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The Effect of Rolipram on Visual Cortical Plasticity in Amblyopia Model: Experimental Animal Study

Ambliyopi Modelinde, Rolipramin Vizüel Kortikal Plastisite Üzerine Etkisi: Deneysel Hayvan Çalışması

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ABSTRACT Objective: Electrophysiological and electron microscopic investigation of the effect of rolipram on visual cortical plasticity in a monocular deprived rat model. **Material and Methods:** Twenty-four female Wistar albino rats were randomly assigned into 6 groups, 4 rats in each. The right eyelids of all rats except the control group were sutured to induce amblyopia. We subjected rats at postnatal day 21 to a monocular deprivation (MD) model until postnatal day 45. Then, the deprived eyes of the MD model rats were reopened' cümlesi çıkarılarak 'On day 46, the deprived eyes of the rats were opened.On day 46, the deprived eyes of the rats were opened. Rats were treated with rolipram or vehicle for 10 days. The ultrastructural modifications in the synaptic junction and the changes to the visual evoked potentials (VEP) were analyzed. **Results:** In flash VEP, the P2 latency was prolonged and the P2 amplitude was reduced in monocularly deprived rats. With rolipram treatment, the P2 latency became shorter and the amplitude increased. In the monocularly deprived rats, the ultrastructural analysis showed that the synapses in the pyramidal neurons were impaired, the synaptic clefts were expanded, the synaptic active zones were shorter, and the thicknesses of the postsynaptic density were decreased. However, rolipram treatment improved all of the structural indices in the MD rats. **Conclusion:** This study shows that rolipram provides structural and functional improvement in visual cortex in amblyopia model in rats. Rolipram shows promise in the treatment of amblyopia and more research is needed.

Keywords: Amblyopia; phosphodiesterase inhibitor; neuronal plasticity; evoked potentials, visual

ÖZET Amaç: Ratlarda, deneysel monooküler deprivasyon ambliyopisi modelinde rolipramın, vizüel kortikal plastisite üzerindeki etkisinin elektrofizyolojik ve elektron mikroskobik olarak incelenmesidir. **Gereç ve Yöntemler:** Yirmi dört Wistar albino cinsi rat, her grupta 4 rat olacak şekilde rastgele 6 gruba ayrıldı. Kontrol grubu dışındaki ratlar dışında tüm ratların sağ göz kapakları 21-45. günleri arası sütüre edilerek monooküler deprivasyon modeli oluşturuldu. Kırk altıncı günde tüm ratlara 10 gün boyunca rolipram veya rolipramı çözmek için kullandığımız sıvı uygulandı. Sinaptik kavşaktaki ultrastrüktürel modifikasyonlar ve görsel uyarılmış potansiyellerdeki (VEP) değişiklikler analiz edildi. **Bulgular:** Deprivasyon ambliyopisi modelinde VEP'te P2 latansının uzadığı, amplütüdün azaldığı ve rolipram ile tedavi sonrası ise latansın kısaldığı ve amplitüdün arttığı ve kontrol grubuna yaklaştığı görüldü. Deprivasyon ambliyopisi modelinde piramidal nöronlardaki sinapsların ultrastrüktüral yapısının bozulduğu, sinaptik yarığın genişlediği, sinaptik aktif zonun uzunluğunun kısaldığı, postsinaptik dansite kalınlığının azaldığı ve rolipram ile tedavi sonrası ise bu değerlerin kontrol grubuna yaklaştığı görüldü. **Sonuç:** Bu çalışma, rolipramın ratlardaki ambliyopi modelinde, vizüel kortekste yapısal ve fonksiyonel iyileşmeyi sağladığını göstermektedir. Rolipram, ambliyopi tedavisinde umut vadetmektedir ve ileri araştırmalara gerek vardır.

