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Optogenetic stimulation of entorhinal cortex reveals the implication of insulin signaling in adult rat's hippocampal neurogenesis

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ABSTRACT

Adult neurogenesis in the hippocampal dentate gyrus plays a critical role in learning and memory. Projections originating from entorhinal cortex, known as the perforant pathway, provide the main input to the dentate gyrus and promote neurogenesis. However, neuromodulators and molecular changes mediating neurogenic effects of this pathway are not yet fully understood. Here, by means of an optogenetic approach, we investigated neurogenesis and synaptic plasticity in the hippocampus of adult rats induced by stimulation of the perforant pathway. The lentiviruses carrying hChR2 (H134R)-mCherry gene under the control of the CaMKII promoter were injected into the medial entorhinal cortex region of adult rats. After 21 days, the entorhinal cortex region was exposed to the blue laser (473 nm) for five consecutive days (30 min/day). The expression of synaptic plasticity and neurogenesis markers in the hippocampus were evaluated using molecular and histological approaches. In parallel, the changes in the gene expression of insulin and its signaling pathway, trophic factors, and components of mitochondrial biogenesis were assessed. Our results showed that optogenetic stimulation of the entorhinal cortex promotes hippocampal neurogenesis and synaptic plasticity concomitant with the increased levels of insulin mRNA and its signaling markers, neurotrophic factors, and activation of mitochondrial biogenesis. These findings suggest that effects of perforant pathway stimulation on the hippocampus, at least in part, are mediated by insulin increase in the dentate gyrus and subsequently activation of its downstream signaling pathway.

1. Introduction

The hippocampus, a structure located in limbic system, plays a key role in the formation of episodic and spatial memory in the adult brain (Persson et al., 2018). However, the mechanisms of learning and memory formation in the hippocampus are complex. Synaptic plasticity

Abbreviation: DG, dentate gyrus; Ent, entorhinal cortex; MEnt, medial entorhinal cortex; NSCs, neural stem cells; SGZ, subgranular zone; CA, cornu ammonis; CNS, central nervous system; IRs, insulin receptors; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; DBS, deep brain stimulation; IHC, immunohistochemistry; MWM, Morris water maze; qPCR, quantitative polymerase chain reaction; DCX, doublecortin; mTOR, mammalian target of rapamycin; S6K, ribosomal protein S6 kinase; NT3, neurotrophin 3; GDNF, glial cell derived neurotrophic factor; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; GAP43, growth associated protein 43; PBS, phosphate-buffered saline; PFA, paraformaldehyde; IF, immunofluorescence; NGS, normal goat serum; IRSs, insulin receptor substrates; S6K1, ribosomal protein S6 kinase 1; MAPK, mitogen-activated protein kinas; ERK, extracellular-signal-regulated kinas; CREB, cAMP-response element binding protein; SRF, serum response factor; MEF-2, myocyte enhancer factor..

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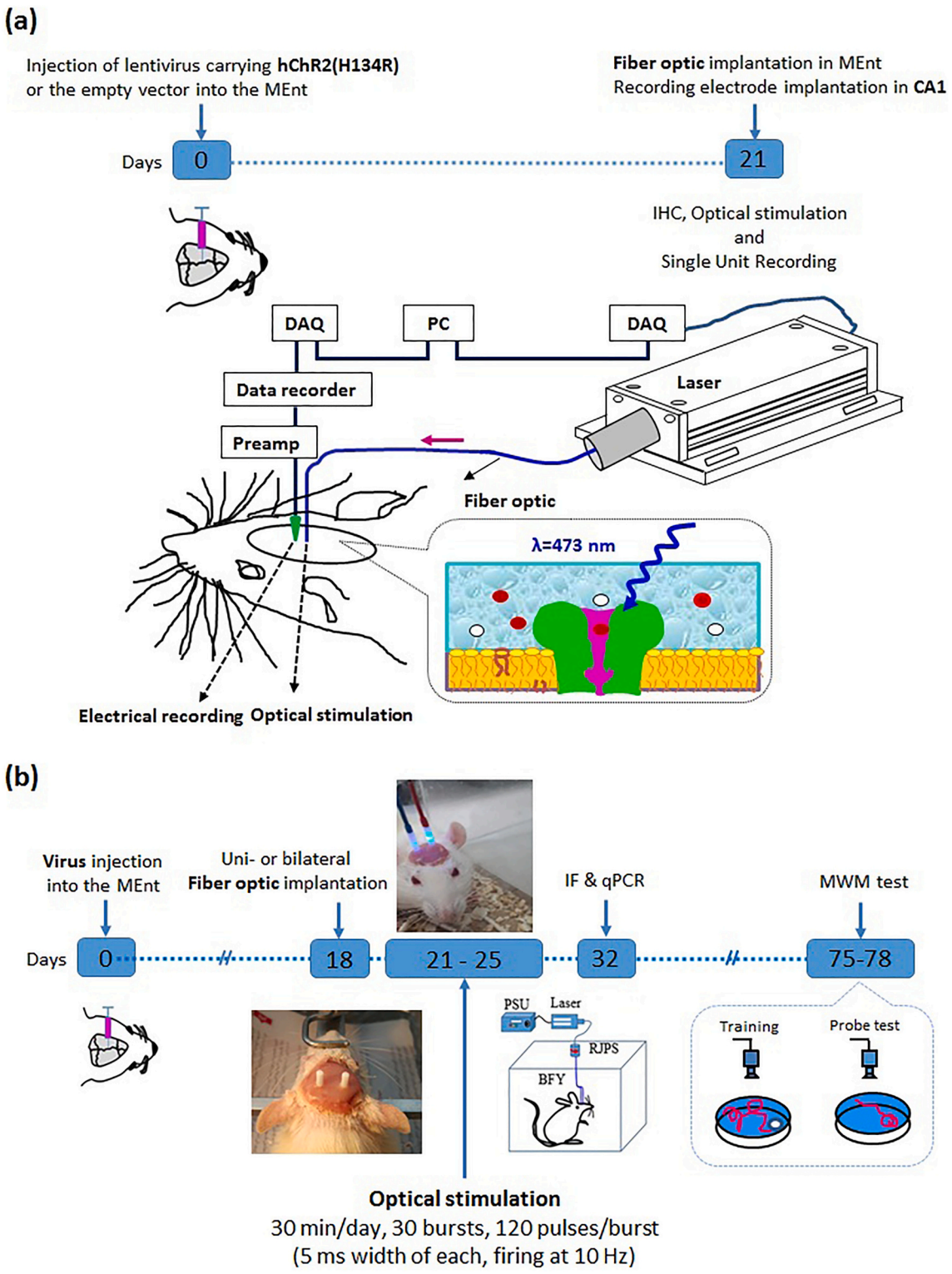


Fig. 1. Schematic diagram of experimental design. Lentiviruses carrying the hChR2 (H134R)-mCherry gene or the control construct were uni- or bilaterally injected into the medial entorhinal cortex (MEnt) on the beginning day of experiments. (a) Verification of opsin expression by immunohistochemistry (IHC) staining of mCherry reporter gene in MEnt and single unit recording of hippocampal CA1 neurons in response to the optical stimulation of MEnt. (b) Five consecutive days of unilateral optical stimulation of MEnt and performing immunofluorescence staining (IF) and quantitative PCR (qPCR), 7 days after the last stimulation. Spatial memory was assessed 50 days after a course of five-day bilateral optical stimulation of MEnt.

changes (Stuchlik, 2014) and neurogenesis in the dentate gyrus (DG) region extensively contribute to these processes (Deng et al., 2010; Yau et al., 2014). Adult neural stem cells (NSCs) in the subgranular zone (SGZ) of the DG proliferate and give rise to immature neuroblasts, which subsequently differentiate into mature granule neurons that send their dendrites to the molecular layer of the DG and extend their axons, called mossy fibers, into the cornu ammonis (CA) 3 region (Braun and Jessberger, 2014; Chavoshinezhad et al., 2019a). CA3 pyramidal cells, in

turn, project to the CA1 neurons through Schaffer collateral pathway and eventually promote new memories (Basu and Siegelbaum, 2015; Stepan et al., 2015). Neurogenesis in the adult brain is regulated by crosstalk between the extrinsic factors and a variety of intrinsic factors including hormones (Zhang and Zhang, 2018), trophic factors (Zhang and Zhang, 2018), mitochondrial metabolism (Beckervordersandforth, 2017), and neurotransmitters/ neuromodulators released from local and long-distance neuronal circuitries (Song et al., 2016). While hippocampus and DG receive many afferents from several areas of the brain, the fibers originating from the cells in layers II and V of entorhinal cortex (Ent) provide the main input to DG (Canto et al., 2008; Ge et al., 2017). One of the major Ent projections to DG is glutamatergic fibers known as the perforant pathway (Canto et al., 2008; Ge et al., 2017). Glutamate is the predominant excitatory neurotransmitter in the adult brain correlated with the hippocampal neurogenesis (Berg et al., 2013; Schlett, 2006). Metabotropic glutamate receptors are highly expressed in NSCs (Gerevini et al., 2004; Melchiorri et al., 2007; Zhang and Zhang, 2018). Moreover, it has been indicated that stimulation of the Ent promotes survival, proliferation, differentiation, and maturation of NSCs in the DG, and consequently enhances spatial memory (Kitamura et al., 2010; Ronaghi et al., 2019; Stone et al., 2011; Yun et al., 2018). However, the underlying molecular mechanisms as well as neuromodulators involved in the neurogenic effects of this pathway are not well known.

