Optogenetic Modulation of TrkB Signaling in the Mouse Brain

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Edited by Kai Zhang

Abstract

Optogenetic activation of receptors has advantages compared with chemical or ligand treatment because of its high spatial and temporal precision. Especially in the brain, the use of a genetically encoded light-tunable receptor is superior to direct infusion or systemic drug treatment. We applied light-activatable TrkB receptors in the mouse brain with reduced basal activity by incorporating Cry2PHR mutant, Opto-cytTrkB(E281A). Upon AAV mediated gene delivery, this form was expressed at sufficient levels in the mouse hippocampus (HPC) and medial entorhinal cortex (MEC) retaining normal canonical signal transduction by the blue light stimulus, even by delivery of noninvasive LED light on the mouse head. Within target cells, where its expression was driven by a cell type-specific promoter, Opto-cytTrkB(E281A)-mediated TrkB signaling could be controlled by adjusting light-stimulating conditions. We further demonstrated that Opto-cytTrkB(E281A) could locally induce TrkB signaling in axon terminals in the MEC-HPC. In summary, Opto-cytTrkB(E281A) will be useful for elucidating time- and region-specific roles of TrkB signaling ranging from cellular function to neural circuit mechanisms.

Introduction

Receptor tyrosine kinases (RTKs) represent a highly conserved signal transduction system that responds to extracellular stimuli, such as growth factors and hormones. Typically, ligand binding to the extracellular domain of RTKs induces autophosphorylation of tyrosine kinase (TK) domains, which leads to activation of downstream signaling molecules [1]. One subfamily of the RTK family is the tropomyosin-related kinases (Trks). There are three Trk subtypes, TrkA, B, and C, which are activated by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), respectively. Binding of neurotrophins to their major receptor is followed by phosphorylation of TK domains, which activates canonical signaling via the mitogen-activated protein kinase kinase (MEKK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt, and phospholipase Cγ1 (PLCγ1)/Ca²⁺/ pathway [2].

TrkB is mainly expressed in neurons and a small subpopulation of glial cells [3]. Its major ligand, BDNF, impacts various neuronal functions, such as the development of young neurons, addiction, synaptic plasticity, and learning and memory [4–6]. The BDNF/TrkB signaling activation matters, with BDNF/TrkB signaling playing a critical role in late-phase LTP (L-LTP); this implies that neuronal communication flows not just through neural circuits but also via receptor-ligand-based delivery of information [7]. By using two-photon fluorescence lifetime imaging microscopy, a recent study addressed this more directly, providing visual evidence that TrkB activation and autocrine BDNF signaling induce rapid structural long-term potentiation...
(sLTP) via a single dendritic spine [8]. Moreover, a study using Bdnf- and Ntrk2-knockout rodent models also demonstrated a role for presynaptic and postsynaptic BDNF/TrkB signaling in LTP induction and maintenance [9]. Temporal aspects of BDNF/TrkB signaling also contribute to differences in cellular changes. For example, Ji et al. demonstrated that acute and gradual changes in BDNF concentration induce different changes in signaling and neuronal plasticity in cultured neurons and acute hippocampal slices [10]. Also, an investigation of time points for BDNF/TrkB signaling, which is critical in synaptic plasticity and TrkB-mediated LTP consolidation, suggested that the timing of TrkB signals linking neuronal plasticity to behavior is distinct at presynaptic and postsynaptic sites [11].

Until now, studies of TrkB signaling have relied on genetic-based approaches, such as genetically mutated models [12–18], truncated forms of TrkB (TrkB-T1) [19] and overexpression of a TrkB trafficking regulator [20]. Methods for activating or de-activating endogenous TrkB receptors using specific agonists, antagonists, or inhibitors are also routinely used [21,22]. However, these approaches are limited in that they cannot be regulated within specific regions (spatial) or activated or de-activated at a certain time point (temporal). In other words, the infusion of chemicals or ligands into a target brain region is not guaranteed to direct them to the desired duration or contain the spread within expected target areas. Moreover, it is not possible to prevent it from indiscriminately affecting different types of cells, which also express the Trk receptor, as do other brain cells, such as microglia [23]. Although numerous studies have demonstrated a role for BDNF/TrkB signaling in the brain, the lack of a tool for activating BDNF/TrkB signaling in a spatiotemporal manner has hindered in vivo studies of the role of BDNF/TrkB signaling. Thus, there is a need for greater confidence in the specificity of BDNF/TrkB signal activation.

With the advent of optogenetics, the trend in neuroscience studies is toward direct stimulation or inhibition of specific regions and cell populations in the brain [24,25]. Additionally, photosensitive proteins are also now being used for modulating cell signaling, as well as altering neural plasticity for behavior change [26–28]. The photolyase homology region (PHR) domain of cryptochrome2 protein from Arabidopsis thaliana is one of the most widely used phototropins found in optogenetic modules, which induce or inhibit molecular functions in mammalian cells [29–35]. For instance, OptoTrkB, in which the PHR domain is conjugated to the C-terminus of TrkB protein, induced the formation of filopodia in cultured neurons when activated by blue light [31]. Moreover, taking advantage of optogenetic TrkB activation our group demonstrated that axon specification of a cultured young neuron is dependent on TrkB signaling [36]. Thus, OptoTrkB could also be considered suitable tools for neuroscience investigations in vivo level.

