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EphB2 Receptor Expression in Histological Layers of Rat Hippocampus

Sıçan Hipokampüsünün Histolojik Katmanlarında Ephb2 Reseptör Ekspresyonu

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Abstract: The hippocampus is one of the main brain regions affected by major diseases such as depression, Alzheimer's disease, Parkinson's disease, and epilepsy. Different histological sub-areas of the hippocampus are reported to be affected by various pathological processes. Eph receptors are members of a family of transmembrane receptors involved in processes necessary for neuronal plasticity. Among these receptors, EphB2 plays a role in both morphological and synaptic plasticity in regulating LTP in the hippocampus. In this study, we aimed to investigate the expression intensities of the EphB2 receptor, which is essential in hippocampal processes in histological substrates in the hippocampus. In brain sections from rats, EphB2 was immunofluorescently stained, and fluorescence intensities in hippocampal areas were evaluated. According to our results, the expression levels of EphB2 receptors vary in the lower layers of the hippocampus. The dentate gyrus was the anatomical layer with the highest expression of EphB2. In all areas, higher EphB2 clustering was detected in cellular layers compared to other layers. Differences in the expression of EphB2 in different layers may be related to behavioural disorders that develop in various nervous system pathologies. Our results may be useful for future studies that will provide a better understanding of molecular pathways in morphological and synaptic plasticity.
Keywords: EphB2 Receptor, Hippocampus, Immunofluorescence, Neuroplasticity, Receptor Protein-Tyrosine Kinases

Özet: Hipokampus, depresyon, Alzheimer hastalığı, Parkinson hastalığı ve epilepsi gibi önemli hastalıklardan etkilenen ana beyin bölgelerinden biridir. Hipokampüsün farklı histolojik alt alanlarının çeşitli patolojik süreçlerden etkilendiği bildirilmektedir. Eph reseptörleri, nöronal plastisite için gerekli süreçlerde yer alan bir transmembran reseptör ailesinin üyeleridir. Bu reseptörler arasında EphB2, hipokampüste LTP'nin düzenlenmesinde hem morfolojik hem de sinaptik plastisitede rol oynar. Bu çalışmada, hipokampal süreçlerde önemli olan EphB2 reseptörünün hipokampüsteki histolojik substratlarda ekspresyon yoğunluklarını araştırmayı amaçladık. Sıçanlardan alınan beyin kesitlerinde EphB2 immünofloresan olarak boyanmış ve hipokampal alanlardaki floresan yoğunlukları değerlendirilmiştir. Sonuçlarımıza göre, EphB2 reseptörlerinin ifade düzeyleri hipokampüsün alt katmanlarında değişiklik göstermektedir. Dentat girus, EphB2 ekspresyonunun en yüksek olduğu anatomik katmandı. Tüm bölgelerde, hücresel katmanlarda diğer katmanlara kıyasla daha yüksek EphB2 kümelenmesi tespit edilmiştir. Farklı katmanlarda EphB2 ekspresyonundaki farklılıklar, çeşitli sinir sistemi patolojilerinde gelişen davranış bozukluklarıyla ilişkili olabilir. Sonuçlarımız, morfolojik ve sinaptik plastisitedeki moleküler yolakların daha iyi anlaşılmasını sağlayacak gelecekteki çalışmalar için faydalı olabilir.
Anahtar Kelimeler: EphB2 Reseptörü, Hipokampüs, İmmünofloresan, Nöroplastisite, Reseptör Protein-Tirozin Kinazlar

Ethics Committee Approval: Ethical approval was obtained from Bursa Uludağ University Animal Experiments Local Ethics Committee dated 07.11.2023, decision number 2023-12/09.

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1. Introduction

The hippocampal formation consists of four distinct regions: the dentate gyrus (DG), the hippocampus divided into three subdivisions (CA1, CA2, and CA3), the subicular complex (subiculum, presubiculum, and parasubiculum), and the entorhinal cortex. These regions are connected by unique and largely unidirectional projections organized in well-defined laminae [1]. The hippocampus is the subject of intense study in neuroscience because of its critical role in memory. Numerous studies showing that the hippocampus is affected in various diseases such as depression, Alzheimer's disease and Parkinson's disease, and epilepsy [2-4] have resulted in a growing interest in subfields of the hippocampus [5].