Anahtar Kelimeler: Ambliyopi; fosfodiesteraz inhibitör; nöral plastisite; uyandırılmış potansiyeller, görsel

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The visual experience in the critical period dramatically affects the neuronal connections in the primary visual cortex. Monocular deprivation (MD) during the crucial period in rats has been shown to have an impact on the morphology, physiology, biochemistry, and function of the visual system. MD during the crucial time causes the deprived eye to become amblyopic and reduces visual acuity and contrast sensitivity. Visual cortical plasticity becomes limited with age; although the underlying mechanisms are not well understood. Currently, there is no certain treatment for adult amblyopia. However, some experimental studies have demonstrated synaptic plasticity reactivation in the adult visual cortex.[1,2]

Myelin maturation, maturation of intracortical inhibition, and condensation of the extracellular matrix molecules to the perineuronal nets are of critical importance in the developmental plasticity of the visual cortex.[3-5]

Synaptic plasticity in the visual cortex is associated with the mitogen-activated protein kinase cascade. This cascade is regulated by the ERK/MSK/CREB signaling pathway (ERK: Extracellular signal-regulated kinase, MSK: mitogen-and-stress-activated kinase).[6] The cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is phosphorylated and activated by the cAMP-dependent protein kinase (PKA).[7,8] CREB is the primary mediator of cAMP-induced transcription. CREB formation results in histone acetylation and phosphorylation, activating the genes that provide plasticity.[6] CREB is known to increase gene expression and play a role in ocular plasticity.[6]

Myelin maturation is also important in the visual cortex's developmental plasticity. Myelin inhibitors [myelin-Nogo (Nogo-A), myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein (OMgp)] have been blamed for the inhibition of axonal regeneration after nervous system damage.[9] Some studies have aimed at inhibiting the NgR complex to which the myelin inhibitors bind, whereas other studies have aimed at increasing cAMPs and CREBs through phosphodiesterase (PDE) inhibition, thereby successfully reversing the adverse effects of myelin inhibitors on axonal plasticity.[8-11] Neurotrophins such as nerve growth factor and brain-derived neurotrophic factor bind to the Trk tyrosine kinase receptors, leading to the autophosphorylation of the receptor and thus activating ERK.[12] ERK activation results in the inhibition of the phosphodiesterase 4 (PDE4) enzyme, preventing cAMP degradation and leading to increased cAMP levels.[12] The increased amounts of cAMP are able to cope with the myelin inhibition, while CREB activity helps cope with the myelin inhibition.[12]

Rolipram is a first-generation, cAMP-specific PDE4 inhibitor. Rolipram leads to increased levels of intracellular cAMP. Rolipram passes through the blood-brain barrier in insubstantial amounts when administered orally or subcutaneously. Rolipram is proven to improve learning and memory functions through cAMP/PKA/CREB modification.[8,13-15] Rolipram is further known to reinforce hippocampus dependent memory and to reduce age-related memory loss.[7,16,17] In addition, it has been shown to improve neuronal development and axonal regeneration in the presence of myelin inhibitors.[9,12] Finally, rolipram has been shown to interact with processes of synaptic tagging.[18]

Because of these effects, rolipram may be effective in treating amblyopia via cortical plasticity through cAMP/PKA/CREB activation and myelin inhibition in a MD amblyopia model. In the current investigation, electrophysiology and electron microscopy were used to examine the structural and functional impacts of rolipram on the reactivation of the visual cortex.

MATERIAL AND METHODS

A total of 24 female albino Wistar rats ranging in age from postnatal day (P) 21to P45 were used in this study. The 4-rat groups were housed in typical cages and given unlimited food. The humidity was kept under control, while the temperature was maintained at 21±2°C. Fluorescent lighting with a 12-hour light/dark cycle illuminated the rooms (06:00 am-06:00 pm). The study was authorized by the Kocaeli University Medical Faculty's Ethics Committee (date: December 27, 2011, no: HADYEK 13/7-2011). The Helsinki Declaration's principles were followed during the study's execution.

Rats were randomly divided into 6 groups, 4 rats in each: normal animals (Nor), monocularly deprived animals without any treatment (MD), normal animals treated with vehicle [Nor+dimethylsulphoxide (DMSO)] or rolipram (Nor+rolipram), and MD animals treated with vehicle (MD+DMSO) or rolipram (MD+rolipram).