Nevertheless, certain hurdles must be overcome if OptoTrkB for modulating signal transduction in the brain is to be effective. The first is achieving broad expression of targeted brain regions via adenovirus-associated virus (AAV)-mediated gene transduction. Second, it is necessary to show that the tool is versatile in vivo without causing perturbations in endogenous signaling. Finally, spatiotemporal control is required for the activation of signaling in specific cell types and regions, such as dendritic spines and axon terminals.

We expected that optogenetic TrkB activation under the AAV-mediated expression in the hippocampus would induce target cell-specific signal transduction. Also, we anticipated that stimulating time and axon-specific induction of TrkB signaling would be controlled by adjusting blue light illumination time and regions, which is in contrast with direct BDNF infusion in the hippocampus. Here, we demonstrated transient or sustainable canonical signal activation via optogenetic control of the TrkB receptor in the hippocampal dentate gyrus (DG). This tool exhibited less basal activity under the no-light stimulating condition by conjugating the PHR mutant. Also, it showed distinct signal activation/deactivation properties compared with ligand infusion in that only the optogenetic method promptly regulated signal turn ON and OFF. Finally, we showed that optogenetic activation of the TrkB receptor could be accomplished exclusively in regions of interest, including cell types and terminals projecting directly from the medial enthorinal cortex (MEC) to the hippocampus (HPC).

**Results**

**AAV-mediated optogenetic TrkB receptor activation in the mouse hippocampus**

To accommodate the AAV packaging limit (~4.9 kb) and prevent its activation by endogenous ligands, we recruited the cytoplasmic domain of rat TrkB (cytTrkB; amino acids 454–821) instead of full-length TrkB. A myristoylation sequence (Lyn signal peptide) was added at the N-terminus of cytTrkB to promote localization of cytTrkB to the plasma membrane, and a Cry2PHR domain was conjugated to the end of cytTrkB. Finally, an HA-tag sequence was added to the C-terminus to facilitate the detection of its expression in immunohistochemistry (Fig. 1A). Because genes transduced via AAV-mediated delivery require much longer to achieve full expression (~2–3 weeks) than genes transiently expressed in cultured neurons, it is necessary that
Fig. 1. Optogenetic TrkB activation in mouse hippocampal DG GCs. (A) Upper images: Structure of the AAVJ8-CaMKIIa::Opto-cytTrkB(E281A)-HA construct. Lower images: Virus was injected into the DG, after which 3 weeks were allowed for recovery from surgery and vector expression. (B) Injected mouse brains were sectioned and immunostained for brain cell-specific markers. White arrows show GAD67-, Iba1- and GFAP-positive cell bodies. (C) Upper image: AAV injection with implantation of a fiber optic cannula on the DG and induction of pERK1/2 in DG GCs by 473-nm blue laser light stimulation. Lower images: Activation of Opto-cytTrkB(E281A)-HA by light stimulation. (D) Light sensitivity of Opto-cytTrkB(E281A)-HA. Light was administered at intensities of 50, 100, 500 μW/mm², and 1 mW/mm², and the percentage of pERK1/2-positive neurons was calculated as described in Methods. Data are presented as means ± s.e.m. (error bars); $F = 0.2957$, $p = 0.8279$ versus the light-stimulated Opto-cytTrkB(E281A)-HA group (one-way ANOVA). n.s., no significant difference. The number of filled circles corresponds to the number of mice used in the experiment. Slice images were obtained using 20× and 60× lenses. All scale bars: 50 μm.
optogenetic tools show reduced basal activity in the brain. Our group recently developed a Cry2PHR mutant, Cry2PHR(E281A), by structure prediction and showed that it exhibits reduced basal activity (spontaneous activity without light stimulation) upon linking to the target protein [53]. To evaluate the basal activity, we infected hippocampal excitatory neurons with AAVs expressing Cry2PHR (Opto-cytTrkB-HA), the Cry2PHR(E281A) mutant (Opto-cytTrkB(E281A)-HA), or virus without the cytTrkB domain (Lyn-Cry2PHR(E281A)-HA). Three weeks after injection, we measured the level of phosphorylated ERK1/2 (pERK1/2) in hippocampal lysates in the absence of light stimulation by Western blotting (Supplementary Fig. 1). Mice expressing Opto-cytTrkB containing Cry2PHR showed an increase in basal pERK1/2 compared with Lyn-Cry2PHR(E281A)-HA, but no significant change in basal pERK1/2 was detected in mice expressing Opto-cytTrkB containing the E281A mutant. We next sought to adjust the AAV injection titer so as to yield moderate virus expression in the mouse brain. To this end, mice were injected in the hippocampus with 0.3–0.4 μl of Opto-CytTrkB(E281A)-HA at doses of 2 × 10^{10} or 2 × 10^{11} genome copies (GC)/ml and sacrificed 3 weeks after injection (Supplementary Fig. 2). No HA-tag signal was detected following injection of 2 × 10^{10} GC/ml, indicating that this titer is too low to express an adjustable amount of Opto-cytTrkB(E281A)-HA. In contrast, the HA-tag was detectable following injection of 2 × 10^{11} GC/ml in the absence of elevated basal pERK1/2 or proBDNF synthesis compared with naïve mice. Therefore, AAV-mediated delivery of Opto-cytTrkB(E281A)-HA at a dose of 2 × 10^{11} GC/ml in the mouse hippocampus produced appropriate expression levels without changing basal pERK1/2 levels.

