Morphine Promotes Astrocyte- Preferential Differentiation of Mouse Hippocampal Progenitor Cells via PKCε-Dependent ERK Activation and TRBP Phosphorylation

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ABSTRACT

Previously we have shown that morphine regulates adult neurogenesis by modulating miR-181a maturation and subsequent hippocampal neural progenitor cell (NPC) lineages. Using NPCs cultured from PKCε or β-arrestin2 knockout mice and the MAPK/ERK kinase inhibitor U0126, we demonstrate that regulation of NPC differentiation via the miR-181a/Prox1/Notch1 pathway exhibits ligand-dependent selectivity. In NPCs, morphine and fentanyl activate ERK via the PKCε- and β-arrestin-dependent pathways, respectively. After fentanyl exposure, the activated phospho-ERK translocates to the nucleus. Conversely, after morphine treatment, phospho-ERK remains in the cytosol and is capable of phosphorylating TAR RNA-binding protein (TRBP), a cofactor of Dicer. This augments Dicer activity and promotes the maturation of miR-181a. Furthermore, using NPCs transfected with wild-type TRBP, S1A, and S1D TRBP mutants, we confirmed the crucial role of TRBP phosphorylation in Dicer activity, miR-181a maturation, and finally the morphine-induced astrocyte-preferential differentiation of NPCs. Thus, morphine modulates the lineage-specific differentiation of NPCs by PKCε-dependent ERK activation with subsequent TRBP phosphorylation and miR-181a maturation.

SIGNIFICANCE STATEMENT

Although it is well-known that addictive drugs such as morphine modulate neurogenesis, the mechanism by which morphine regulates miR181a/Prox1/Notch1 remains elusive. We identified the roles of PKCε and phosphorylated ERK is this process by using hippocampal neural progenitor cells (NPCs) isolated from PKCε knockout mice. We also used the MEK inhibitor U0126 to block morphine-induced astrocyte-preferential differentiation. Cytosolic phospho-ERK is capable of phosphorylating TRBP, a Dicer cofactor that promotes maturation of miR-181a. In summary, this study demonstrates that morphine’s modulation of NPC differentiation is mediated by PKCε-dependent ERK activation and subsequent TRBP phosphorylation and miR-181a maturation.

INTRODUCTION

During adult neurogenesis in the hippocampus, neural progenitor cells (NPCs) in the subgranular zone give rise to neuroblasts and immature neurons, which migrate a short distance into the granule cell layer of the dentate gyrus [1, 2]. The immature or mature neurons differentiated from neuroblast cells and NPCs then integrate into the existing hippocampal circuitry [3–6]. This process is regulated by a variety of factors [6, 7], including several addictive drugs such as morphine [8, 9], heroin [10, 11], methamphetamine [12, 13], cocaine [14, 15], alcohol [16], and cannabinoids [17]. In vivo and in vitro evidence suggests that agonists of opioid receptors are important regulators of adult neurogenesis, especially in cell fate specification. For example, chronic morphine treatment increased expression of glial fibrillary acidic protein (GFAP) in the hippocampus [18]. Chronic treatment of NPCs with antagonists of µ-opioid receptor (OPRM1) and δ-opioid receptor (OPRD1) increased neuronal differentiation while decreasing their differentiation into both astrocytes and oligodendrocytes [19].

Previously, we observed that morphine and fentanyl differentially regulated the activity of neurogenic differentiation 1 (NeuroD1), a basic helix-loop-helix transcription factor...
essential for the differentiation and maturation of newborn neurons [20], via their regulation of miR-190 [21]. This differential regulation results in divergent effects on neurogenesis, as morphine, but not fentanyl, attenuates neural differentiation and memory retention [22]. In addition, we also demonstrated that morphine, but not fentanyl, was capable of regulating adult neurogenesis by controlling miR-181a and subsequent NPC lineages. This effect was mediated by the Prox1/Notch1 pathway as demonstrated by an increase in Notch1 levels in the morphine- but not fentanyl-treated NPCs, and blocked by expression of Notch1 siRNA [23]. These studies suggest that morphine could modulate various stages of adult neurogenesis by its action on various miRs. However, the mechanism leading to the regulation of miR-181a levels and subsequently NPC fate determination remains unresolved.