General anesthesia was administered to the rats with intraperitoneal ketamine hydrochloride (70 mg/kg), xylazine hydrochloride (3.5 mg/kg), and acepromazine maleate (0.7 mg/kg).[19] The right eyelids of the 21-day-old rats were sutured and monocularly deprived.[20] The sutures were not sewn tight so as not to disturb eyelid development.[20] The deprivation was planned between P21 and P45.[10,21] During the deprivation, the sutures were checked on a daily basis. The eyelids of the 4 subjects whose sutures were opened during the dark cycle were re-sutured under anesthesia. The subjects whose eyelids were opened during the light cycle were planned excluded from the study and no animal have been excluded.[20] The eyelid sutures of postnatal 46 days-old subjects were opened, and treatment with intraperitoneal rolipram or vehicle was initiated. Rolipram (Sigma-Aldrich®, R6520, 25 mg) was prepared in distilled water including 2% DMSO at a dose of 3 mg kg^{-1}.[8]

The rats were administered intraperitoneal rolipram everyday at the same time (10:00 am-12:00 am) for ten days. The control groups were administered vehicle (distilled water containing 2% DMSO) everyday at the same time at the same volume.

When the treatment was complete, flash visual evoked potentials (VEP) were taken via Sharon's method for all of the rats and the results were evaluated (n=4 rats in each group).[22] For VEP recording, Ag-AgCl recording electrodes were placed in the occipital region using conductive gel. The reference electrode was placed in the midline of the Fpz region. For stimulation, a flash stimulation with a frequency of 2 Hz was used by closing the left eye. The distance was adjusted to create 30 cm between the flash stimulation and the subjects. For each experiment, analyses were conducted with Neuropack Nihon Kohden, MEB-5504 K, Tokyo, Japan, averaging 100 stimuli, 250 milliseconds of analysis time, 1-100 Hz amplification bandwidth, and 0.5-200 Hz signal filter. To measure the flash VEP wave amplitudes and latencies, artifact elimination was first performed in the analysis of the recordings. In all groups, high-quality and reliable results were obtained. Data analysis identified one peak of visual evoked potentials, wheras second positive peaks were noted with P2. The amplitudes and latencies for P2 were measured. The latency of P2 waves was recorded in milliseconds (ms), and the amplitude of P2 waves was recorded in micro-volts (μV). The values of the potential waves in the VEP responses were expressed as "mean±standard deviation." Only positive response was considered, and the VEP responses for the subject P2 peaks were taken under evaluation (n=4).

After obtaining flash VEP, deep anesthesia was applied to the rats through ketamine (100 mg/kg) and xylazine hydrochloride (20 mg/kg), and transaortic perfusion was performed (perfusion with fixative containing 2.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.1% picric acid in pH 7.3, with 0.1 M HEPES buffer). Following the fixation, decapitation and removal of the brains were performed. The brains were kept within the fixative all night at 4°C to conduct the post-fixation process. After flushing in 0.1 M HEPES buffer (pH 7.3), coronal slices were cut at a thickness of 300 μm via Leica VT 1000 S vibratome (Wetzlar, Hesse, Germany). To look at the connections in the pyramidal neurons in layers II-III of the binocular zone of the primary visual cortex opposite the eye that is being deprived of vision, the respective area of the occipital lobe was removed under stereomicroscope and post-fixation was applied in 1% osmium tetroxide/1.5% potassium ferricyanide (1:1 in distilled water). The slices were flushed in the distilled water and then stained in the darkness at room temperature for 30 minutes with 0.5% uranyl acetate. The tissue was then dehydrated in graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812 for 24 h at 60°C. Semi-thin sections were obtained at a thickness of 1 μm from the tissue blocks via Leica Ultracut S ultramicrotome. The sections were colored with toluidine blue, and suitable areas for the electron microscopic examinations were established by analyzing via light microscope. Thin sections (60-100 nm in thickness) from

these areas were left to dry over 200-mesh nickel grids coated with grid coating pen (Coat-Quick 'G' pen, Kiyota International; Elk Grove, IL, USA). After the contrast technique with uranyl acetate and lead citrate was applied to the grids containing the thin sections, analysis was performed via microscope (JEOL 1200 EXII).