We next sought to attain promoter-specific expression and activation of Opto-cytTrkB(E281A)-HA in the hippocampal DG. To this end, 0.3–0.4 μl of the Opto-cytTrkB(E281A)-HA viral construct under the control of the CaMKIIα(0.4) promoter was injected into the DG. After 3 weeks from the surgery, Immunohistochemical experiments were performed. The images showed that Opto-cytTrkB(E281A)-HA was expressed in DG granule cells (GCs) (Fig. 1A). To determine whether the construct was expressed in other cell types in the DG, hippocampal sections were immunostained for CaMKII (calcium/calmodulin-dependent protein kinase II), GAD67 (glutamate decarboxylase 67 kD), Iba1 (ionized calcium-binding adaptor molecule 1) and GFAP (glial fibrillary acidic protein)—specific markers for excitatory neurons, GABAergic neurons, microglia, and astrocytes, respectively. HA-tag signals were colocalized with CaMKII-positive DG GCs, but not with other cell-specific markers (Fig. 1B). To verify the potential for activating TrkB signaling in DG GCs with blue light stimulation, we implanted a cannula optic fiber (200 μm diameter) into the outer molecular layer (DV: 1.5 mm) followed by stimulation with a 473-nm laser. Because continuous, prolonged light stimulation with strong intensity light has the potential to cause phototoxicity [37], we utilized a pulse-type light stimulation strategy rather than a continuous stimulation paradigm. Stimulation with a 1 mW/mm^2 laser for 1 s per 5 s pulse was sufficient to activate the MAPK/ERK pathway in DG GCs (Fig. 1C). Next, we tested light stimulation at powers less than 1 mW/mm^2. Interestingly, Opto-cytTrkB(E281A)-HA could be activated at powers as low as 50 μW/mm^2 with no noticeable change in the percentage of pERK1/2-positive neurons (Fig. 1D). No induction of pERK1/2 was observed in mice injected with Lyn-PHR(E281A)-HA virus and stimulated with 1 mW/mm^2 light, indicating that pERK1/2 induction was not caused by light stimulation itself. Given that this construct could be activated by very low light intensity, we speculated that it might be activated by noninvasive blue light stimulation delivered by an LED light source (cage lid light stimulation) instead of requiring an implanted optic fiber (Fig. 2A) [38]. Indeed, we found that noninvasive blue light (~1 mW/mm^2), delivered continuously for 0.5 h, was sufficient to induce pERK1/2 with a slight increment of mBDNF (Fig. 2B and C). In contrast, mice exposed to room light from a white fluorescence lamp showed no significant pERK1/2 signal. These results demonstrate that Opto-cytTrkB(E281A)-HA can be expressed in a cell type-specific manner and is sufficient to activate canonical TrkB signaling, even at a low light intensity.

**Optogenetic modulation of TrkB signaling**

The Cry2PHR-based optogenetic tool does not have a fast turn OFF feature (~15min for dissociation from dimer or oligomer of Cry2PHR) [39]. Also, its characteristics, as shown Fig. 1D, activating Opto-cytTrkB(E281A)-HA cannot be adjustable with manipulating light intensity. Thus, a new strategy for signal turn ON and OFF is needed for the PHR based RTKs systems. To overcome this limitation and establish new signal-modulating conditions, we tested various light-pulse types and ultimately selected two types of pulses for transient and sustained signal transduction (Supplementary Fig. 3). For sustained signal transduction, we selected a condition of 1 s pulse per 5 s continuous pulse-type. It was expected that light stimulation for 1 s would be sufficient for transient signaling induction. However, we found that light stimulation for 30 s was actually better for activating TrkB signaling in DG GCs. Next, we tested whether these two types of simulating conditions can drive transient or sustained TrkB signaling (Fig. 3A). Transient pERK1/2 induction was detected at 0.5 h after a 30-
s stimulation and decreased over time (Fig. 3B). In contrast, continuous pulse-type stimulation drove sustained pERK1/2 induction for over 4 h. To verify prolonged activation of TrkB signaling and test deactivating times under transient-stimulation conditions, we set variable stimulation durations of 0–9 h. Continuous pulse-type stimulation was sufficient to induce pERK1/2, even after 9 h (Fig. 3C). The deactivating time point for pERK1/2 induction by 30-s blue light stimulation was between 1 and 2 h. We also assessed the phosphorylation of S6 ribosomal protein (pS6), a target of the PI3K/AKT pathway downstream of TrkB signal activation (Supplementary Fig. 4). As expected, pS6 activation could be modulated in a transient or sustained manner using our light pulse strategy. We then monitored the expression of the immediate early gene, activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) (Supplementary Fig. 5), which plays an essential role in the learning and memory process by regulating AMPAR trafficking in dendritic spines [40]. Interestingly, newly expressed Arc was detected beginning after 0.5 h and peaked at 2 h. Moreover, although the percentage of Arc-positive neurons in DG GCs differed between transient and continuous pulse-stimulation conditions, expression, and degradation time points were similar in both conditions, with very weak expression detected after 4 h.