Erythropoietin-producing human hepatocellular (Eph) receptors were discovered in 1987 and are the largest known family of receptor tyrosine kinases [6]. Their ligands are membrane-bound ephrins. There are Eph receptors (nine Eph A receptors [Eph A1 - A8 and Eph A10], five Eph B receptors [Eph B1 - B4 and Eph B6]) and eight ephrin ligands (five ephrin-A ligands [ephrin-A1 - A5] and three ephrin B ligands [ephrin B1 - B3]) [7,8]. Eph-ephrin signaling is essential in the development and homeostasis of the nervous system. In the developing nervous system, Eph-ephrin signaling generally leads to changes in the cytoskeleton to regulate neuronal progenitor proliferation, cell migration, and guidance of axonal growth cones towards their synaptic targets and synapse formation. Many Eph-ephrin-mediated effects on axon guidance and synaptic plasticity occur through forward signaling through the Rho family of GTPases, resulting in cytoskeleton modification [9].

Eph receptors are closely associated with signaling pathways in the postsynaptic membrane that affect the amount of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and regulate LTP [16], NMDAR localization and function [10,11]. The formation and morphology of dendritic spines depend on EphB proteins; EphB1, EphB2, and EphB3 work together to form mature spines. The failure to form dendritic protrusions is due to the inability of actin to accumulate in the spinae [12].

Studies have shown that abnormal expression of EphB2 contributes to central nervous system diseases. Reduction of hippocampal EphB2 has been reported to be associated with impaired cognitive function in a mouse model of Alzheimer's disease [13, 14]. EphB2 also affects emotional learning and memory [15-17]. EphB2 directly interacts with NMDAR and mediates tyrosine phosphorylation of NMDAR receptor subunits. Loss of EphB2 results in a reduction in long-term potentiation and synaptically localized NMDAR function, which in turn causes neuronal dysfunction [18].

Histological studies have shown that hippocampal subregions can be particularly affected by different conditions, leading to a growing interest in subregional assessment [19]. Because of the importance of EphB2 receptors in LTP and synaptogenesis, it is obvious that the expression and localisation of these receptors in the hippocampus would provide a better understanding of the subject. Therefore, we aimed to investigate the expression intensity of EphB2 receptors on histological substrates in the hippocampus.

2. Materials and Methods

a. Animals

The Wistar albino experimental animals used in the study were obtained from Bursa Uludağ University DEHYUAM. 4-month-old rats weighing 200-225 g were used. Prior to the experiments, the estrous cycle was determined from vaginal swabs and female rats in diestrus were used. Rats were housed on a 12 h dark/12 h light cycle with 2/3 rats per cage and had unlimited access to water and food.

b. Tissue Processing

The rats were anaesthetised by inhalation of a high dose of ether. An abdominal incision was enlarged and the chest opened. A cannula connected to a peristaltic pump was inserted through the left ventricle into the aorta, and an incision was made at the right auricle. The rats' circulatory systems were accessed transcardially and perfusion fixation was carried out with cold phosphate-buffered 4% paraformaldehyde (PFA) after the blood had been washed with saline (0.9% NaCl).

After fixation, the skulls of the rats were carefully opened, and the removed brains were postfixed in 4% paraformaldehyde for 24 hours at +4^{o}C. The brain tissue was then washed in tap water. Brain regions were trimmed coronally. Tissues were placed in cassettes, and routine alcohol-xylol solutions were used. Paraffin blocks were then prepared by embedding the tissues in paraffin in the rostrocaudal direction with the help of a paraffin casting device (Diapath). According to the coordinates on the Paxinos rat brain atlas (Paxinos,

and Watson 2009), 5 µm paraffin coronal brain sections were taken from approximately Bregma -2.80 mm and Bregma -4.30 mm areas where the hippocampus was located (Figure 1).