Pyramidal neurons in layers II-III of the binocular area of the primary visual cortex, which is contralateral to the deprived eyes, recorded synapse forms. Jones' and Güldner's methods were used to calculate and statistically analyze the width of the synaptic cleft, the length of the synaptic active zone, and the thickness of the postsynaptic density (PSD).[23,24] Synaptic parameters were measured and calculated using the Image J 1.47 program. Each rat (n=4) had 50 synapses chosen for them. The curvatures of the synaptic faces were also evaluated qualitatively.

SPSS for Windows version 16 (SPSS Inc., Chicago, IL, USA) was used to do the statistical analysis of the data. Data were expressed as mean±standard error of mean (SEM). The one-way analysis of variance (ANOVA) test was used to evaluate the differences between the 3 amblyopic groups and the 3 control groups. The differences between groups were assessed pairwise using the least significant difference t-test test. Using the unpaired student's t-test, the paired comparisons between the control and amblyopic groups were evaluated. Statistics were deemed significant if the p value was less than 0.05.

RESULTS

Latencies and amplitudes of the deprived eyes as measured by flash VEP (Figure 1). When the P2 latency and amplitude means were compared, latency was found to be prolonged and amplitude reduced

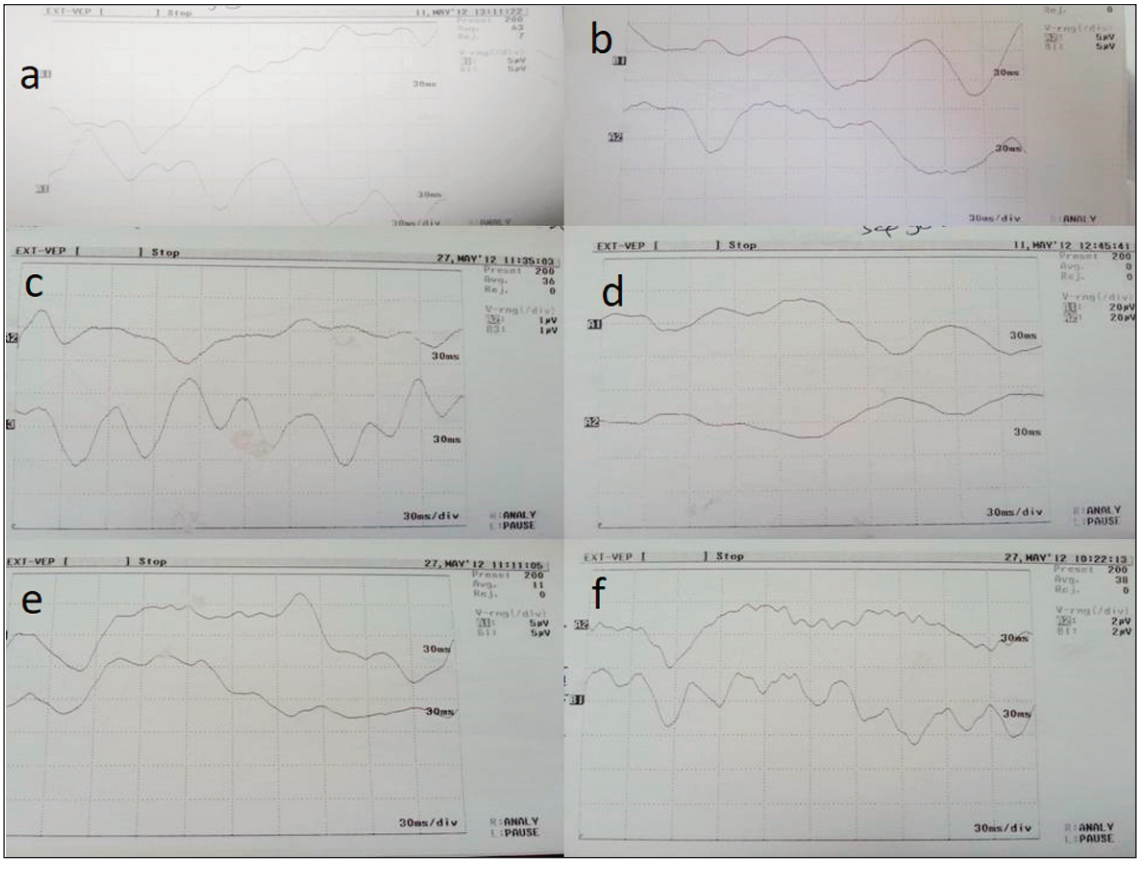

FIGURE 1: Representative waveforms of flash visual evoked potential (F-VEP) in rats. The images: **a)** Nor; **b)** Nor+DMSO; **c)** Nor+rolipram; **d)** MD; **e)** MD+DMSO; **f)** MD+rolipram.
VEP: Visual evoked potentials; MD: Monocular deprivation; DMSO: Dimethylsulphoxide.

TABLE 1: In the MD group rats, prolonged P2 latency and reduced P2 amplitude were observed in VEP, suggesting impaired visual function. Following treatment with rolipram, the MD rats showed decreased F-VEP latency and significantly increased amplitude [data Are mean±SEM (n=4 eyes per group)].