Optogenetic activation of receptors offers the critical advantage of spatiotemporal activation and deactivation of signal transduction compared to treatment with a ligand, which cannot be withdrawn (deactivated) in vivo. To reinforce this concept, we examined TrkB signaling following treatment with exogenous TrkB ligand. For these experiments, a guide cannula was implanted into the DG, and after allowing mice 3 weeks to recover, human recombinant BDNF (hrBDNF) was infused via the implanted cannula. Infusion of hrBDNF induced pERK1/2 within 0.5 h, an effect that was maintained over 4 h (Fig. 3E). To verify that TrkB signaling induced by hrBDNF infusion is sustained, we sacrificed animals at different time points (0.5–9 h) and assessed pERK1/2 levels. Even 9 h after hrBDNF infusion, strong pERK1/2 signals were detected in hippocampal DG GCs (Fig. 3F). No significant pERK1/2 induction was detected in saline (PBS)-treated
Fig. 3. Modulation of TrkB signaling in the DG by blue light stimulation and BDNF infusion. (A) Schema showing light-stimulation protocols for transient and sustained signal transduction using the optogenetic TrkB receptor. A 30-s light pulse (transient) or a continuous series of 1 s pulses every 5 s (sustained) was given, and mice sacrificed after 0.5, 1, 2, 4, or 6 h. Continuous light stimulation was additionally performed up to 9 h. Sham light (injection with no light stimulation) was given to a group of mice in the dark. (B, C) Brains that had been stimulated by a transient pulse, continuous series of
groups at 0.5 or and 2 h after infusion. These results demonstrate that transient or sustained TrkB signaling can be generated by the selection of the appropriate light pulse strategy, which is advantageous compared with direct ligand infusion in the brain.

**Induction of axonal TrkB signaling using Opto-cytTrkB**

Signal activation using the direct ligand-treatment method has limited utility in the brain because a given brain region contains not only neuronal cell bodies but also has other types of cells and projecting axon terminals from different regions. Thus, an exogenously applied ligand cannot be directed to activate only target cells in the infused brain regions. However, the genetic expression of photoactivatable receptors driven by the appropriate promoter can achieve high-resolution neuronal expression, selectively activating proximal (soma, dendrites) or distal (axons) receptors in neurons. To test terminal-specific activation, we constructed an AAV virus-containing reversed Opto-cytTrkB(E281A)-HA under a double-inverted open reading frame (DIO) containing the CaMKIIα(0.4) promoter (AAV-CaMKIIα::DIO-Opto-cytTrkB(E281A)-HA) and a retrograde Cre virus (AAV2-retro-hSyn1::Cre) [41] (Fig. 4A). AAV2-retro-Cre and AAV-DIO-Opto-cytTrkB(E281A)-HA were injected into the hippocampal lacunosum layer with the molecular layer of the DG and MEC, respectively. Three weeks after injection, Opto-cytTrkB(E281A)-HA was mainly expressed in neurons in layers 2 and 3 of the MEC, and their axons were detected in the hippocampus (Fig. 4B). Notably, mice in which the HPC was illuminated with a blue light at 500 μW/mm² for 0.5 h showed increased pS6 levels in the MEC (Fig. 4C and D) compared with the unstimulated group (normal room light). However, no significant pERK1/2 and pS6 signals were detected from neuronal somas in HPC, where the light was delivered for TrkB signaling activation of the MEC terminal (Supplementary Fig. 6). To validate that hrBDNF infusion in the hippocampus can induce signal activation at distal regions where neurons project to the hippocampus, we infused hrBDNF or saline 1 day after infusion of CTB-488 used to retrogradely label neurons (Fig. 4E and Supplementary Fig. 7A). Mice were sacrificed 0.5 h after the infusion and brain sections were stained by pS6. The images and quantified data showed that hrBDNF infusion in the HPC not only induced S6 phosphorylation in the hippocampal neurons (Supplementary Fig. 7B), it also induced S6 phosphorylation in MEC neurons that project to the HPC (Fig. 4F and G), indicating that infusion of ligand into the target brain region activates downstream signaling in projecting neurons, as well as cells at the infusion site. In conclusion, light stimulation was capable of inducing TrkB signaling in axon terminals where local cells and projecting axon terminals co-exist.

**Discussion**

Receptor/ligand-mediated signal transduction is the prototypical process by which cells respond to ever-changing extracellular conditions. In the brain, variations in the extracellular environment drive communication between cells and result in alterations in cellular functions, which manifest not only in the form of changes in neural circuits through ion channel-induced neural activation, but also internal changes in signal transduction that influence outcomes with respect to morphology, development, plasticity, and even behavior [42–44].