c. Immunofluorescence

Immunofluorescence labelling method was used to determine the expression of EphB2 receptor proteins in the hippocampal areas of coronal serial brain sections taken on poly-L-lysine slides. Firstly, antigen retrieval pretreatment was performed in 98°C oven in pH 6.0 sodium citrate buffer. To prevent non-specific binding, incubation with 10% horse "blocking" serum was performed for 2 hours at room temperature. To evaluate EphB2 expression in hippocampal tissue, mouse-anti-EphB2 antibody (sc-130752, Santa Cruz Biotechnology, USA) was prepared in PBS buffer at 1/200 dilution and used as primary antibody. Sections were incubated with primary antibody at +4°C for 2 nights. As secondary antibody: FITC-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, UK) was prepared at 1/800 dilution in PBS and incubated for 2 hours at room temperature. Washes were performed and the dried sections were covered with coverslips by applying DAPI-containing covering material.

d. Analysis of Immunofluorescence Images

Sections were photographed using a fluorescence attachment photomicroscope (Olympus BX-50, microscope and Olympus DP71 CCD colour camera) for semi-quantitative assessment of receptor expression in the histological layers of CA1, CA3 and DG of the labelled hippocampus. All images were photographed uniformly for each magnification, with standardised exposure time and ISO setting to minimise data variation. Photographs were transferred to Image-J software and converted to 8-bit grayscale images. The sections obtained from each subject were analysed, and the fluorescence radiation intensity was measured and recorded by randomly selecting equivalent areas for each histologic layer of the hippocampus using Image-J software. The arithmetic mean of the measurements was calculated by Graphpad (Version 9.5.0) software.

3. Results

According to immunofluorescence findings, the expression distribution of EphB2 followed the laminar organization pattern of the hippocampus and expression levels differed between anatomical areas and histological layers.

In CA1, EphB2 expression was detected in the soma and appendages (Figure 2A). In the CA1 area of the hippocampus, the stratum oriens (Figure 2B), the cellular layer stratum pyramidale (Figure 2C) and the stratum radiatum (Figure 3D) were evaluated. Overall, the highest fluorescence intensity of EphB2 expression was observed in the stratum pyramidale (together with the whole-area averages of receptor clusters in neuron somas). It was lower in the oriens and radiatum layers (other fibre subfields of CA1 pyramidal cells) compared to this layer (Figure 2E).

In the CA3 area, EphB2 expression was detected in the soma and extensions (Figure 3A). In the CA3 area of the hippocampus, the stratum pyramidale (Figure 3B), stratum lucidum (Figure 3C) and stratum radiatum (Figure 3D) were evaluated. In the CA3 area of the EphB2 receptor, the histologic layer with the highest irradiance was the stratum pyramidale containing pyramidal cell somata, followed by the other layers, stratum lucidum and stratum radiatum (Figure 3E).

In the dentate gyrus (Figure 4A), the molecular layer (Figure 4B), the granular layer containing cell somata (Figure 4C), and the polymorphic layer known as the polymorphous cell layer (Figure 4D) were evaluated. EphB2 receptor protein expression was highest in the granular layer containing cell somas, followed by the molecular layer and polymorphic layer (Figure 4E).

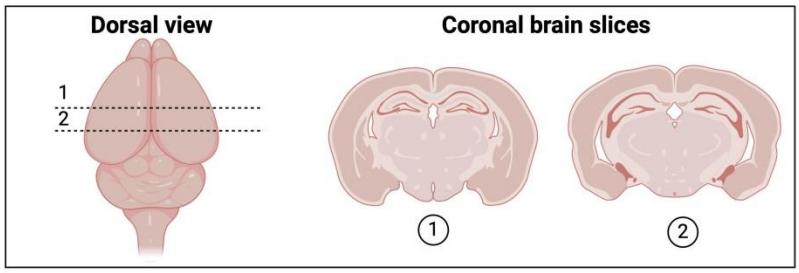

Figure 1. Schematization of hippocampus evaluations in the rat brain. Preparation of hippocampus sections. 1 and 2 approximately represent the Bregma -2.80 mm and -4.30 mm levels. Illustration created by the author on www.biorender.com.