MicroRNAs (miRNAs) are a class of small RNAs expressed in most somatic tissues. They negatively regulate gene expression in a variety of eukaryotic organisms by base-pairing with the 3′ untranslated region of their target mRNAs [24, 25]. The miRNA genes are transcribed by RNA polymerase II (Pol II), thus generating the primary miRNA (pri-miRNA) with a local hairpin structure [25]. The pri-miRNA then undergoes nuclear processing initiated by a nuclear RNase III, Drosha, which cleaves the stem-loop to release a small hairpin-shaped RNA termed precursor miRNA (pre-miRNA) [26]. Pre-miRNA is exported to the cytosol, where Dicer, another RNase III, cleaves pre-miRNA near the terminal loop and liberates the mature miRNA [27]. During this process, Dicer is often regulated and accompanied by a double-strand RNA-binding protein (dsRBP) [28], such as the HIV TAR RNA-binding protein (TRBP) in humans [29]. Mature miRNAs then direct sequence-specific silencing of target mRNAs through effector complexes that contain Argonaute family proteins [30]. It is widely accepted that miRNA maturation is a crucial step in modulating neurogenesis. For example, it was demonstrated that Drosha regulates neurogenesis by controlling expression of neurogenin 2 (Ngn2) and NeuroD1 [31]. Similarly, Dicer is required for regulating cortical stem cell multipotency [32] and limiting the time span of cortical neurogenesis [33]. As our previous studies indicate that morphine treatment does not alter the pri- and pre-miR-181a levels in NPCs [23], it is likely that morphine modulates the maturation of miR-181a by regulating Dicer and its cofactors.

TRBP was first identified as a cellular protein that facilitates the replication of human immunodeficiency virus and inhibits the activation of protein kinase R [34, 35]. As a member of dsRBPs, TRBP also contributes to pre-miRNA processing as a cofactor of Dicer. The Dicer-TRBP complex is essential for both miRNA processing and assembly of the RNA-induced silencing complex, which is required for miRNA biogenesis and post-transcriptional gene silencing [29, 35, 36]. The activity of TRBP is regulated by ERK-mediated phosphorylation of its serine residues. TRBP phosphorylation stabilizes the miRNA-generating complex and upregulates Dicer expression by stabilizing the Dicer protein, thus promoting the maturation of certain miRNAs such as miR-17 and miR-20a [37]. This has been demonstrated in transgenic mice, as apigenin, a flavonoid, impairs miR-103 maturation by inhibiting the ERK-TRBP phosphorylation cascade [38]. Agonist-selected signaling of OPRM1 by morphine and fentanyl results in distinct pathways of ERK activation [21, 39]. Since ERK-induced TRBP phos-

### MATERIALS AND METHODS

#### Animals

Eight-week-old wild-type C57BL/6 male mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA, http://www.criver.com) 2 weeks before experiments. β-Arrestin2 KO mice (C57BL/6J background) were gifts from Dr. Robert Lefkowitz. PKCε KO mice bred from pairs of hybrid (50% C57BL/6, 50% 129S4) mice heterozygous for the mutant PKCε gene were gifts from Dr. George Wilcox [40, 41]. Animal maintenance and procedures were conducted in accordance with Institutional Animal Care and Use Committee policies at University of Minnesota.

#### NPC Expansion and Differentiation

Primary cultures and differentiation of mouse hippocampal neurons were carried out as previously described [23]. For cell expansion, cells are cultured as neurospheres in proliferation medium containing 10 μg/ml epidermal growth factor (EGF) and 10 μg/ml FGF2. For cell differentiation, glass coverslips were coated with 1 mg/ml Matrigel for 2 hours at room temperature. Neurospheres (less than five passages) were triturated to form a single-cell suspension and cultured in the complete differentiation medium for approximately 4 days. EGF and FGF2 were obtained from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.com). NeuroCult NSC Medium for proliferation and differentiation, and the Enzymatic Dissociation Kit were purchased from STEMCELL Technologies (Vancouver, Canada, http://www.stemcell.com). Matrigel was from BD Biosciences (San Jose, CA, http://www.bdbiosciences.com).

#### In Vivo Morphine Treatment

Eight-week-old wild-type (C57BL/6) mice, β-arrestin2 KO (C57BL/6J) mice, and PKCε KO (50% C57BL/6, 50% 129S4) mice were administered morphine by subcutaneous implantation of one morphine pellet (75 mg free base per mouse) for 4 days. Control animals were implanted with placebo pellets. Mice were anesthetized lightly with isoflurane during the implantation process. The tablets for implantation were obtained from National Institute on Drug Abuse (Bethesda, MD).