Unlike TrkB activation strategies based on direct infusion or systemic treatment, Opto-cytTrkB offers a number of advantages in vivo: (1) it has a simple ON-OFF mechanism, (2) it does not require a ligand or chemical agonist (e.g., BDNF, 7,8-DHF) for activation, and in fact is immune to the effects of endogenous ligands owing to depletion of the extracellular domain, (3) it allows activation of specific axon terminals of target neurons, and (4) an optogenetic rescue model can be generated from genetically mutated models. In this study, we demonstrated the feasibility of the light-controllable TrkB receptor, Opto-cytTrkB(E281A)-HA, showing that it can be adapted to the study of spatial and temporal aspects of TrkB signaling. One of the important features of this tool is the incorporation of the Cry2PHR mutant Cry2PHR(E281A), which reduces basal activity (measured as pERK1/2 levels) in the absence of blue light stimulation. Moreover, we found a viral titer range (2–4 × 10⁻¹¹ GC/ml) that showed proper expression levels in
Fig. 4. MEC-HPC axon activation of TrkB signaling by light stimulation and BDNF infusion. (A) Upper images: Schematic illustration of the AAV constructs, AAV9-CaMKIIα::DIO-Opto-cytTrkB(E281A)-HA and AAV2-retro-hSyn1::Cre. Lower images: Illustration of injection sites for labeling neurons projecting to the hippocampus (HPC) from the MEC, showing injection of AAV9-CaMKIIα::DIO-Opto-cytTrkB(E281A)-HA into the MEC and AAV2-retro-hSyn1::Cre into the ipsilateral HPC; implanted fiber-optic cannula is also shown. (B) Virus expression in MEC-HPC neurons. Images were obtained from coronal and sagittal sections of the HPC and MEC. (C, D) Blue light was delivered 1 s every 5 s for 0.5 h into the ferrule implanted on the HPC. Retrograde TrkB activity in the somas of MEC neurons was quantified by immunostaining sections for pS6. The percentage of pS6-positive neurons was calculated as described in Methods. Data are presented as means ± s.e.m. (error bars); ****p < 0.0001 (two-tailed t-test). (E) CTB-488 injected HPC (left) and retrograde-traced neurons (right). (F) MEC slices from hrBDNF- or saline (PBS)-injected mice were immunostained for pS6. (G) Quantification of pS6-positive cells in hrBDNF- or saline-mice. The analysis is the same as that in (D). Data are presented as means ± s.e.m. (error bars); *p = 0.0296 (two-tailed t-test). The number of filled circles in (D) and (G) corresponds to the number of mice used in the experiments. Slice images were obtained using 20× and 60× lenses. Scale bars: 200 μm (B and E) and 50 μm (C and F).
the mouse hippocampus without causing increases in basal pERK1/2. We further found that overexpression of the cytosolic TK domain by Opto-cytTrkB(E281A)-HA caused no remarkable changes in proBDNF levels compared with control (PBS-injected) mice, confirming that Opto-cytTrkB(E281A)-HA has no adverse effects in neurons [45]. An ancillary benefit of this system is that Opto-cytTrkB(E281A)-HA is activatable by non-invasive light stimulation from an LED attached under the home cage lid, a sensitivity that was anticipated based on our previous studies [38,53] showing that very weak intensity light (50 μW/mm²) was sufficient to activate Opto-cytTrkB(E281A)-HA. Although shown activation in low-light power, it has a possibility that 50 μW/mm² was not yet weak enough for adjusting Opto-cytTrkB(E281A)-HA action by light power. Also, there would be the chance that upstream of pERK1/2, the signal pathway becomes decreased through manipulating light power without affecting phosphorylation of ERK1/2. For clarifying, further experiment using intenimetric sensors for GTPase families is needed with a real-time stimulation of Opto-cytTrkB(E281A)-HA in the mouse brain [46]. There was also no concern about the potential effects of rearing mice under room light conditions, given that we found no significant induction of pERK1/2 under these conditions. Collectively, these observations support the conclusion that Opto-cytTrkB(E281A)-HA is useful for noninvasive activation of TrkB signaling.

An advantage of optogenetics for in vivo applications is the simplicity of its signal turn-ON/tum-OFF mechanism; this contrasts with the application of exogenous ligand, which cannot be withdrawn. This facile ON/OFF control also allows prolonged signal transduction to be induced by continuous light stimulation. This was verified by injecting Opto-cytTrkB(E281A)-HA virus in the DG followed by stimulation with light, which induced pERK1/2 and pS6, indicative of continuous signal transduction. Induction of the immediate-early gene Arc was also detected. Arc expression was maximal at ~2 h after light stimulation, but was decreased and significantly degraded after 4 h. This result is consistent with previous studies showing that Arc expression decreases gradually after 4 h in the DG owing to negative feedback regulation of Arc mRNA by the Arc protein itself [47,48]. It is also in accord with the fact that there is a critical time window in BDNF/TrkB signaling-related LTP induction in the perforant path that requires Arc [47]. By comparison, a 30s light stimulation was sufficient to induce canonical TrkB signaling that deactivated over time. In contrast, infused BDNF continued to induce signaling, even after the passage of a substantial amount of time. pERK1/2 induction was detected 4 h after infusion of 0.25 μg of hrBDNF into the hippocampus, a time when optogenetically induced transient signaling had become deactivated and continued to be observed for at least 9 h after infusion. This result indicates that infusing hrBDNF is not a suitable method for elucidating short-term specific roles of BDNF/TrkB signaling. Thus, studying the role of TrkB signaling in the brain requires the use of optogenetic receptor activation with high time resolution when ligand treatment is incapable.