In the central nervous system (CNS), insulin possesses neuromodulatory effects and also participates in nutrient homeostasis, synaptic transmission, and neural plasticity (Blázquez et al., 2014). Expression of insulin receptors (IRs) has been widely shown in the hippocampus, cerebral cortex, striatum, olfactory bulbs, and cerebellum (Duarte et al., 2012). However, synthesis of insulin in the brain is not a consensus. In several human and rodent's studies, insulin mRNA has been detected in the hippocampus (Devaskar et al., 1994; Kuwabara et al., 2011; Mehran et al., 2012), cortex (Csajbók and Tamás, 2016; Molnár et al., 2014), hypothalamus (Lee et al., 2020; Madadi et al., 2008), and olfactory bulb (Kuwabara et al., 2011; Mehran et al., 2012). Additionally, it is shown that insulin can be stored in the adult rat brain in synaptic vesicles within nerve endings and released under depolarizing conditions (Clarke et al., 1986; Wei et al., 1990). Insulin binding to the IR leads to autophosphorylation of the IR followed by tyrosine phosphorylation of the insulin receptor substrate (IRS) protein family which initiates phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT) pathway (Han et al., 2008; Taniguchi et al., 2006). Involvement of insulin and its downstream signaling pathway in cognitive function as well as proliferation and differentiation of NSCs has been the subject of several studies (Lee et al., 2016; McNay et al., 2010; Ziegler et al., 2015).

We previously reported that the deep brain stimulation (DBS) of the Ent in the adult rats increases the DG neurogenesis and facilitates the spatial learning and memory through insulin signaling pathway (Ronaghi et al., 2019). DBS influences not only presynaptic and postsynaptic terminals but also affects glial cells and produces local excitatory or inhibitory responses at the network level in the target tissue (Fenoy et al., 2014). Recently, it has been shown that activated glial cells release neurotransmitters and neuromodulators modulating the neuronal activity. Thus, the DBS-induced response is heterogeneous and results from interactions between neuronal and non-neuronal cells (Jakobs et al., 2019; McIntyre and Anderson, 2016; Vedam-Mai et al., 2012).

Optogenetics is a well-approved approach for neuromodulation (Knafo and Wyart, 2015; Pama et al., 2013). In this technique, genetically modified cells expressing light-sensitive channels are modulated by light (Kim et al., 2017). This technique is a suitable method to activate or inhibit the specific neuronal cells to monitor the sub-cellular events resulted from photostimulation (Repina et al., 2017). In the present study, we used optogenetics to investigate molecular mechanisms involved in the adult rat's hippocampal neurogenesis and synaptic plasticity following Ent optostimulation.

2. Materials and methods

2.1. Animals

Adult male Wistar rats, weighing 240–260 g, were obtained from a breeding colony in Neuroscience Research Center, Shahid Beheshti University of Medical Sciences. Animals were cared under the standard laboratory conditions (12 h light/dark cycle, the temperature of 21 ± 2 °C) with free access to food and water. All experiments were carried out based on the guide for the care and use of laboratory animals (National Institutes of Health Publication No. 80–23, revised 1996) and confirmed by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.SM.REC.1394.147).

2.2. Lentivirus preparation

The plasmid, pLenti-CaMKIIa-hChR2(H134R)-mCherry-WPRE, a gift from Karl Deisseroth (Addgene plasmid # 20943; http://n2t.net/addgene:20943; RRID:Addgene_20,943) (Zhang et al., 2007) was co-transfected into Lenti-X 293T cells (Clontech, USA, #632180) with ViraPower TM Lentiviral Expression kit (Invitrogen, USA, #k497000). Culture supernatant containing virus particles was harvested at 48 h, filtered, and subsequently ultracentrifuged at 50×10^3 g for 3 h. Then, the lentiviral pellet was dissolved in the phosphate buffered saline (PBS), at a titer of approximately 10^9 TU/ml aliquoted, and stored at −80 °C until use.

2.3. Surgery and experimental design

Rats were intraperitoneally anesthetized with a mixture of ketamine and xylazine (80 and 20 mg/kg, respectively) and placed into a stereotaxic apparatus for the intracerebral injections (Stoelting, USA). A volume of 3 μl of the concentrated lentivirus suspension, carrying hChR2 (H134R), was injected into the MEnt (AP: −6.84 mm, ML: ±4.6 mm, DV: 8.8 mm based on the Paxinos and Watson atlas (Paxinos and Watson, 2007), using 30-gauge needle connected to a Hamilton syringe through a polyethylene tubing at the rate of 0.5 μl/min. Control animals received the same lentiviral vector, expressing only mCherry reporter gene (cloned in our lab). In the part I of experiments, to assess the transduction efficiency, the animals were sacrificed at day 21 after injection and the mCherry expression was determined in the Ent region by the immunohistochemistry (IHC) staining (n = 3/group). Moreover, the activity of pyramidal neuron in CA1 region was investigated by the single unit recording to validate the hippocampal response to the blue laser light stimulation of the Ent (n = 3/group, Fig. 1a). After verification of optogenetic stimulation, our study was continued in parts II and III (Fig. 1b). In the part II, animals received a unilateral injection of lentiviral suspension in the Ent, after18 days, the optical fiber was implanted in the Ent region. After recovery, the laser light stimulation was started on day 21 and continued daily until day 25 of the experiment. Seven days after the last laser stimulation (day 32), the animals were sacrificed and prepared for the histological and molecular assessments in the hippocampus (n = 3/group). In the part III of experiments, animals received a bilateral injection of lentiviral suspension in the Ent and were subjected to the laser light stimulation on days 21–25. Spatial memory was evaluated using the Morris water maze (MWM) test, 50 days after the last laser light stimulation, day 75 (n = 6/group).

2.4. Optical stimulation and single-unit recording

On day 21 post-injection, rats were anesthetized with the urethane (1.2–1.5 g/kg, i.p.) and were placed in the stereotaxic apparatus. Fiber optic was implanted in the right MEnt at the same coordinates that virus was injected and a tungsten microelectrode (shaft diameter; 127 μm, tip exposure; 1–3 μm and tip impedance; 5MΩ) (HFC Inc., Bowdoin ME, USA) was placed in the pyramidal cells layer of CA1 (AP: −3.3 mm, ML:

Table 1
Primer sequences used in quantitative polymerase chain reaction (qPCR).

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Nestin	GAGTTCTCAGCCTCCAGCAG	GGAGCAGGAGAAGCAAGGTC
Ki67	CGGCGAGCCTCAAGAGATA	CGTGCTGTTCTACATGCCC
DCX	TTGCTGCTAGCCAAGGACTG	GGAAGGGGAAAGCTATGTCTG
Insulin	ATCTTCAGACCTTGGCACTGG	GTAGAGGGAGCAGATACTGGT
IR	GGTGTAGTGGCTGTCACATT	GAGCGGAGGAGTCTTCATT
IRS2	GCTAAGCATCTCCTCAGAATGGA	GACTTCTTGTCCCATCACTTGAAA
PI3K	ATGTGTATGGACCCGGAAGG	AGCCATCTGCCTCCACGTTAG
Akt	ACGTAGCCATTGTGAAGGAGG	TGCCATCATTCTTGAGGAGGAA
mTOR	AGAACCTGGCTCAAGTACGC	AGGATGGTCAAGTTGCCGAG
S6K	ATGGAACAGTCACGCACACA	AGACTCCACCAATCCACAGCAC
NT3	ACTCTCCTCGGTGACTCTTATGC	GACACAGAACTACTACGGCAACAG
GDNF	CCTCTGCGACCTTTCCCTCTG	GCTGACCAGTGACTCCAATATGC
NGF	GAACAACATGGACATTACGCTATGC	CCCAATAAAGGCTTTGCCAAGGAC
BDNF	CAGAACAGAACAGAACAGAACAGG	CGATTAGGTGGCTTCATAGGAGAC
PGC1α	GTCCAGGTCATTCACATCAAGTTC	GTGCAGCCAAGACTCTGTATGG
NRF1	GTTGCATCTCCTGAGAAGCG	AAATTGGGCCACATTACAGGG
TFAM	CATTCAGTGGGCAGAAGTC	AGAGTTGTCATTGGGATTGG
GAP43	CGGGCACTTTCCTTAGGTTTG	TGCAGAAAGCAGCCAAGCT
Reelin	GTCGTCCTAGTAAGCACTCGC	ACCTTCGCCTTCGGTTGTAG
β-actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACACTCC

+2.2 mm, DV: 2.4 mm) according to the rat brain atlas (Paxinos and Watson, 2007). Then, the electrode was finely driven through the CA1 pyramidal cell layer using a manual microdrive until a well-defined spike activity with a signal-to-noise ratio of more than two was isolated from the background noise. The signals were amplified 10,000 times using a differential amplifier (DAM-80; WPI, Sarasota FL, USA), and the band-pass filter was 0.3–10 kHz and digitalized at 50 kHz sampling rate and 12-bit voltage resolution using a data acquisition system (D3109; WSI, Tehran, Iran). After finding a pyramidal neuron with stable firing frequency and spike amplitude, a baseline activity was recorded for 20 s. Then, the laser light stimulation was performed, and the recording was continued for more 40 s. All recordings were saved on a computer for later offline analyses (Haghparast et al., 2012; Riahi et al., 2015). All-or-none spike events were detected using a window discriminator (W3205; WSI, Tehran, Iran) based on the spike amplitude. The spike frequencies were counted and displayed online in time bins of 1000 ms over the entire recording period by an online-sorter homemade software (Spike; ScienceBeam, Tehran, Iran). Data of the discriminated spikes as well as the whole data streams (including undetected spikes and background activities) of all recordings were also saved on a computer device for analyses by plexon offline sorter and neuroexplorer (Plexon Inc., Dallas TX, USA).