#### Immunoblotting

Immunoblotting was performed as described previously [39]. Briefly, chemiluminescence was detected using the ECF Reagent (GE Healthcare, Little Chalfont, U.K., http://www.bdbiosciences.com) and the fluorescence intensity was measured with Storm 860 Molecular Imager (GE Healthcare). The intensity of individual bands was determined with ImageQuant software (GE Healthcare). U0126 was purchased from EMD Millipore (Billerica, MA, http://www.emdmillipore.com). Antibodies are listed in Supporting Information Table S1.

#### Quantitative Reverse Transcription PCR

The total RNAs were extracted and reverse transcribed with the miScript system (Qiagen, Hilden, Germany, http://www.qiagen.com).
Quantitative reverse transcription PCR (qRT-PCR) was performed according to the instructions in the miScript system, which included a SYBR Green PCR kit (Qiagen). GAPDH was used as an internal control. Primer sets used in qRT-PCR assays are listed in Supporting Information Table S2.

DNA Construct Transfection
DNA constructs of wild-type, SΔA, and SΔΔ TRBP were sub-cloned into the pcDNA vector with 3×Flag tag at N terminal, and were gifts from Dr Qinghua Liu’s lab [37]. The over-expression of wild-type, SΔA, and SΔΔ TRBP were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, http://www.lifetechnologies.com) according to the manufacturer’s instructions. Briefly, the DNA-lipid complex solution containing 7.5 μl transfection reagent and 2.5 μg DNA was added to each well of a six-well plate. The cells were incubated in the transfection medium for 24 hours before being used for subsequent experiments. Transfection efficiency was determined to be ≥60% by quantifying the number of green fluorescent protein (GFP⁺) cells in transfection studies using a GFP expression vector.

Immunocytochemistry and Cell Quantification
Immunocytochemistry was performed as described previously [42]. Labeled cells were mounted on slides with DAPI Fluoromount G (SouthernBiotech, Birmingham, AL, http://www.southernbiotech.com) and visualized using an upright microscope (model DM5500 B; Leica, Germany) with a CCD camera. Cells were counted using ImageJ (NIH, Bethesda, MA, http://imagej.nih.gov/ij/index.html). The total cell numbers were counted based on DAPI-positive particles and those of each cell type were counted according to indicated markers. Double positive cells were confirmed by overlapping color and counted manually by the “Cell Counter” plugin. The final data represent at least four individual experiments. For each single experiment, at least three individual fields of a single sample were selected randomly to reach a total number of at least 1,000 cells. Antibodies are listed in Supporting Information Table S1.

Data Analysis
All statistical and curve-fitting analyses were performed using GraphPad Prism 6.0 software (Graphpad Software, La Jolla, CA, http://graphpad.com). Data represent mean ± SEM of at least three separate experiments. Statistical significance was determined by one-way ANOVA followed by post hoc comparison using Dunnett’s tests.

Results
Morphine and Fentanyl Activate ERK via Differential Pathways
We previously reported that morphine, but not fentanyl, modulates NPC lineages by upregulating miR-181a levels [23]. However, the mechanism underlying this ligand-biased regulation has not been determined. It is likely that the reported pathway-selective ERK activation exhibited by morphine and fentanyl [21, 39] also exists in NPCs, and such pathway selectivity is the cause for the observed differential regulation of miR-181a processing. As shown in Supporting Information Figure S1, although the maximal ERK activation induced by either agonist was observed at 10 minutes, their EC₅₀ values are significantly different (3.1 ± 1.2 × 10⁻⁸ M for morphine and 4.1 ± 1.7 × 10⁻¹⁰ M for fentanyl). Using NPCs from β-arrestin2 (βArr2) and PKCε knockout (KO) mice, we found that 1 μM morphine did not activate ERK in NPC isolated from PKCε KO cells, but was retained in NPCs isolated from βArr2 KO mice. As such, morphine did not increase levels of phosphorylated PKC substrates in NPCs from PKCε KO mice (Fig. 1A, 1B). Meanwhile, 10 nM fentanyl induced ERK phosphorylation in NPCs from PKCε KO but not those from βArr2 KO mice (Fig. 1A, 1B). The siRNA-mediated knockdown of PKC subtypes indicated that PKCε, but not PKCζ, mediated morphine-induced ERK activation. In contrast, knocking down of either subtype had no significant effect on fentanyl’s ERK activation (Fig. 1C–1F). In addition, morphine- and fentanyl-activated ERK exhibited different cellular locations. Fentanyl-activated ERK showed significant nuclear translocation, whereas morphine-activated ERK mainly in the cytosol (Supporting Information Fig. S2). These results indicate distinct differences between ERK activation pathways activated by morphine and fentanyl in NPCs, similar to our previous observations with other cell lines [39].