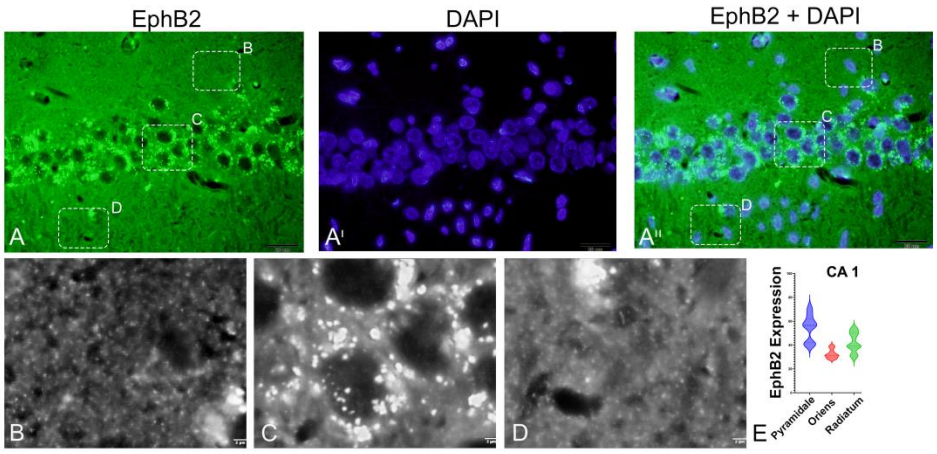

Figure 2. Representative photographs of immunofluorescence intensity of CA1 according to the histological regions evaluated (A-Aıı). Receptor expression at high magnifications, stratum oriens (B), stratum pyramidale (C), stratum radiatum (D). Average EphB2 immunofluorescence intensities of CA1 histological layers (E). The high magnification images show representative images of EphB2 in CA1 in 8-bit greyscale format of pixel intensities at fixed brightness and threshold.

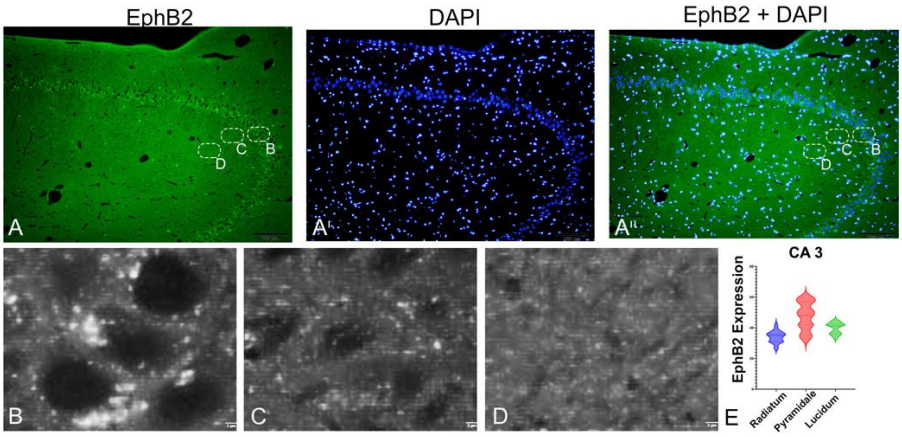

Figure 3. Representative photographs of immunofluorescence intensity of CA3 according to the histological regions evaluated (A-Aıı). Receptor expression at high magnifications, stratum pyramidale (B), stratum lucidum (C), stratum radiatum (D). Average EphB2 immunofluorescence intensities of CA3 histological layers (E). The high magnification images show representative images of EphB2 in CA3 in 8-bit greyscale format of pixel intensities at fixed brightness and threshold.