	Nor	Nor+DMSO	Nor+rolipram	MD	MD+DMSO	MD+rolipram
P2 latency (±SD) (ms)	60.45±10.95	65.22±13.7	65.03±3.8	73.17±8.54	75.82±8.76	60.18±7.65
P2 amplitude (±SD) (μV)	3.78±1.66	3.38±1.95	3.38±1.78	2.28±1.31	2.34±1.25	4.71±1.84

DMSO: Dimethylsulphoxide; MD: Monocular deprivation; VEP: Visual evoked potentials; SEM: Standard error of mean.

in the monocularly deprived group (Figure 2, Table 1) (When both P2 latency and P2 amplitude were compared in Groups Nor and MD, the unpaired student's t-test was p=0.026, p=0.028, respectively). No difference was observed between the groups for the non-monocularly deprived groups (Nor, Nor+DMSO, Nor+rolipram) (one-way ANOVA, p>0.05) P2 latency became shorter and the amplitude increased in group MD+rolipram when compared to the groups MD and MD+DMSO (one-way ANOVA, p<0.05). Additionally, there was no statistically significant difference between P2 latency and amplitude between the Nor and MD+rolipram groups (unpaired student's t-test, p=0.95 and p=0.72 respectively) (Figure 2).

We examined the structural characteristics of synaptic connections using electron micrographs in order to examine the impact of rolipram on the synaptic plasticity of the primary visual cortex in rats after monocular deprivation (Figure 3). The width of the synaptic cleft, the length of the synaptic active zone, and the thickness of the PSD were calculated.

Ultrastructural analysis showed that the synapses in the pyramidal neurons were impaired, the synaptic clefts expanded, the synaptic active zones shorter, and the thicknesses of the PSD decreased in MD group when compared with the normal group (Nor vs. MD, unpaired student's t-test, p<0.05) (Figure 4, Table 2).

However, rolipram treatment improved every structural index in the monocularly deprived rats (MD vs. MD+DMSO vs. MD+rolipram, one-way ANOVA, p<0.05).

The width of the synaptic cleft, the length of the synaptic active zone, and the thickness of the PSD were similar in the 3 normal groups (one-way ANOVA, p>0.05).