Morphine Promotes Astrocyte-Preferential Differentiation via PKCε-Mediated ERK Phosphorylation
To confirm the roles of different ERK activation pathways on NPC differentiation, we examined whether they were related to the modulation of NPC lineages. Using βIII-tubulin (Tuj1), GFAP, and O4 as markers for neurons, astrocytes, and oligodendrocytes, respectively, there were equal percentages of cells stained positive for these markers after differentiation of NPCs isolated from wild-type, PKCε, and β-Arr2 KO mice (Fig. 2A, 2B). In wild-type mice, morphine treatment resulted in a large percentage of GFAP⁺ cells (59.7% ± 6.8%) but very few Tuj1⁺ neurons (5.3% ± 2.2%), with no change in O4⁺ cells (35.0% ± 5.1%). We observed similar results in NPCs from β-Arr2 KO mice. However, this morphine effect was absent in NPCs isolated from PKCε KO mice, as reflected in nonpreferential differentiation of the NPCs (Fig. 2A, 2B). This suggests that PKCε activation is essential in morphine-induced astrocyte-preferential NPC differentiation.

As ERK is activated downstream of PKCc and β-arrestins, we then examined its role in morphine-modulated NPC differentiation. Using the MAPK/ERK kinase inhibitor U0126, we were able to block ERK activation in NPCs. As shown in Figure 2C, 2D, pretreatment with 10 μM U0126 for 1 hour blocked the effect of morphine by decreasing the percentage of GFAP⁺ cells (from 57.3% ± 7.2% to 36.3% ± 3.8%) and increasing that of Tuj1⁺ neurons (from 8.9% ± 2.8% to 36.7% ± 4.0%). The immunofluorescence results were further confirmed by examining lineage-specific markers, βIII-tubulin, GFAP, and myelin basic protein (oligodendrocyte marker) using qRT-PCR (Supporting Information Fig. S3). These results indicate that PKCc-mediated ERK phosphorylation plays a major role in morphine-induced astrocyte-preferential differentiation of NPCs.

Morphine Promotes TRBP Phosphorylation and Dicer Expression via OPRM1
Previously, we reported that morphine’s control of miR-181a levels occurs via OPRM1-mediated regulation of microRNA processing [23]. We examined whether the morphine effect is
due to modulation of specific mRNA processing enzymes. The levels of Drosha, Dicer, TRBP, and ERK in NPCs were determined after 1 μM morphine or 10 nM fentanyl for 24 hours, with or without pretreatment of 10 μM OPRM1-specific antagonist Cys2-Tyr3-Orn5-Pen7-amide (CTOP) for 1 hour. As indicated in Figure 3, morphine significantly increased both Dicer expression (2.10-6.0.12-folds) and TRBP phosphorylation (3.23-6.0.24-folds), but had no significant effect on the level of Drosha. Fentanyl, conversely, did not show any significant effect on the levels of all three proteins despite activating ERK. The effect of morphine was blocked in the presence of CTOP, indicating the involvement of OPRM1. Conversely, the mRNA levels of TRBP and Dicer remained stable in the presence of both ligands (Fig. 3E), indicating an effect of protein stabilization but not transcriptional regulation. These results suggest that morphine regulates microRNA processing in NPCs by promoting both TRBP phosphorylation and Dicer expression at via OPRM1.

Morphine Promotes TRBP Phosphorylation and Dicer Expression via PKCe-Mediated ERK Activation

We have shown that morphine activates ERK via PKCe (Fig. 1) and promotes TRBP phosphorylation and Dicer expression (Fig. 3) in NPCs. Since it was reported that TRBP phosphorylation and Dicer expression are ERK mediated, we thus hypothesize that morphine-induced regulation of TRBP and Dicer is mediated by the PKCe-ERK pathway. We first examined the effect of 1 μM morphine or 10 nM fentanyl treatment in the protein levels of TRBP and Dicer in NPCs isolated from wild-type, PKCe KO, or β-arrestin2 KO mice. Whereas the ability of morphine to increase TRBP phosphorylation (from 3.34-6.0.21-folds to 0.95-6.0.23-folds) and TRBP phosphorylation (from 2.38-6.0.24-folds to 1.13-6.0.29-folds) was eliminated in NPCs isolated from PKCe KO mice, it remained unaffected in those isolated from β-arrestin2 knockout mice (Fig. 4A, 4B). Similarly, when the NPCs were pretreated with 10 μM U0126 for 1 hour in order to block ERK