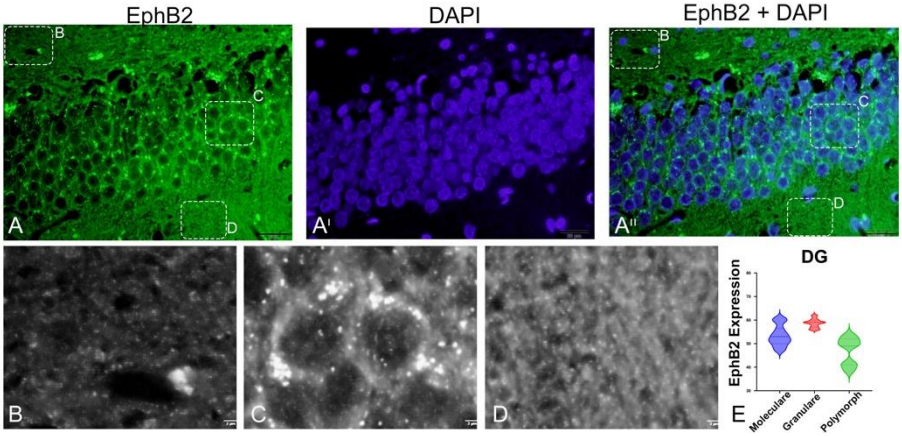

Figure 4. Representative photographs of immunofluorescence intensity of DG according to the histological regions evaluated (A-A^{II}). Receptor expression at high magnifications, stratum moleculare (B), stratum granulare (C), plymorph (D). Average EphB2 immunofluorescence intensities of DG histological layers (E). The high magnification images show representative images of EphB2 in DG in 8-bit greyscale format of pixel intensities at fixed brightness and threshold.

4. Discussion

At the hippocampus first information from the entorhinal cortex via the perforant pathway enters the granular cells of the dentate gyrus (Stratum moleculare). It is then transmitted via the temporoammonic pathway (Stratum radiatum) to the distal apical dendrites of CA1. The second consists of connections to the pyramidal cells of CA3 via mossy fibrils to the dentate gyrus (Stratum lucidum). Finally, CA3 is connected to CA1 via the Schaffer collateral pathway (Stratum oriens and stratum radiatum). This loop can be summarised as a simplified view of hippocampal circuits, given the multiple interconnected pathways of the subfields [20, 21]. Due to the different neuronal regions that hippocampal layers contain and the various physiological events in which they participate, the amount of EphB2 receptors in these regions at the histological level may be valuable for understanding different physiological outcomes.

There are a few studies that have examined hippocampal expression of the EphB2 receptor immunohistochemically [22,23] however, these have not investigated the localisation of these proteins in histological subfields in the hippocampus. EphB2 is highly abundant at excitatory synapses in the adult brain. It involves dendritic branching, synapse development, spine development, and maintenance [24]. EphB/Ephrin-Bs are of great interest for their role in regulating neuronal maturation and synaptic plasticity. EphB2 has an essential role in dendritic spine morphogenesis in hippocampal neurons, which can mediate the clustering of endogenous syndecan-2 or actin polymerization via Rho-family guanosine triphosphatases [25-27]. EphB receptors play an essential role in maintaining dendritic spine morphology, so a loss or reduction in EphB2 results in the formation of immature spinae that lack synaptic activity [28,29]. EphB2-deficient mice have been reported to develop impaired synaptic transmission and plasticity [14] and reduced motility of postsynaptic dendritic filopodia during synapse formation in vitro [30]. It has been reported that EphB2 is densely localized in the dendritic regions of granule neurons of the CA1 and dentate gyrus of the hippocampus, especially in sub-compartments with high NMDA receptor expression [31]. With the findings of our study, we have once again supported and updated the known data on the localization and expression levels of EphB2 receptors in both cellular and hippocampal tissue in the female rat hippocampus We detected EphB2 expression in various layers of CA1, CA3 and DG regions and fluorescence in cell bodies representing receptor clusters in a punctate fashion in neuronal extensions. Our results showed that the DG is the anatomical layer with the highest expression of EphB2. We also found the highest expression rate in the lower layers of the cornu ammonia. However, using dual staining, including neuronal and glial markers, in the cellular evaluation of EphB2 will further our results.

In conclusion, the widespread expression patterns of Eph receptors, their involvement in various critical cellular events and their unique mode of action are of interest in biological and medical fields. According to our results, the expression levels of EphB2 receptors differ across anatomical regions of the hippocampus and their substrates (cellular and molecular layers). Synaptic relationships in related layers and molecular pathways implicated in EphB2 may be important for understanding the diverse behavioral and pathological consequences of receptor sensitization. A better understanding of molecular pathways in morphological and synaptic plasticity will contribute to the experience of neuropsychiatric and neurodegenerative diseases and the discovery of novel therapeutics.

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