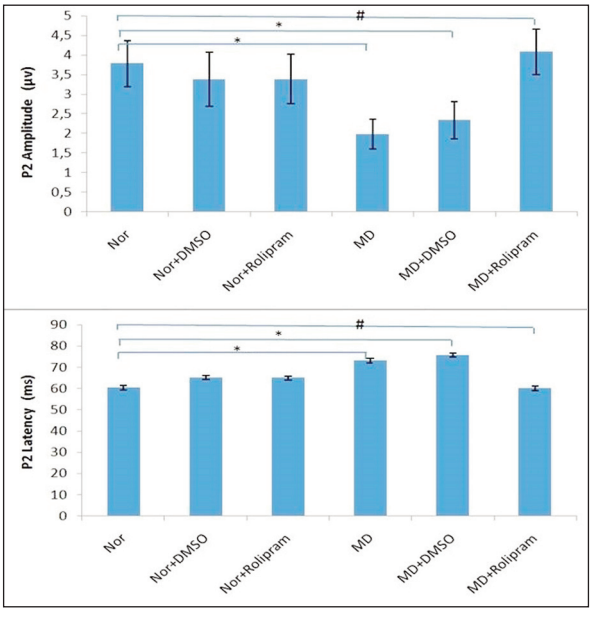

FIGURE 2: In the MD group rats, prolonged P2 latency and reduced P2 amplitude were observed in VEP, suggesting impaired visual function. Following treatment with rolipram, the MD rats showed decreased F-VEP latency and significantly increased amplitude [data are mean±SEM (n=4 eyes per group)] (*p<0.05, **p<0.01, #p>0.05 by student's t-test).
MD: Monocular deprivation; VEP: Visual evoked potentials; SEM: Standard error of mean.

The curvature of the synaptic faces of the Nor and MD groups were also evaluated qualitatively. It was observed that the synaptic interface curvatures of the synapses in the pyramidal neurons were reduced in cases of amblyopia, while the curvature recovered with the rolipram treatment.

DISCUSSION

Essential components of synaptic plasticity are the structural changes at the synaptic junction.[25] Mor-

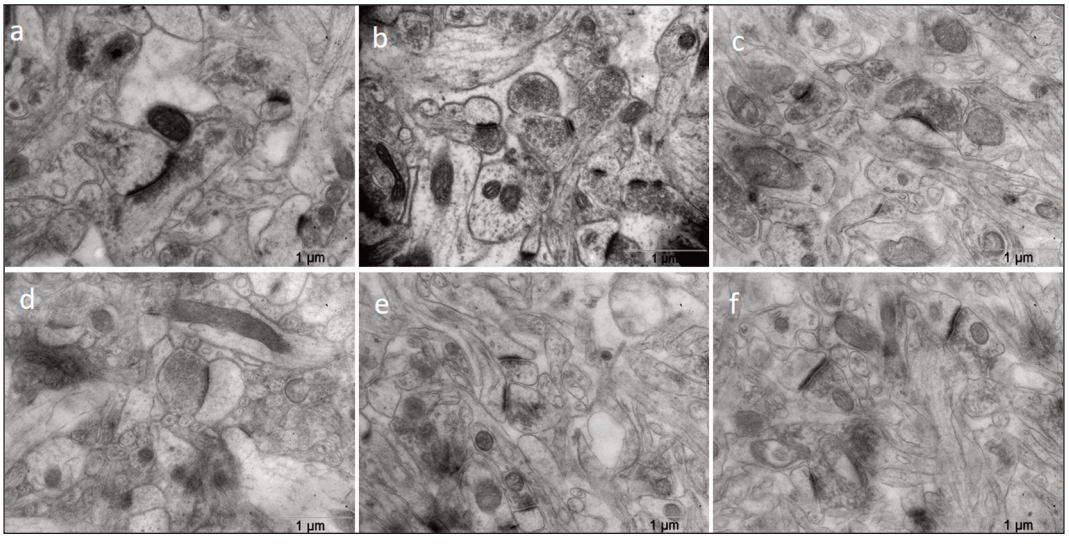

FIGURE 3: The electron microscopic appearance of the ultrastructure of the synapses in the pyramidal neurons in layers II-III of the binocular zone of the primary visual cortex contralateral to the deprived eye in different groups. The images: **a)** Nor; **b)** Nor+DMSO; **c)** Nor+rolipram; **d)** MD; **e)** MD+DMSO; **f)** MD+rolipram. Scale bar = 1 µm.
MD: Monocular deprivation; DMSO: Dimethylsulphoxide.