Finally, we tested whether Opto-cytTrkB(E281A)-HA can be activated at axon terminals, not in the local area, but long-range projections like those of the medial entorhinal cortex-hippocampus (MEC-HPC) circuit. For expression and labeling exclusively in neurons that project to the HPC, retrograde Cre virus was injected in the HPC and DIO-Opto-cytTrkB(E281A)-HA was injected in the ipsilateral MEC. Because of differences in MAPK/ERK activation by TrkB signaling between the axon terminal and soma [49], (TrkB activation at axon terminals induces ERK5), activation of TrkB signaling could be evaluated by staining for pS6 in MEC sections. pS6 was significantly induced in mice in which the HPC was stimulated by blue light, indicating that light stimulation of axon terminals expressing Opto-cytTrkB(E281A)-HA selectively and effectively induces retrograde TrkB signaling in MEC-HPC projecting neurons. In contrast, the infusion of BDNF in the HPC activated both hippocampal and MEC neurons.

The endogenous TrkB receptor is prevalent on the plasma membrane of neurons, where it plays distinct roles depending on its subcellular location [50,51]; it is also expressed in nonneuronal cells in the brain, highlighting the importance of controlling cell-type-specific activation at distinct target regions in the brain. The optogenetic TrkB activation system described here provides a tool for brain research that allows transient or sustained activation of TrkB-mediated signaling in a cell type- and region-specific manner.

Materials and methods

Vector construction

The pAAV-CK(0.4) GW vector (Addgene ID: 27226) and pAAV-Ef1a-DIO-EYFP vector (Addgene ID: 27056) were used for the construction of the main viral vectors. The CMV::Lyn-cytTrkB-Cry2PHR-mCitrine construct was digested with the restriction enzymes AgeI and NotI to eliminate mCitrine [36]. This fluorescent protein sequence was replaced with an HA-tag, incorporating AgeI and NotI sites at each end of the sequence, 5'-ACCGGTATATCCATATGATGTCCAGATTATGCTTAAAGCGGCCGC-3'. This construct was used as a template for polymerase chain reactions (PCR) in AAV vector construction. The PCR-amplified Lyn-cytTrkB-Cry2PHR-HA sequence was
cloned into pAAV-CaMKII(0.4)GW at KpnI and EcoRI sites by Gibson assembly. The Cry2PHR(E281A) mutant was generated by site-directed mutagenesis. The resulting construct, pAAV-CaMKII(0.4)::Lyn-cryTrkB-Cry2PHR(E281A)-HA, was termed pAAV-CaMKII(0.4)::Opto-cryTrkB(E281A)-HA. The control vector, pAAV-CaMKII(0.4)::Lyn-cryTrkB(E281A)-HA, was prepared by removing the cryTrkB-Cry2PHR(E281A)-HA sequence from pAAV-CaMKII(0.4)::Lyn-cryTrkB-Cry2PHR(E281A)-HA by cleavage with EcoRI and HindII and replacing it with Cry2PHR(E281A)-HA. The pAAV-Ef1a::DIO-EYFP vector was cut with MluI and XbaI to remove Ef1a, which was replaced with CaMKIIα(0.4) from the pAAV-CaMKII(0.4)GW vector. pAAV-CaMKIIα::DIO-Opto-cryTrkB(E281A)-HA was constructed by PCR-amplification of the Lyn-cryTrkB-Cry2PHR(E281A)-HA sequence, followed by digestion with Nhel and Ascl, and substitution of the EYFP sequence from pAAV-CaMKIIα(0.4)::DIO-EYFP. Last, the ATG sequence, which is near the C-terminal lxx2272 site of pAAV-CaMKIIα::DIO-Opto-cryTrkB(E281A)-HA, was changed to CTG, causing a change in translation from the Lyn sequence when inverted by Cre recombine. pAAV-hSyn1::Cre was constructed by replacing CaMKIIα from pAAV-Cry2PHR(Cry2PHR(E281A)-HA, was injected at rate of 0.3 μl/min with a glass pipette. For broad expression in the cortical and hippocampal neurons, we recruit AAV2/5, AAVDj/8, and AAV9 serotypes [52]. For retrograde trafficking experiments, 0.3 μl of AAV2-retro-hSyn1::Cre virus was injected in the hippocampal lacunosum layer containing the molecular layer of the DG (AP, –2.1; ML, 1.4; DV, 1.55), and 0.6 μl of AAV9-CaMKIIα::DIO-Opto-cryTrkB(E281A)-HA was injected into the ipsilateral MEG (AP, –4.7; ML, 3.0; DV, 2.5) using the same injection methods. A 200-μm–diameter zirconia cannula optic ferrule (Doric Lenses) was implanted in the right side of the DG (AP, –2.1; ML, 1.4; DV, 1.5) and fixed with dental cement. For retrograde activation, the same cannula optic ferrule was implanted in the ipsilateral DG (AP, –2.1; ML, 1.4; DV, 1.4) against MEC. For guide cannula implantation, a 26G guide cannula was implanted on the right side of the DG (AP, –2.1; ML, 1.4; DV, 1.4) and fixed with dental cement. The guide cannula was capped with a dummy cannula projecting 0.2 mm from the tip of the guide cannula.