2.5. Fiber optic implantation and optical stimulation

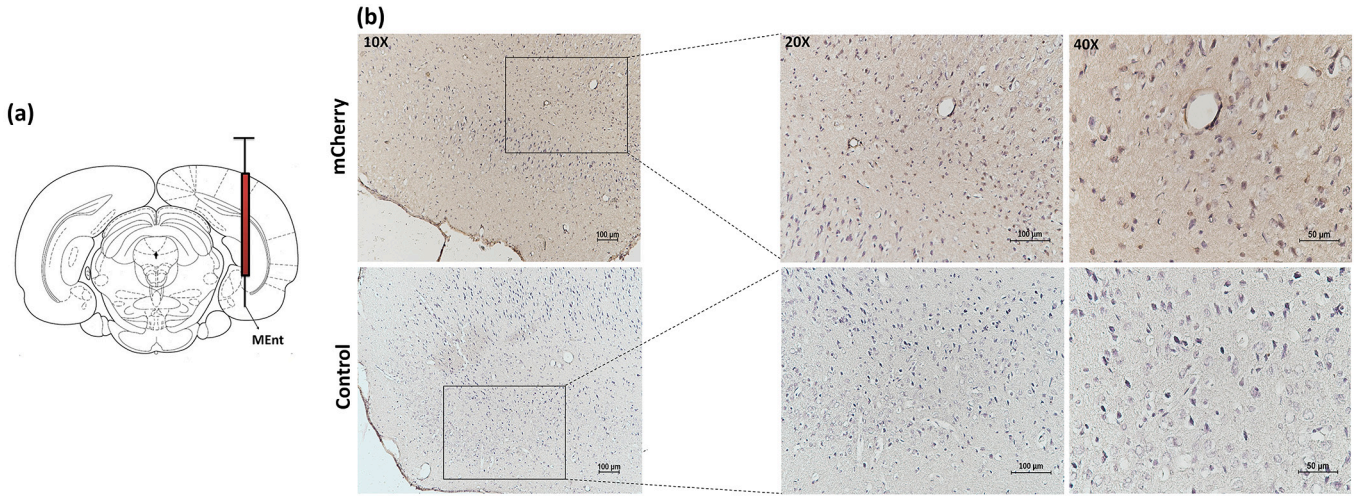
Eighteen days after lentivirus injection, the rats were anesthetized and underwent stereotaxic surgery for the uni- or bilateral optical fiber implantation (Wu et al., 2015). Optical fibers (200 μm core diameter, 0.5 NA, Thorlabs) with ceramic ferrule were implanted into the same coordinates of virus injection and mounted with acrylic dental cement on the skull. Three days after surgery, animals received a 30 min stimulation session per day for 5 consecutive days. The session had 30 bursts, each burst contained 120 pulses (5 ms width of each, firing at 10 Hz), and the inter-burst interval was 48 s (Song et al., 2017). The 473 nm blue laser light was generated by a CNI laser system equipped with a rotary joint patch cable (RJPSF2, Thorlabs) to enable the rat to rotate freely in the chamber. The optical commutator was connected to a bifurcated multimode fiber optic cable (BFYL2LF01, Thorlabs) for simultaneous in vivo optical stimulation of two targets in the brain areas, also known as Y-cables. Two ports of Y-cables were connected with a ceramic sleeve (ADAL1, Thorlabs) to surgically implanted LC ceramic ferrule. Rats were lightly anesthetized by isoflurane for connecting the Y-cables to the dual LC fiber optic cannula. In each experiment, the laser was turned on for approximately 40 min to stabilize. Subsequently, the photo-stimulation process began. Control rats injected with empty virus also received optical fiber implantation into the EC and laser light stimulation.

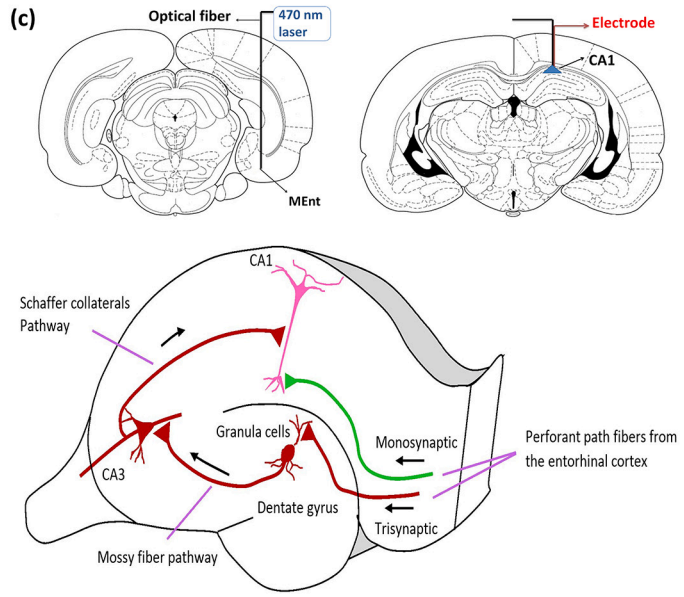
2.6. RNA extraction and quantitative polymerase chain reaction (qPCR)

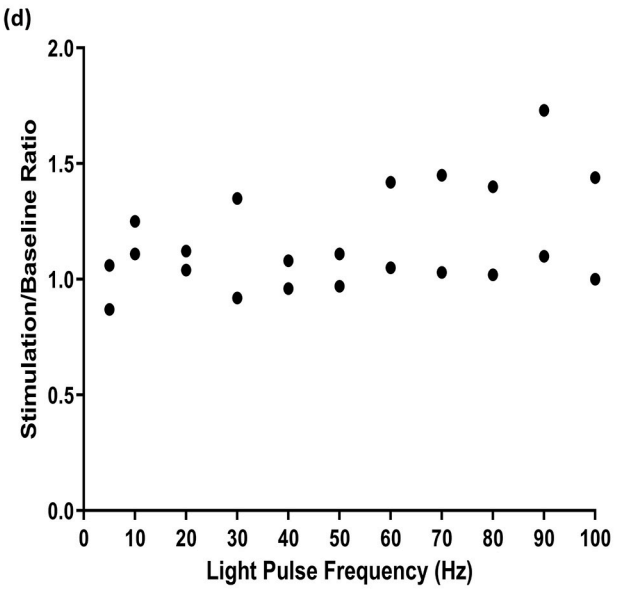
On day 32 of experiments, 3 rats from the control and optical stimulation groups were sacrificed. The hippocampus tissue was immediately dissected, frozen in liquid nitrogen, and kept at −80 °C until qPCR analysis. The total RNA was extracted using YTzol reagent (Yekta Tajhiz Azma, Iran, #YT9063). Purity and concentration of all extracted RNA samples were analyzed spectrophotometrically by NanoDrop 2000 (Thermo Scientific, USA). Then, cDNA was synthetized by PrimeScript™ RT reagent kit (Takara, Japan, #RR037A) and used as a template to quantify the expression of Nestin, Ki67, doublecortin (DCX), insulin (Ins1 and Ins2), IR, IRS2, PI3K, AKT, mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (S6K), neurotrophin-3 (NT3), glial cell derived neurotrophic factor (GDNF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), growth associated protein 43 (GAP43), and Reelin genes. The specific primers were designed using Allele ID 7, as presented in Table 1. The qPCR was performed by SYBR Green qPCR Master Mix (2×) (Ampliqon, Denmark) and ABI StepOne (Applied Biosystems, USA) instrument under cycling conditions: activation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s (Chavoshinezhad et al., 2019b). All genes were normalized to β-actin as a house-keeping gene. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative changes in expression of the genes mentioned above (Livak and Schmittgen, 2001).