Figure 1. Morphine and fentanyl activate ERK via differential pathways. (A): ERK phosphorylation was tested in wild-type, PKCe KO, or β-arrestin2 KO neural progenitor cells (NPCs) cultured in differentiation media by immunoblotting after treatment of 1 μM morphine or 10 nM fentanyl for 10 minutes. PKC activities were determined with immunoblotting of PKC phosphorylated substrates. β-Actin was used as the internal control. C: control; M: morphine; F: fentanyl. (B): Quantification of ERK phosphorylation as shown in (A), calculated as folds of control. *, p < .05 compared to wild-type cells treated with the same agonist. Data are the mean ± SEM of four independent experiments. (C): ERK phosphorylation was tested in NPCs transfected with control siRNA, PKCa siRNA, and PKCe siRNA by immunoblotting after treatment of 1 μM morphine 10 minutes. (D): Quantification of ERK phosphorylation as shown in (C), calculated as folds of control. *, p < .05 compared to cells transfected with control siRNA. Data are the mean ± SEM of four independent experiments. (E): ERK phosphorylation was tested in NPCs transfected with control siRNA, PKCa siRNA, and PKCe siRNA by immunoblotting after treatment of 10 nM fentanyl for 10 minutes. (F): Quantification of ERK phosphorylation as shown in (E), calculated as folds of control. Data are the mean ± SEM of four independent experiments. Abbreviation: KO, knockout.
phosphorylation, the ability of morphine to regulate TRBP phosphorylation (from 3.36-60.25-folds to 1.36-60.16-folds) and Dicer expression (from 2.10-60.12-folds to 1.23-60.13-folds) was also eliminated (Fig. 4C, 4D). Fentanyl, conversely, did not exhibit any significant effects on the NPCs isolated from either the wild-type or the knockout mice. Thus, morphine promotes TRBP phosphorylation and Dicer expression via PKCε-mediated ERK activation.

**TRBP Phosphorylation Mediates Dicer Expression and Activation of the miR-181a/Prox1/Notch1 Cascade**

As TRBP phosphorylation has been shown to stabilize the miRNA-generating complex [37], we next investigated the functionality of phospho-TRBP on Dicer expression and the miR-181a pathway in NPCs. NPCs were transiently transfected with wild-type, phospho-mutant (serine-to-alanine, SΔA), and phospho-mimic (serine-to-aspartate, SΔD) TRBP DNA constructs. All cells were treated with 1 μM morphine or 10 nM fentanyl for 24 hours and both mRNA and protein levels of Dicer and TRBP were examined. Although all cell lines expressed similar levels of Dicer and TRBP mRNAs (Fig. 5A), the expression of Dicer protein was inhibited when TRBP phosphorylation was blocked in SΔA cells (Fig. 5B, 5C). The capability of morphine to increase Dicer protein was eliminated in SΔA cells (from 2.07-60.24-folds in wild-type cells to 0.80-60.19-folds), showing the same level as those in control and fentanyl-treated groups. In SΔD cells, however, TRBP phosphorylation was enhanced in control and fentanyl-treated groups, but not in morphine-treated cells (Fig. 5B, 5C). Meanwhile, Dicer expression was also increased in control and fentanyl-treated cells, with levels similar to those observed in morphine-treated cells. These results suggest that morphine-
induced increase in Dicer expression is mediated by TRBP phosphorylation.

Since phospho-TRBP and Dicer are major components of the microRNA-generating complex, we investigated whether the elevated miR-181a level in NPCs induced by morphine is a direct consequence of morphine-mediated increase in phospho-TRBP. qRT-PCR analysis was carried out to quantify miR-181a, Prox1, and Notch1 in NPCs transfected with wild-type or SΔA, SΔD mutant TRBP. As shown in Figure 5D, expression of SΔA significantly increased Prox1 mRNA level and decreased miRNA-181a and Notch1 mRNA levels in NPCs in regardless of agonist treatment. Conversely, expression of SΔD decreased Prox1 mRNA level and increased miRNA-181a and Notch1 mRNA levels in NPCs without morphine treatment, but significantly affected the levels in morphine-treated NPCs (Fig. 5D). Taken together, these data suggest that the morphine-induced increase in TRBP phosphorylation and subsequent Dicer expression is critical in facilitating miR-181a maturation and modulation of Prox1 and Notch1 mRNA levels in NPCs.