SDS-PAGE and Western blot

AAV-injected mice were anesthetized, and their brains were removed in a dark room under a dim red light on ice to minimize light stimulation and phosphatase activity. Isolated brains were cut into 1 mm sections, and hippocampal regions were cut with a biopsy punch (Kai Medical). The samples were fixed in liquid nitrogen immediately after punching. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were prepared in lysis buffer (Invitrogen) containing phosphatase inhibitor (PhosSTOP; Sigma) and boiled in sample buffer (Bio-sang). Proteins in boiled samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (iBlot; ThermoFisher), primary antibodies, diluted as indicated in Tris-buffered saline-Tween-20 containing 1% casein, and incubated with secondary antibodies, conjugated secondary antibody (LI-COR) diluted in blocking buffer and washed with TBS-T. Fluorescence images were obtained using an Odyssey CLx system (LI-COR).

hrBDNF infusion

Three weeks after guide cannula (Plastics1) implantation, 0.25 μg of hrBDNF (ThermoFisher), dissolved in 0.5 μl of PBS, was infused at a flow rate of 0.1 μl/min via an internal cannula projecting 0.2 mm from the tip of the guide cannula. The internal cannula was removed 5 min after the end of the infusion and re-capped with a dummy cannula until the mice were sacrificed. For control experiments, 0.5 μl of PBS was infused under the same conditions.

**Surgery and AAV injection**

Mice were anesthetized with 200 mg/kg of 2,2,2-tribromoethanol dissolved in 400 μl of PBS and injected with AAVs in the hippocampal DG (stereotaxic coordinates: anteroposterior (AP), –2.1; mediolateral (ML), 1.4; dorsoventral (DV), 1.7). A total of 0.3–0.4 μl of AAVDJ/8-CaMKIIα::Opto-cryTrkB-HA, AAVDJ/8-CaMKIIα::Opto-cryTrkB(E281A)-HA or AAV2/5-CaMKIIα::Lyn-PHR(E281A)-HA was injected at rate of 0.1 μl/min with a glass pipette. For broad expression in the cortical and hippocampal neurons, we recruit AAV2/5, AAVDj/8, and AAV9 serotypes [52]. For retrograde trafficking experiments, 0.3 μl of AAV2-retro-hSyn1::Cre virus was injected in the hippocampal lacunosum layer containing the molecular layer of the DG (AP, –2.1; ML, 1.4; DV, 1.55), and 0.6 μl of AAV9-CaMKIIα::DIO-Opto-cryTrkB(E281A)-HA was injected into the ipsilateral MEG (AP, –4.7; ML, 3.0; DV, 2.5) using the same injection methods. A 200-μm–diameter zirconia cannula optic ferrule (Doric Lenses) was implanted in the right side of the DG (AP, –2.1; ML, 1.4; DV, 1.5) and fixed with dental cement. For retrograde activation, the same cannula optic ferrule was implanted in the ipsilateral DG (AP, –2.1; ML, 1.4; DV, 1.4) against MEC. For guide cannula implantation, a 26G guide cannula was implanted on the right side of the DG (AP, –2.1; ML, 1.4; DV, 1.4) and fixed with dental cement. The guide cannula was capped with a dummy cannula projecting 0.2 mm from the tip of the guide cannula.

**SDS-PAGE and Western blot**

AAV-injected mice were anesthetized, and their brains were removed in a dark room under a dim red light on ice to minimize light stimulation and phosphatase activity. Isolated brains were cut into 1 mm sections, and hippocampal regions were cut with a biopsy punch (Kai Medical). The samples were fixed in liquid nitrogen immediately after punching. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were prepared in lysis buffer (Invitrogen) containing phosphatase inhibitor (PhosSTOP; Sigma) and boiled in sample buffer (Bio-sang). Proteins in boiled samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (iBlot; ThermoFisher), primary antibodies, diluted as indicated in Tris-buffered saline-Tween-20 containing 1% casein, and incubated with secondary antibodies, conjugated secondary antibody (LI-COR) diluted in blocking buffer and washed with TBS-T. Fluorescence images were obtained using an Odyssey CLx system (LI-COR).

hrBDNF infusion

Three weeks after guide cannula (Plastics1) implantation, 0.25 μg of hrBDNF (ThermoFisher), dissolved in 0.5 μl of PBS, was infused at a flow rate of 0.1 μl/min via an internal cannula projecting 0.2 mm from the tip of the guide cannula. The internal cannula was removed 5 min after the end of the infusion and re-capped with a dummy cannula until the mice were sacrificed. For control experiments, 0.5 μl of PBS was infused under the same conditions.
Light stimulation

Mice were handled three consecutive days before the experiment day to minimize the chance of adverse effects (e.g., fiber optic cannula detachment from the skull). A 200-μm-diameter fiber optic coupled to a 473-nm laser (MBL—III—473 nm; CNI) via an optic patch cord was used for light stimulation. Light intensity was measured at the tip of the optic fiber using a power meter (#P120D; Thorlab). A custom-designed LED cage lid that fits in the mouse home cage lid was used for noninvasive blue light stimulation (LCI, Customized; Republic of Korea), at a power of ~1 mW/mm² [38]. Unstimulated mice were housed under room light conditions (300–500 lux from a fluorescent lamp).