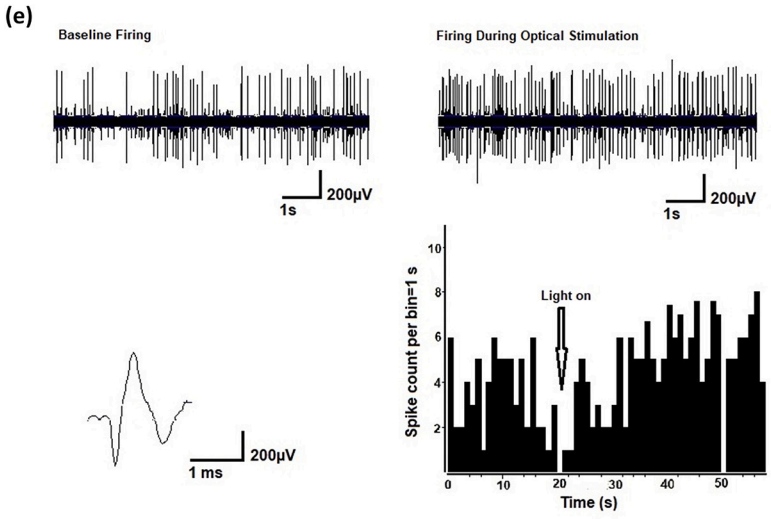
2.7. Histological assays

Immunohistochemistry (IHC) staining was performed to detect the mCherry immunoreactivity in the Ent. On day 21 post-injection, rats were anesthetized ($n = 3$) and transcardially perfused with cold PBS (0.1 M; pH 7.4) followed by 4% paraformaldehyde (PFA, Merck, Germany). The brains were harvested, post-fixed in PFA overnight, and cryoprotected in 30% sucrose (Merck, Germany) for 48 h at 4 °C, and then were embedded, frozen in OCT (optimal cutting temperature) compound (Bio optica, Italy, #059801), and kept at −80 °C. Frozen coronal sections (10 μm) were prepared using a cryostat apparatus (Sci Lab, English) along the anterior-posterior axis and placed on gelatin-coated slides. The sections were fixed with acetone, quenched in the 1% hydrogen peroxide, permeabilized in the 0.2% Triton X-100 (Merck,









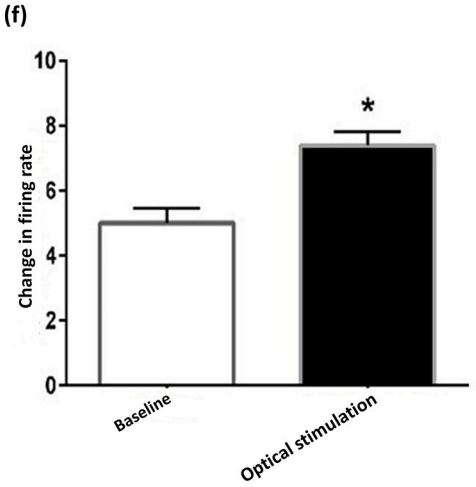


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Fig. 2. Lentivirus-mediated expression of hChR2 in entorhinal cortex and verification of optogenetic stimulation. Schematic Lentivirus injection site and Lentiviral-mediated expression of mCherry protein in MEnt of adult rats on day 21 after injection, scale bar: 100 μm in ×10, ×20 and 50 μm in ×40 objective, respectively (a, b). Schematic representation of light stimulation and single unit recording loci (c). Different light pulse frequencies (5–100 Hz) were tested and correlation between light pulse frequencies and changes in the firing rate of neurons to baseline has been shown ($r = 0.7$, $P < 0.05$) (d). An example that shows the pattern of baseline spontaneous firing of one CA1 pyramidal neurons and same neuron during optical stimulation of MEnt cortex, an expanded waveform of a spike recorded extracellularly from the CA1 pyramidal neuron and histogram representing spike count per time bins of 1 s over 20 s baseline recording and during 40 s light stimulation (e). As an example, the firing rate of the CA1 pyramidal neuron was increased significantly relative to the baseline firing after light stimulation (10 Hz) of MEnt cortex. Values are presented as means ± SEM ($n = 2$ neuron from two rats). Unpaired *t*-test was used for statistical analysis. *$P < 0.05$ vs. baseline (f).

Germany), blocked, incubated overnight at 4 °C with the rabbit anti-mCherry antibody (1100, Abcam, # ab167453), and then with the EnVision™ + Dual Link System-HRP (Dako Denmark A/S, Glostrup, Denmark) for 1 h at room temperature (Ronaghi et al., 2019). Immunoreactivity was visualized by treatment with the 3, 3′-Diaminobenzidine tetrahydrochloride (DAB, Dako, Denmark) solution followed by nuclear counterstaining with the hematoxylin dye. After dehydration using an ascending series of ethanol, the sections were cleared in xylene and then cover-slipped. No primary antibody-treated sections were used as control. The images were captured with the Nikon light microscope (E600; Objective: ×10, ×20, and ×40).

On day 32 of the experiments, 3 rats from control and optical stimulation groups were subjected to immunofluorescence (IF) staining. The frozen sections of hippocampus were fixed with acetone (Merck, Germany) for 20 min, then were permeabilized in the 0.2% Triton X-100 (Merck, Germany) and blocked with 10% normal goat serum (NGS) (Sigma, USA, prepared in 0.2% Triton X-100) for 1 h at room temperature. Then, the sections were incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. The following primary antibodies were used: rabbit anti-Nestin (1:100, Abcam, #ab93157), rabbit-anti Ki67 (1:100, Abcam, #ab66155) and rabbit anti-DCX (1:200, Abcam, #ab77450). On the next day, immunoreactivity was detected and visualized after incubating the sections in the goat anti-rabbit IgG FITC conjugated (1:100, Sigma, USA, #F1262) for 1 h at room temperature followed by nuclear counterstaining with the DAPI reagent (Sigma, USA) (Ronaghi et al., 2019). The images were obtained by the Olympus invert fluorescence microscope (IX71; Objective: ×20).

2.8. Morris water maze (MWM) test

Fifty days after bilateral optogenetic stimulation, the learning and long-term spatial memory performance were measured by MWM test. The apparatus consisted of a pool (150 × 60 cm) filled with water (23 °C) to a depth of 45 cm. The pool was arbitrarily divided into four quadrants and a black round platform (11 cm in diameter) was located in the center of the target quadrant, 2 cm beneath the water surface. The pool was surrounded by the spatial cues in the constant positions throughout the experiment. The training and probe trials were video tracked (Panasonic Inc., Japan) and analyzed using the Ethovision software (version XT7, Netherlands). Animals were trained over 3 days with three trials per day (inter trial interval, 30 s). On each trial, the rats were released into the pool, facing the wall, in one of four defined quadrants (the order of each trial was selected randomly throughout training). Rats were given 60 s to swim and find the platform. The trial was accomplished once the rats found the platform and stayed there for 20 s, or 60 s had elapsed that in this case rats were guided to the platform by the experimenter (Ronaghi et al., 2019). Latency to find the platform (escape latency) was recorded in the training sessions as an index of spatial learning (Adeli et al., 2017). The day after last training session, the probe session was performed in the pool without the platform, with a starting point in the quadrant opposite to the former platform position, for a period of 60 s. The time spent in the target quadrant was recorded in the probe session as measures of the spatial memory performance (Chavoshinezhad et al., 2019a).

2.9. Statistical analysis

All Data were analyzed using 16th version of SPSS software. The data of single unite recording were analyzed by un-paired *t*-test. One-way ANOVA followed by Tukey's post hoc test was applied to compare the molecular findings between groups. Moreover, the data of the MWM were analyzed using two-way ANOVA with repeated measures (training days), and un-paired t-test (probe session). $P < 0.05$ was considered statistically significant. Data are reported as the means ± SEM.

3. Results

3.1. Lentivirus-mediated expression of hChR2 in the Ent and verification of optogenetic stimulation

In this study, the efficiency of hChR2 expression was evaluated in the Ent region, 21 days after transduction. In the lentivirus construct, both hChR2 and mCherry genes were under the control of the CaMKII promoter, and the expression of hChR2 gene was monitored by the mCherry reporter gene. As shown in the Fig. 2a and b, a strong expression of mCherry protein was detected in the Ent. Moreover, the single unit recording results revealed that optical stimulation of the excitatory neurons of Ent enhanced the firing rate of pyramidal neurons of CA1 in comparison to the basal activity (Fig. 2d–f, $P < 0.05$).

3.2. Optogenetic stimulation of MEnt promoted neurogenesis in the hippocampus

Seven days after unilateral optogenetic stimulation of MEnt, we assessed the mRNA and protein levels of Nestin (a NSC marker) (Strojnik et al., 2007), Ki67 (a proliferation marker) (Henry et al., 2009), and DCX (a neuroblast marker) (Vergni et al., 2009) in the hippocampus. The day 7 post stimulation was selected as an optimum time point to track neurogenesis process in SGZ based on the previous evidence (Kee et al., 2007; Kempermann et al., 2015). In IF staining, Nestin, Ki67, and DCX-positive cells enhanced in the SGZ neurogenic niche and ectopically in the hilus of ipsilateral DG in the optically stimulated rats compared to control (Fig. 3a, c and e). Interestingly, as shown in Fig. 3a and e, an increase in the intensity of Nestin and DCX immunoreactivity was also identified in the contralateral DG of optically stimulated group versus control. However, the changes were less prominent in the contralateral side than in the ipsilateral. Despite the bilateral increase in the Nestin and DCX-positive cells in the DG of optically stimulated group compared to control, we did not detect an increased expression of Ki67 protein in the contralateral DG (data not shown).

In qPCR test, as shown in Fig. 3b, d, and f, the mRNA levels of Nestin, Ki67, and DCX bilaterally elevated in the hippocampus of optically stimulated group compared with the corresponding sides of control. However, the changes were not statistically significant on the contralateral side. One-way ANOVA analysis indicated that optogenetic stimulation of the excitatory neurons in the MEnt significantly increased the mRNA levels of Nestin and DCX in the ipsilateral hippocampus of optically stimulated group versus the corresponding side of control group, and also to the contralateral side of optically stimulated group (Fig. 3b and f, $P < 0.05$ for Nestin, $P < 0.01$ and $P < 0.05$ for DCX, respectively).

