that the basic regulatory pathways for modulating the responsiveness of the stress axis may be an evolutionary conserved mechanism in vertebrates.

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