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde (PFA) dissolved in PBS, after which brains were removed and postfixed in 4% PFA for 24 h at 4 °C. Fixed brains were cut into 40–45-μm sections using a vibratome (VT1200S; Leica). Slices were incubated in blocking buffer (5% normal goat serum, 0.3% Triton-X100 in PBS) under free-floating conditions for 1.5–2 h, followed by incubation in staining solution (2.5% normal goat serum, 0.15% Triton-X100 in PBS) containing anti-HA (1:1000; Cell Signaling Technology, C29F4 and 6E2), anti-pS6 (1:500; Cell Signaling Technology, 4858), anti-CaMKII (1:400; Abcam, ab22609), anti-GAD67 (1:400; Millipore, MAB5406), anti-lba1 (1:1000; Wako, 019–19741) or anti-GFAP (1:4000; Abcam, ab7260) primary antibodies for 18–24 h at 4 °C. Thereafter, slices were washed with PBS containing 0.3% Triton X-100. For fluorescence imaging, the slices were incubated in a blocking solution containing secondary antibodies (ThermoFisher) for 75 min at room temperature (RT), and then washed with PBS containing 0.3% Triton X-100. For pERK1/2 staining, mice were perfused with ice-cold 4% PFA dissolved in PBS immediately after the end of light stimulation, and isolated brains were postfixed under the same conditions. For Arc staining, mice were perfused with 4% PFA at RT, and brains were removed and sectioned. Thereafter, for pERK1/2 or Arc staining, 40–45-μm sections were pretreated with 1% NaBH₄ in PBS for 15 min at RT, then washed first with PBS followed by 10% EtOH/3% H₂O₂ solution in PBS for 20 min at RT, and a final wash with PBS. After incubating in blocking buffer for 1.5–2 h, pretreated slices were incubated with anti-pERK1/2 (1:10,000; Cell Signaling Technology, 9101) and anti-Arc (1:500; Santa Cruz Biotechnology, sc-17839) primary antibodies for 36–48 h and washed with TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20). Slices were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:1000; ThermoFisher, G-21234) or anti-mouse (1:500; ThermoFisher, 31430) secondary antibody, diluted as indicated in blocking solution, for 75 min at RT, followed by washing with TNT buffer. For signal amplification, slices were treated TSA-Cy3 solution (PerkinElmer) for precisely 10 min and washed immediately after the reaction. For subsequent staining steps, slices were re-blocked with a blocking solution, after which the same protocols described above were performed. Slices were imaged using a Nikon C2 confocal microscope.

Data analysis

Imaging analyses were performed using NIS-element AR imaging software (Nikon), and all single-cell analyses were performed by manually counting. For detailed imaging analysis of pERK1/2-, pS6- and Arc-positive cells in samples from virus-injected mice, cells in which the mean fluorescence intensity was greater than 500 arbitrary units (a.u.) only in the soma were counted and evaluated for each positivity of target proteins. The percentage of pERK1/2-positive cells was calculated as (pERK1/2⁺/HA-tag⁺/HA-tag⁻) × 100 (%). For image analysis of tissues from hrBDNF-infused mice, cells located at a distance of 100–500 μm were analyzed, a spread made necessary by the efficiency of infusion. Data calculation and statistical analyses were performed using Prism Software (GraphPad). Data were analyzed by one-way analysis of variance (ANOVA) or two-tailed t-test as appropriate. The number of samples corresponds to the number of individual data points (filled circles) on each graph.

Acknowledgments

We are grateful to Sungsoo Kim for the discussion and manuscript supporting. We thank Minju Kim for the illustration of Graphic Abstract. This work was supported by a grant from the Institute for Basic Science, Daejeon, Republic of Korea, (no. IBS-R001-D1) and KAIST Institute for the BioCentury, Daejeon, Republic of Korea.

Author contribution

J.H performed all the experimental designs, experiments, and data analysis. J.H and W.D.H wrote the manuscript.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2020.01.010.

Received 23 October 2019; Received in revised form 22 December 2019;
Keywords: optogenetics; Ntrk2; BDNF; TrkB; Cry2PHR

Abbreviations used:

Cry2PHR, cryptochrome2 photolyase homology region; MEC, medial entorhinal cortex; HPC, hippocampus; RTK, receptor tyrosine kinase; TK, tyrosine kinase; BDNF, brain-derived neurotrophic factor; AAV, adeno-associated virus; DG, dentate gyrus; GCs, granule cells; DIO, double inverted open reading frame; LTP, long-term potentiation; 7,8-DHF, 7,8-dihydroxyflavone; CTB-488, cholera toxin B-Alexa488 conjugate; LED, light emitting diode.

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