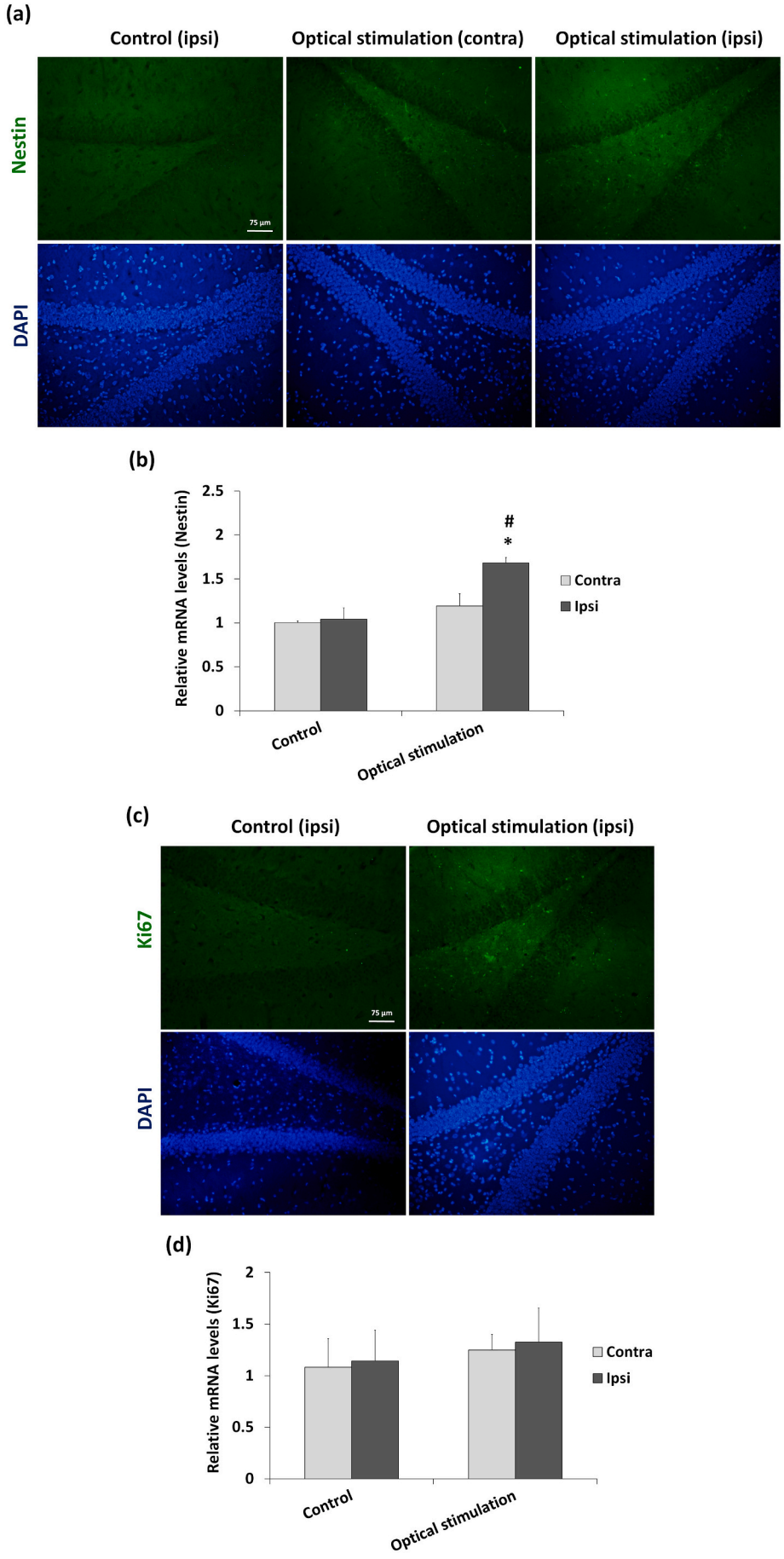

Fig. 3. The effect of unilateral optogenetic stimulation of the MEnt on neurogenesis markers in the hippocampus. Representative micrographs of immunofluorescence staining of Nestin (a), Ki67 (c) and Doublecortin (DCX) (e) in the ipsilateral (ipsi) and contralateral (contra) hippocampus of the control and optically stimulated groups. Cell nuclei were counterstained with DAPI. Scale bar: 75 µm. qPCR data analysis of Nestin (b), Ki67 (d) and DCX (f) in hippocampus of ipsi- and contralateral hemispheres in the control and optically stimulated groups. Data are reported as the means ± SEM ($n = 3$/group). One-way ANOVA followed by Tukey's post hoc test was used for statistical analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. corresponding control group. $^{\#}P < 0.05$ between two sides in the optically stimulated group.

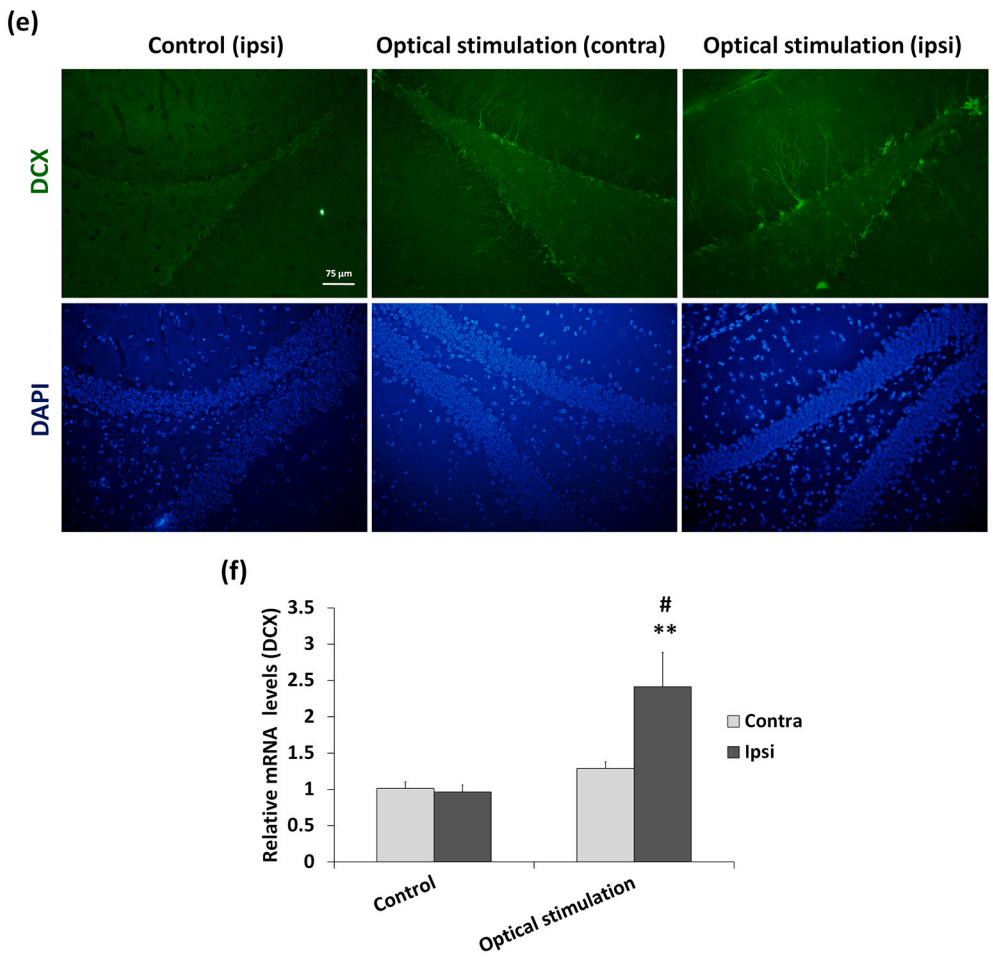

Fig. 3. (*continued*).

Furthermore, a slight increase but not statistically significant change in the mRNA level of Ki67 was found in the ipsilateral hippocampus of optically stimulated rats compared to the corresponding side of the control rats (Fig. 3d, $P > 0.05$).

3.3. Optogenetic stimulation of MEnt enhanced insulin mRNA and activated insulin signaling pathway in the hippocampus

Seven days after unilateral optogenetic stimulation of the Ent, the mRNA levels of insulin and its downstream signaling effectors in the hippocampus were evaluated by the qPCR analysis. PI3K/AKT/mTOR/S6K is one of the important pathways of the IR signal transduction in the brain (Na et al., 2017; Perluigi et al., 2014). The insulin binding to the IR results in autophosphorylation of the IR followed by tyrosine phosphorylation of the insulin receptor substrates (IRSs). Phosphorylated IRS binds to the several effector molecules such as p85 or p55 regulatory subunit of PI3K and triggers activation of PI3K, which in turn phosphorylates and activates AKT. AKT subsequently activates mTOR, which recruits ribosomal protein S6 kinase 1 (S6K1) to initiate protein translation (Kido et al., 2001). The one-way ANOVA analysis indicated that mRNA levels of insulin, IR, IRS2, PI3K, AKT, mTOR and S6K were significantly enhanced in the ipsilateral hippocampus of optically stimulated rats versus the corresponding side in the control rats (Fig. 4a–g, $P < 0.001$, $P < 0.01$, $P < 0.05$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). Interestingly, levels of PI3K, mTOR and S6K mRNA in the ipsilateral hippocampus were significantly higher than that in the contralateral side of optically stimulated group (Fig. 4d, f, and g, $P < 0.001$). As shown in the Fig. 4g, a significant elevation in S6K mRNA expression was detected in the contralateral hippocampus of the optically stimulated rats in comparison with corresponding side in the control rats ($P < 0.001$).