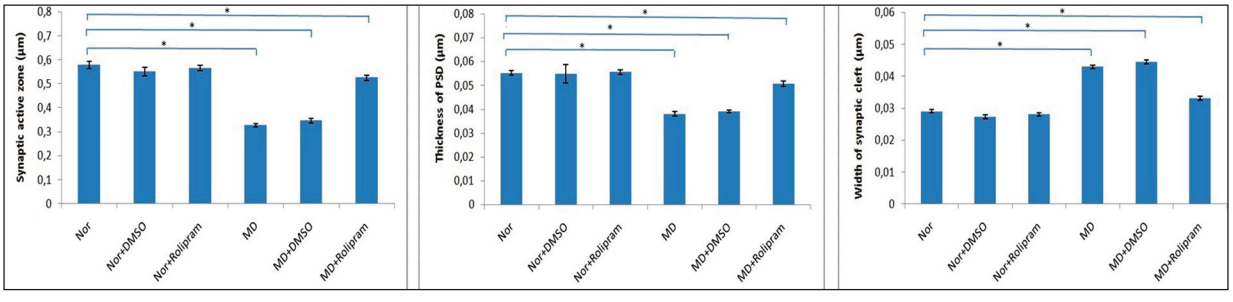

FIGURE 4: Effects of rolipram on the structural modification of synaptic interface in the binocular zone of the primary visual cortex contralateral to the deprived eye. The results are expressed as mean±SEM (n=4 rats per group, 200 synapses studied in each group; comparisons used mean data per animal) (*p<0.05, **p<0.01 by student's t-test).
SEM: Standard error of mean.

phological modifications, such as expanded width of synaptic clefts, reduced thickness of PSD, shortened length of the synaptic active zone, and decreased curvature of the synaptic interface are the main changes affecting axonal conduction.[10] MD revealed that the width of the synaptic cleft increased, the thickness of the PSD decreased, and the length of the synaptic active zone shortened, and that all of these changes were reversed with rolipram treatment.

The primary determinant of synaptic plasticity is the increasing thickness of PSD.[10] The size of the PSD ranges from 250-500 nm in diameter, and from 25-50 nm in thickness, depending on the activity status.[26] In the present study, the mean thickness of the PSD was 55 nm in the Nor group, 38 nm in the MD group, and 50 nm in the MD+rolipram group. Rolipram modified the ultrastructural characteristics of the synapses in the synaptic connections, achieving the reactivation of synaptic plasticity in the monocularly deprived rats. Similarly to the present study, in Luo et al.'s 2011 study, NEP1-40, which has been demonstrated to inhibit the NgR receptor, was administered for 7 days to rats that had been monocularly deprived between P21 and P45. The reactivation of the visual cortical plasticity was found

TABLE 2: Effects of rolipram on the structural modification of synaptic interface in the binocular zone of the primary visual cortex contralateral to the deprived eye. The results are expressed as mean±SEM (n=4 rats per group, 200 synapses studied in each group; comparisons used mean data per animal).

Group	The length of the synaptic active zone (±SD) (μM)	The width of the synaptic cleft (±SD) (μM)	The thickness of the postsynaptic density (±SD) (μM)
Nor	0.57±0.20	0.029±0.006	0.055±0.013
Nor+DMSO	0.55±0.23	0.027±0.008	0.054±0.055
Nor+rolipram	0.56±0.15	0.028±0.006	0.055±0.013
MD	0.32±0.09	0.042±0.007	0.038±0.012
MD+DMSO	0.34±0.11	0.044±0.009	0.039±0.008
MD+rolipram	0.52±0.14	0.033±0.007	0.050±0.015

DMSO: Dimethylsulphoxide; MD: Monocular deprivation; SEM: Standard error of mean.

immediately after the treatment, and the medication was found to be effective.[10]

Increasing evidence shows that the modified spine structure affects synaptic physiology and that the modified spine morphology leads to changes in synaptic strength.[27] Electrophysiological studies have evaluated synaptic strength to reveal the objective visual function. Studies in the literature report that VEP in amblyopia results in generally lower amplitude and prolonged latency, especially in advanced amblyopia.[10,28]