3.4. Optogenetic stimulation of MEnt increased the expression of neurotrophic factors in the hippocampus

One-way ANOVA analysis revealed that NT3 mRNA level was significantly enhanced in both the ipsilateral and the contralateral hippocampus of the optically stimulated rats in comparison with corresponding sides of control (Fig. 5a, $P < 0.01$ and $P < 0.001$, respectively). In contrast, as depicted in Fig. 5b and c, there was only a significant elevation in the mRNA levels of GDNF and NGF in the ipsilateral hippocampus of optically stimulated group versus the corresponding side in the control ($P < 0.05$). Moreover, the changes of BDNF mRNA expression were not statistically significant in both sides of optically stimulated group compared to the control (Fig. 5d, $P > 0.05$).

3.5. Optogenetic stimulation of MEnt increased mitochondrial biogenesis in the hippocampus

As shown in the Fig. 6a–c, the one-way ANOVA analysis indicated that the mRNA levels of PGC1α, NRF1, and TFAM, main regulators of mitochondrial biogenesis, were significantly elevated in the ipsilateral hippocampus of optically stimulated group versus corresponding side of the control (Fig. 6a–c, $P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively). Additionally, the mRNA levels of PGC1α and NRF1 in the ipsilateral hippocampus were significantly higher than that in the contralateral

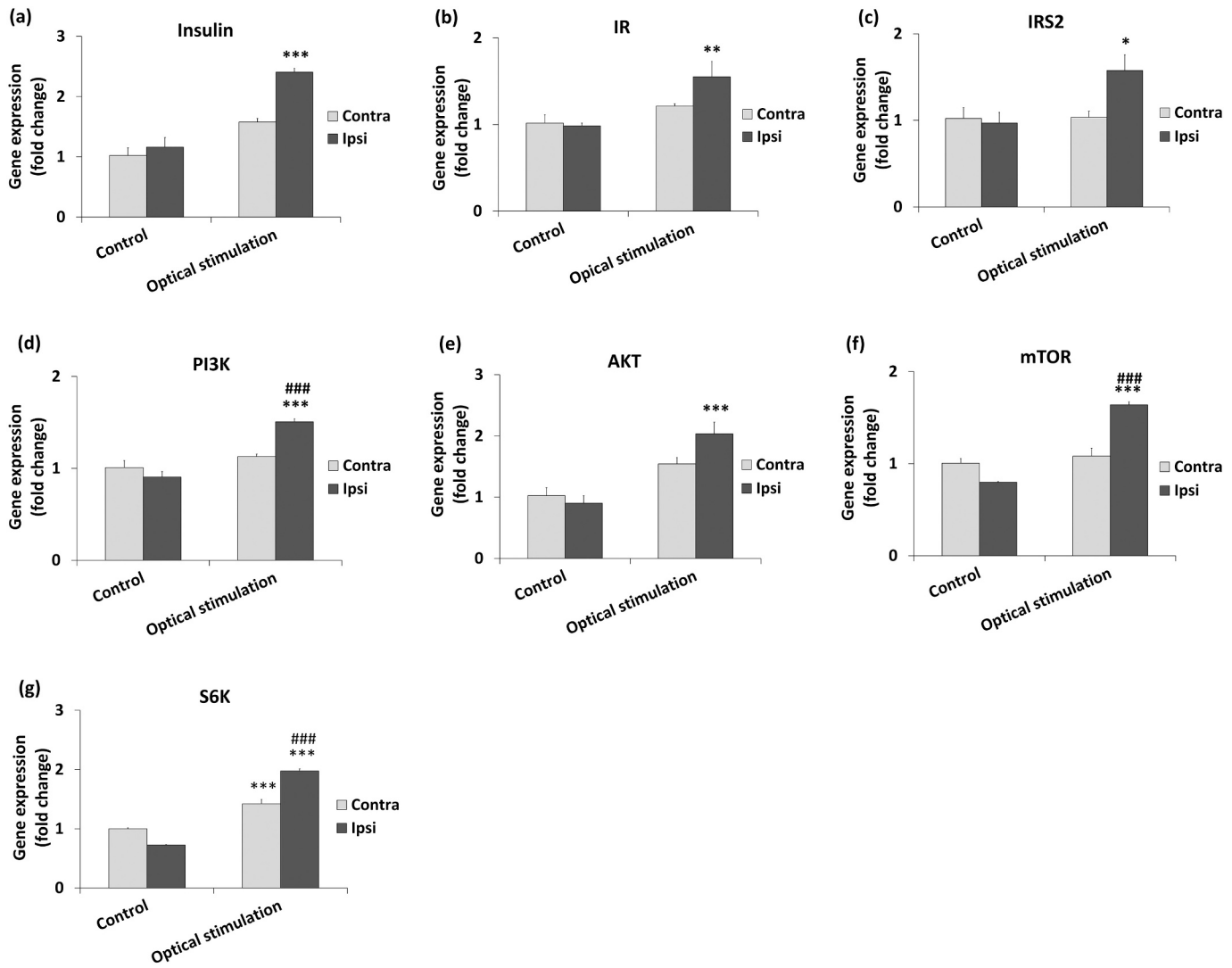

Fig. 4. The effect of unilateral optogenetic stimulation of the MEnt on mRNA expression of insulin signaling components in the hippocampus. The qPCR data analysis of insulin (a), IR (b), IRS2 (c), PI3K (d), AKT (e), mTOR and S6K in the ipsilateral (ipsi) and contralateral (contra) hippocampus of the control and optically stimulated groups. Data are reported as the means ± SEM ($n = 3$/group). One-way ANOVA followed by Tukey's post hoc test was used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs corresponding control group. $^{###}P < 0.001$ between two sides in the optically stimulated group.

hippocampus of the optically stimulated rats (Fig. 6a and b, $P < 0.05$ and $P < 0.01$, respectively). Interestingly, unilateral optogenetic stimulation of the MEnt significantly increased PGC1α expression in the contralateral hippocampus compared to the corresponding side of the control group (Fig. 6a, $P < 0.01$). Similar expression patterns were identified in NRF1 and TFAM mRNA in the contralateral hippocampus of the optically stimulated when compared to corresponding side of the control group, but the changes were not statistically significant (Fig. 6b and c, $P > 0.05$).

3.6. Optogenetic stimulation of MEnt enhanced synaptic plasticity in the hippocampus

Seven days after unilateral optogenetic stimulation of MEnt, the mRNA levels of GAP43 and Reelin were assessed in the hippocampus by qPCR analysis to investigate the synaptic plasticity changes. As shown in the Fig. 7a and b, one-way ANOVA analysis revealed that GAP43 gene expression, but not Reelin, significantly increased in the ipsilateral hippocampus of the optically stimulated rats compared to the corresponding side of the control rats ($P < 0.05$). Also, a slight increase in expression of GAP43 mRNA was found in the contralateral hippocampus of optically stimulated group in comparison with the corresponding side of the control rats (Fig. 7a, $P > 0.05$).

3.7. Optogenetic stimulation of MEnt did not improve spatial learning and memory in the adult rats

In the last step of the study, we were interested to know whether optogenetic stimulation of the MEnt could facilitate learning and memory processes. To ensure the maximum production of new functional neurons, we stimulated animals' MEnt bilaterally. Rats were trained in the water maze 50 days after optogenetic stimulation. This period is an optimum time course for the maturation and functional integration of the newborn granular cells for the spatial learning (Kee et al., 2007; Kempermann et al., 2015). Two-way ANOVA with repeated measures indicated that there was a significant effect of time [$F_{(1.45, 14.53)} = 16.62$, $P < 0.001$] in the training, and the escape latency was decreased on day 3. However, there was no significant effect of the optical stimulation [$F_{(1,10)} = 0.537$, $P = 0.48$] and time×optical stimulation [$F_{(2,20)} = 0.246$, $P = 0.784$] (Fig. 8a). The swimming speed among the experimental groups was also not statistically significant in the training days (data not shown). Furthermore, un-paired *t*-test