P2 latency was prolonged and P2 amplitude was reduced in the MD group when compared with the Nor group (unpaired student's t-test). There was no change in the P2 latency and amplitudes measurements among the normal groups (Nor vs. Nor+DMSO vs. Nor+rolipram, one-way ANOVA, $p>0.05$). With rolipram treatments, latencies were lowered and amplitudes increased, the VEP results of the MD+rolipram group even reached normal levels (Nor vs. MD+rolipram, unpaired student's t-test). The P2 amplitude of the MD+rolipram group tends to be higher than that of Nor group. These values may provide more meaningful results if the treatment dose and duration are changed. Based on these results, it is concluded that rolipram improved visual function by reducing the latency and increasing the amplitude of VEP in the deprived eye. Changes in the VEP accompanied improvement in synaptic plasticity.

The effect of rolipram on memory has been shown to vary in behavioral studies, partly dependent on dosage.[29,30] Therefore, rolipram administration at different doses and durations may be considered to increase the effects of amblyopia treatment and should be further investigated. Rolipram was developed as an anti-depressant and used in clinical trials. Nevertheless, the trials were terminated due to the side effects in some patients, such as nausea and vomiting.[31,32] Immunosuppression and metabolic abnormalities are some other possible adverse effects of PDE4 inhibition (e.g. altered glucose metabolism).[33] However, the treatment of depression requires long-term administration. For amblyopia treatment, rolipram may require administration for only a short period, during which the side effects may be tolerable. Moreover, for the side effects of rolipram, studies may be conducted with PDE4 subtypes. Subcutaneous administration and its effectiveness by passing through the blood-brain barrier in this way is the advantage of rolipram.[8] Rolipram has also been demonstrated to pass through the blood-brain barrier when administered orally. In addition to oral usage, an alternative route of subcutaneous drug administration may be used to eliminate the side effects.

Dendritic spines are highly dynamic structures, and their plasticity varies based on the adaptation to the modifications in neural activity.[34] In rats, the dendritic spine density increases as the eyes are opened, reaching its peak level at adult age. The structural modifications underlying the synaptic plasticity have not yet been exactly clarified. Previous research has shown that during the crucial period, the density of dendritic spines in the pyramidal neurons in the visual cortex located contralateral to the monocularly deprived eye reduced.[10] In the present study, dendritic spine density and the dynamic modifications in the spines might have been evaluated. Additionally, the amount of cAMP and CREB protein could have been

measured by Western Immunoblot Analysis. This is the limitation of our study.

CONCLUSION

The findings of this research show that rolipram provided a structural and functional improvement in the monocularly deprived eyes of rats. The visual acuity improvement is possibly related to the reopening of the occluded eye combined with greater visual plasticity provided by rolipram. However, further experimental studies are required to establish the long-term effects and tolerability of the substance, as well as to determine the presence of side effects. Rolipram represents a new opportunity for neuropharmacological treatments of amblyopia, and the studies in this field are promising.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Esra Emre, Büşra Katre, Özgül Altıntaş;* ***Design:*** *Esra Emre, Büşra Katre;* ***Control/Supervision:*** *Esra Emre, Özgül Altıntaş;* ***Data Collection and/or Processing:*** *Esra Emre, Büşra Katre, Züleyha Şık Sarman, Özlem Tuğçe Çilingir, İlkay Özdemir, Başak Özkan;* ***Analysis and/or Interpretation:*** *Özgül Altıntaş, Hüsnü Efendi, Dilek Akalın, Seyhun Solakoğlu;* ***Literature Review:*** *Esra Emre, Büşra Katre;* ***Writing the Article:*** *Esra Emre;* ***Critical Review:*** *Özgül Altıntaş;* ***References and Fundings:*** *Esra Emre, Büşra Katre, Dilek Akalın, Hüsnü Efendi, Seyhun Solakoğlu;* ***Materials:*** *Esra Emre.*

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