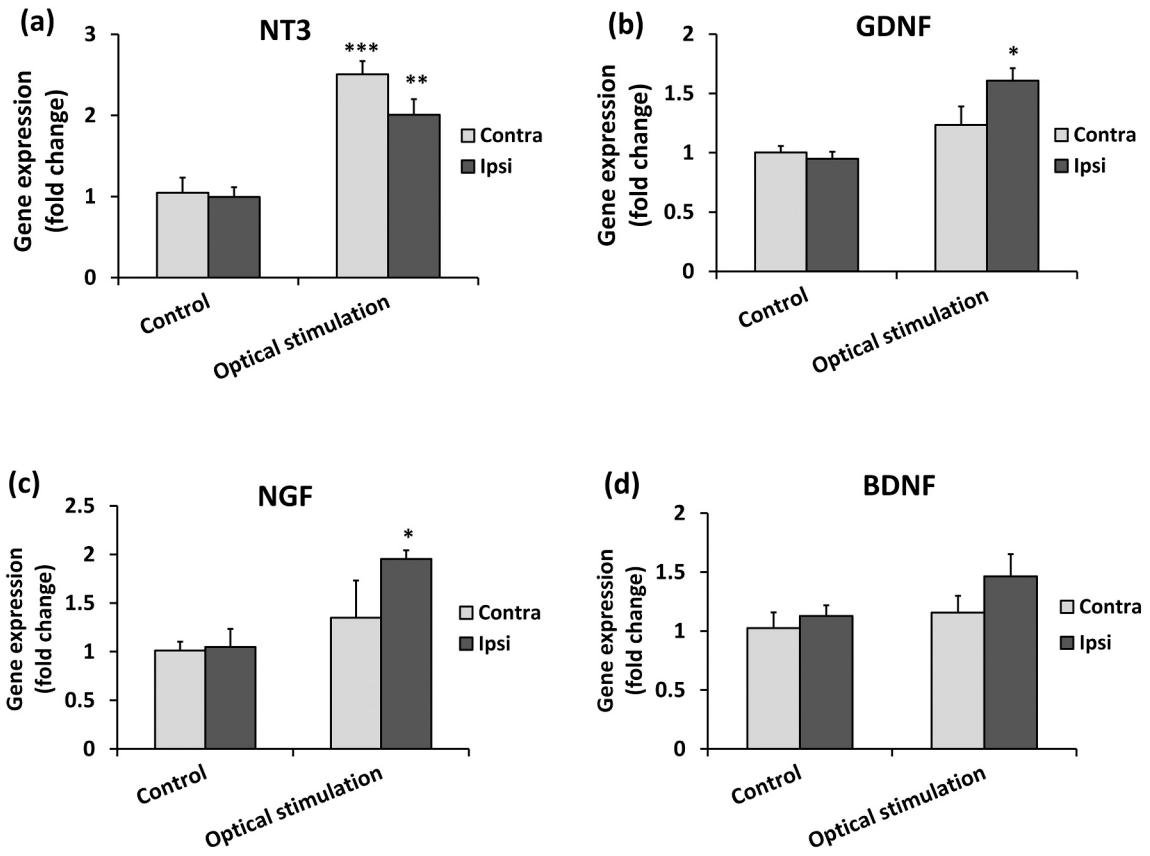

Fig. 5. The effect of unilateral optogenetic stimulation of MEnt on genes expression of neurotrophic factors in the hippocampus. The qPCR findings of NT3 (a), GDNF (b), NGF (c) and BDNF (d) in the ipsilateral (ipsi) and contralateral (contra) hippocampus. Data are reported as the means ± SEM ($n = 3$/group). One-way ANOVA followed by Tukey's post hoc test was used for statistical analysis. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. corresponding control group.

revealed that bilateral optogenetic stimulation had no effect on the time in target zone compared to the control [$t = 0.361$, df $= 10$, $P = 0.725$] (Fig. 8b).

4. Discussion

The entorhinal cortex provides the main afferent input to the hippocampus and is thought to play a critical role in the learning and spatial memory as well as the promotion of neurogenesis (Ronaghi et al., 2019; Stone et al., 2011; Yun et al., 2018). However, the precise role of neuronal networks is unknown yet. Optogenetics is a fundamentally new method that allows explaining the exact function of the neuronal circuits (Deisseroth, 2011; Delbeke et al., 2017; Erofeev et al., 2015). In the present study, our findings showed that optogenetic stimulation of neurons in the MEnt enhanced the firing rates of the CA1 pyramidal neurons in the hippocampus of the adult rats. Excitation of the CA1 neurons following optogenetic stimulation of the Ent region may have occurred through the monosynaptic or trisynaptic pathways (Basu and Siegelbaum, 2015; Silkis, 2011; Yau et al., 2015). In the monosynaptic pathway, the excitatory glutamatergic fibers arising from the Ent projects monosynaptically to the CA1 pyramidal neurons, while activation of the perforant path fibers in the trisynaptic pathway initiates a sequential excitation of the DG, CA3, and CA1 neurons (Basu and Siegelbaum, 2015; Yau et al., 2015). Moreover, it has been reported that excitatory transmission in three main synaptic stations of the hippocampus is mediated by the glutamate (Cammalleri et al., 2019).

The role of glutamate has been previously demonstrated in the survival, proliferation, migration, synapse formation, and integration of newly generated neurons in the hippocampus (Berg et al., 2013; Schlett, 2006; Vicini, 2008). Consistently, we found that optogenetic stimulation of the neurons in Ent as the major source of glutamatergic inputs to the DG, increased NSCs pool as well as their proliferation and differentiation in the ipsilateral hippocampus. It has been previously revealed that DBS of Ent improves spatial memory via enhancing the hippocampal neurogenesis (Ronaghi et al., 2019; Stone et al., 2011). Although neurogenic changes that we have observed, were less pronounced than DBS-induced neurogenic effects in our previous research (Ronaghi et al., 2019), this discrepancy may be attributed to the wide spectrum action of DBS including the anterograde effects, retrograde effects, and effects on non-neuronal cells which all can subsequently impact the hippocampal neurogenesis (Fenoy et al., 2014; Jakobs et al., 2019; McIntyre and Anderson, 2016; Vedam-Mai et al., 2012). In the present study, the ectopic neurogenesis increased in the hilus of the optical stimulated-rats. Hilar ectopic granule cells have been previously identified after the severe continuous seizures (Cho et al., 2015). However, many studies have shown that some non-pathogenic conditions such as intracerebroventricular administration of neurotrophic factors including vascular endothelial growth factor (Jin et al., 2002), BDNF (Scharfman et al., 2005), and insulin-like growth factor 1 (Lichtenwalner et al., 2001) can induce hilar ectopic granule cells.

Insulin, a key neuromodulator in the brain, has been recognized to influence the hippocampal neurogenesis and cognitive function (Spinelli et al., 2019; Taouis and Torres-Aleman, 2019). Some neurons have been shown to release insulin during depolarization (Wei et al., 1990). Interestingly, in parallel with neurogenesis, an increase in insulin mRNA level was detected in the ipsilateral hippocampus of the rats following the Ent optical stimulation. In our previous study, we also observed that insulin receptor antagonist administration to DG inhibited the DBS-induced neurogenesis in the rat hippocampus (Ronaghi et al., 2019). It is unclear whether insulin is released by presynaptic or postsynaptic

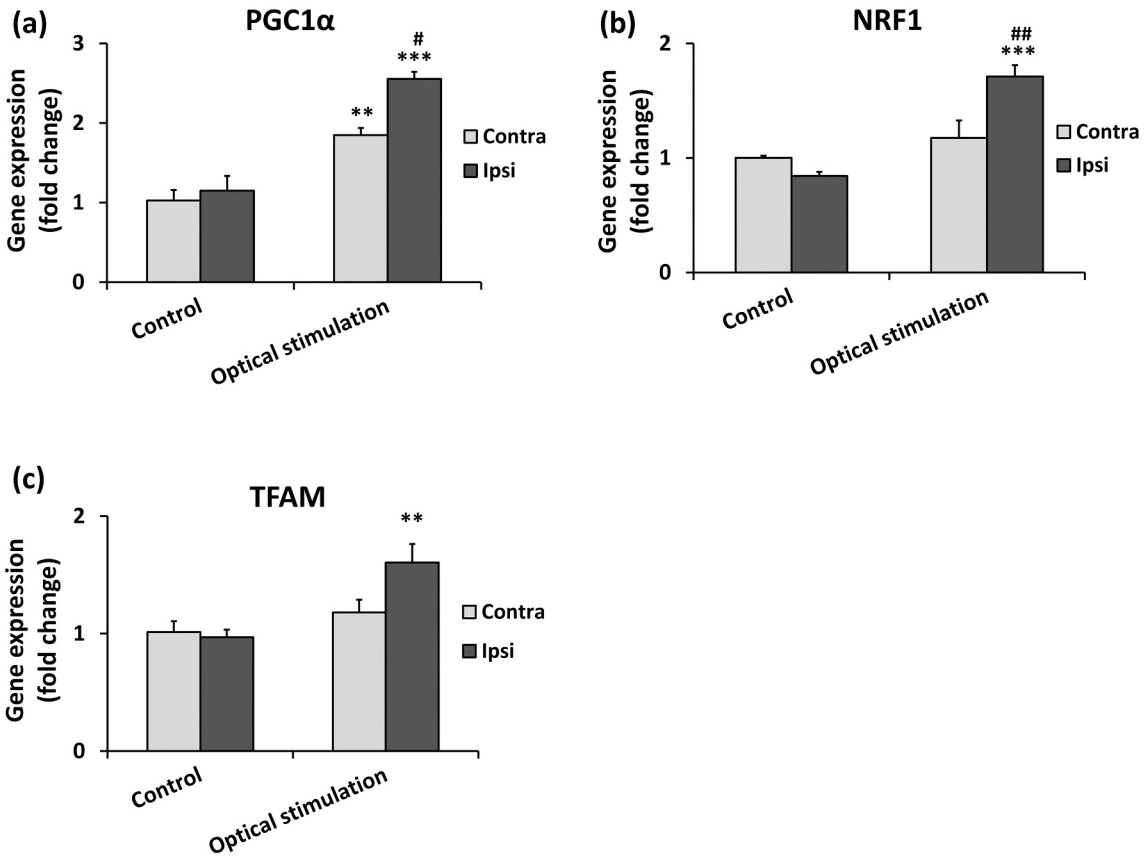

Fig. 6. The effect of unilateral optogenetic stimulation of MEnt on the expression of mitochondrial biogenesis-involved factors in hippocampus. The qPCR findings of PGC1α (a), NRF1 (b), and TFAM (c) in the ipsilateral (ipsi) and contralateral (contra) hippocampus. Data are reported as the means ± SEM ($n = 3$/group). One-way ANOVA followed by Tukey's post hoc test was used for statistical analysis. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. corresponding control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ between two sides in the optical stimulated group.

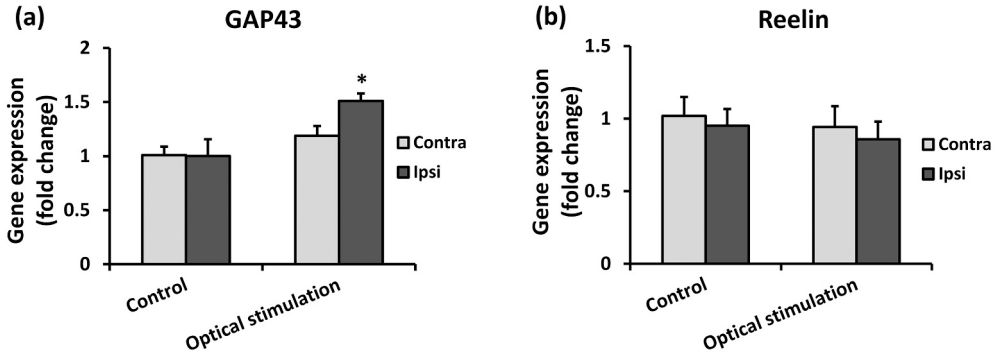

Fig. 7. The effect of unilateral optogenetic stimulation of MEnt on the expression of synaptic plasticity markers in hippocampus. The qPCR data of GAP43 (a) and Reelin (b) in the ipsilateral (ipsi) and contralateral (contra) hippocampus. Data are reported as the means ± SEM ($n = 3$/group). One-way ANOVA followed by Tukey's post hoc test was used for statistical analysis. $^{*}P < 0.05$ corresponding control group.

neurons and it still needs further investigations. Remarkably, we found that the expression of IRs, as well as downstream components of PI3K/AKT/mTOR signaling was increased in the rat hippocampus in response to the Ent optical stimulation. Consistently, a high expression of IRs and its signaling components was discovered in the rat hippocampus following DBS of the Ent region in our previous study (Ronaghi et al., 2019). Elevation of IRs, in spite of high insulin levels in this study, is an observation that can be described by functional and structural properties of IRs in the CNS. While peripheral IRs are downregulated in response to high insulin concentration, their counterparts in the brain do not show such down regulation (Ghasemi et al., 2013). Effects of IRs and its downstream signaling pathways on the proliferation and differentiation of NSCs have been previously reported in several studies (Chirivella et al., 2017; Han et al., 2008; LiCausi and Hartman, 2018; Meng et al., 2018; Rhee et al., 2013; Ronaghi et al., 2019; Ryskalin et al., 2017; Schubert et al., 2003; Vogel, 2013). Wozniak et al. have indicated that the number of insulin receptors increases during cell proliferation and differentiation in the developing brain (Wozniak et al., 1993). Thus, in the present study, high expression levels of IRs may be associated with increased neurogenesis induced by the optogenetic stimulation of the glutamatergic neurons in the Ent. These findings open up a new window for treating neurodegenerative disorders, specifically Alzheimer's

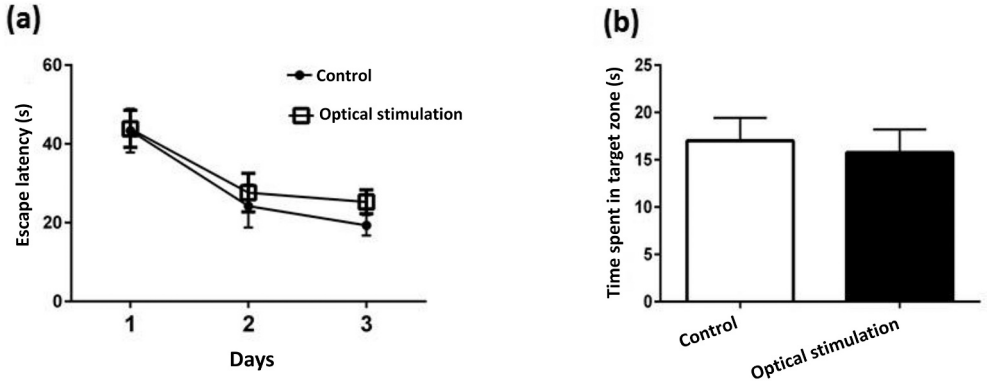

Fig. 8. The effect of bilateral optogenetic stimulation of MEnt on spatial learning and memory in MWM test. Escape latency over three training days (a) and time spent in the target quadrant (b). Data are reported as the means ± SEM ($n = 6$/group). Two-way ANOVA with repeated measures was used for the statistical analysis in the training days and un-paired t-test in the probe session.

disease (AD). The insulin signaling and neurogenesis defects as well as degeneration of the perforant pathway projections have been previously linked to the pathogenesis of AD (Pugazhenthi et al., 2017; Young, 2020). So, optogenetic stimulation of the Ent can be a promising therapeutic approach for improvement of cognitive deficits in AD.

It has been demonstrated that trophic factors and mitochondrial metabolism regulate neurogenesis process in the adult brain (Beckervordersandforth, 2017; Vilar and Mira, 2016). Here, we showed that optogenetic stimulation of the perforant pathway upregulates the expression of neurotrophic factors and components involved in the mitochondrial biogenesis in the rat hippocampus. Although further investigations are required to clarify the mechanisms implicated in this effect, trophic effect of glutamate has been previously indicated (Balazs, 2006). Glutamate receptors activate CaMKII which initiates several signaling cascades including Ras-mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) and PI3K-AKT. The consecutive activation of protein kinase signaling pathways induces the expression of trophic factors through phosphorylation of several transcription factors such as cAMP-response element binding protein (CREB), serum response factor (SRF), and myocyte enhancer factor 2 (MEF-2) (Balazs, 2006). Consistent with this study, we identified an increase in the expression of NT3, GDNF, and NGF in the rat hippocampus following Ent optogenetic stimulation. On the other hand, Activation of MAPK signaling increases PGC-1α gene expression and its activity (Akimoto et al., 2005; Jäger et al., 2007; Jung and Kim, 2014; Knutti et al., 2001; Puigserver et al., 2001). PGC-1α acts as a main regulator of mitochondrial biogenesis by integrating the activity of several transcription factors such as NRF and TFAM (Onyango et al., 2010). Interestingly, we found that optogenetic stimulation of the glutamatergic neurons in Ent induces mitochondrial biogenesis in the rat hippocampus manifested by an increase in the mRNA levels of PGC-1α, NRF, and TFAM.

Moreover, we investigated the changes in the hippocampal synaptic plasticity after optogenetic stimulation of the perforant pathway. Interestingly, an increase in GAP43 mRNA expression, a determinant of neuronal development and plasticity (Holahan, 2015), was detected in the rat hippocampus. Console-Bram et al., have revealed that glutamate upregulates GAP43 mRNA in the cultured cerebellar granule cells (Console-Bram et al., 1998). Additionally, activation of PI3K-AKT-S6K pathway has been shown to increase GAP43 expression in the dorsal root ganglion neurons (Liu et al., 2012). Therefore, increased GAP43 expression in the present study may be induced by the glutamate and/or insulin signaling. This is interestingly noticeable that unilateral optogenetic stimulation of the perforant pathway triggered molecular and histological changes in the hippocampus bilaterally, a phenomenon which has been reported in the electrical stimulation of Ent in rats (Ronaghi et al., 2019). The anatomical connection of Ent with contralateral DG has been previously indicated in rats (van Groen et al., 2002).

In spite of the fact that optogenetic stimulation promoted molecular mechanisms involved in the learning and memory formation in the hippocampus, we found that bilateral optogenetic stimulation of Ent did not facilitate spatial memory formation. Our findings are in contrast to previous works, which suggest pro-cognitive effects of electrical stimulation of Ent (Ronaghi et al., 2019; Stone et al., 2011). High-frequency DBS stimulates additional neural circuits which in turn affect memory directly or indirectly through the influence of neurogenesis or insulin release. The function of other neurotransmitters released from Ent projections including parvalbumin, somatostatin, neuropeptide Y, and substance P on the spatial memory has been previously shown in several studies (Abbas et al., 2018; Artinian et al., 2019; Gøtzsche and Woldbye, 2016; Murray et al., 2011). In our study, it is likely that optogenetic stimulation of perforant pathway at frequency of 10 Hz for 5 days is not sufficient for translation of molecular changes to behavioral changes and it may require higher frequencies or longer duration of optostimulation to enhance the spatial learning and memory.

5. Conclusions

Our findings showed that optogenetic stimulation of MEnt promotes hippocampal neurogenesis and synaptic plasticity concomitant with the increased levels of insulin mRNA, insulin signaling pathway, neurotrophic factors and mitochondrial biogenesis. These findings reveal that viral mediated opsin expression and optical stimulation of the Ent provides an appropriate platform to study hippocampal neurogenesis and also answer our key question that de novo synthesis of insulin as a neuromodulator is implicated in the adult brain neurogenesis.

Author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Progress in Neuropsychopharmacology & Biological Psychiatry*.

Authorship contributions

Conceptualization: L. Dargahi, M.I. Zibaii, F. Motamedi, A. Haghparast. Funding acquisition and supervision: L. Dargahi, M.I. Zibaii, F. Motamedi. Acquisition of data: S. Chavoshinezhad, A. Ronaghi, M.H.

Seyed Nazari, A. Asgari Taei, A. Ghorbani, S. Pandamooz, M.S. Salehi, N. Vailan. Analysis and interpretation of data: S. Chavoshinezhad, A. Ronaghi, L. Dargahi, M.I. Zibaii, A. Haghparast. Drafting the manuscript: S. Chavoshinezhad, S. Pandamooz, L. Dargahi, M.I. Zibaii. Revision and approval of the final version: All authors.

Declarations of interest

None.

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