TRBP Phosphorylation Modulates Morphine-Induced Astrocyte-Preferential Differentiation of NPCs

Since TRBP phosphorylation was shown to be essential for miR-181a maturation and thus regulate the expression of Prox1 and Notch1 (Fig. 6), we then tested its role in lineage determination during NPC differentiation. NPCs were transfected with wild-type, SΔA, or SΔD mutant TRBP and treated with 1 μM morphine for 4 days before Tuj1+ neurons and GFAP+ astrocytes were detected by immunocytochemistry. As shown in Figure 6A, 6B, after transient SΔA transfection, the percentage GFAP+ astrocytes with or without morphine treatment decreased from 59.7% ± 8.9% and 35.8% ± 4.8% (wild type) to 30.1% ± 5.5% and 24.6% ± 4.3%, respectively. Meanwhile, the percentage of Tuj1+ neurons in the presence of morphine increased correspondingly. Conversely, transfection of SΔD increased the percentage of GFAP+ astrocytes to 61.3% ± 9.1%, with concomitant decrease of Tuj1+ neurons (from 29.6% ± 5.6% to 15.3% ± 4.2%). Interestingly, morphine treatment did not further increase the percentage of NPC differentiated into GFAP+ astrocytes. To substantiate these
observations, qRT-PCR analyses were carried out to quantify βIII-tubulin and GFAP. Transfection of SAA significantly increased βIII-tubulin mRNA level and decreased GFAP mRNA level in NPCs treated with or without morphine. In contrast, expression of SAD decreased βIII-tubulin mRNA level and increased GFAP mRNA level in NPCs without morphine treatment, but did not show significant effects in morphine-treated NPCs (Fig. 6C). Taken together, these data suggest that TRBP phosphorylation is a crucial step leading to morphine-induced astrocyte-preferential differentiation.

**DISCUSSION**

Previously, we demonstrated that the miR-181a/Prox1/Notch1 cascade plays an essential role in morphine-induced astrocyte-preferential differentiation.
preferential differentiation [23]. However, the mechanism by which morphine and fentanyl differentially regulate miR-181a expression remains to be clarified. In this study, we demonstrate that morphine and fentanyl activate ERK via PKCe- and PKCb-arrestin-dependent pathways, respectively. This results in differential cellular localization of phosphorylated ERKs, either in the cytosol or in the nucleus. The fact that ERK activated via PKCe-dependent pathway is retained in the cytosol is contrary to the accepted dogma. Hence, we hypothesize that certain cellular factors other than β-arrestins might be involved in the cytosolic retention of phosphorylated ERK. Only the morphine-induced PKCe-mediated and cytosol-localized activated ERK is capable of inducing TRBP phosphorylation, thereby promoting Dicer expression and miR-181a maturation. Fentanyl, however, does not induce these effects despite its ability to activate OPRM1 and ERK. The difference between the two agonists is ultimately reflected in the lineage-specific differentiation of NPCs (Fig. 7). Overall, these results indicate an important role of OPRM1 agonist-selective pathway-dependent signaling in the progenitor cell fate determination.

As indicated in this study, morphine’s inhibition of neurogenesis by modulating miR-181a maturation and NPC differentiation depends on ERK activation. This observation is consistent with our previous studies showing that morphine-induced ERK activation inhibits adult neurogenesis and subsequent contextual memory retention [22]. Such effects can be attributed to the regulation of NeuroD1 level by miR-190 [21], which is in turn regulated by the ERK phosphorylation pathway [39]. These observations begin to elucidate the role of OPRM1’s ligand selective signaling in regulating adult neurogenesis (Fig. 7). ERK activated by morphine remains in the cytosol and does not induce the transcription factor YY1 phosphorylation, thus having no effect on the miR-190 level via the talin2 transcription [43]. Furthermore, cytosolic
phosphorylated ERK promotes the phosphorylation of other cytosolic substrates such as TRBP, which in turn upregulates Dicer expression and facilitates miR-181a maturation. This drives an upregulation of Notch1 expression and astrocyte-preferential differentiation of NPCs [23]. Thus, morphine attenuates adult neurogenesis via dual mechanisms. Its ability to phosphorylate TRBP gives rise to activation of the miR-181a/Prox1/Notch1 pathway, resulting in a decreased number of neurons [23]. In addition, its inability to alter miR-190 level and its inhibition of CaMKIIα activity result in attenuation of NeuroD1 activity [21], which plays a major role in development of the nervous system [20]. By its differential regulation of various miRNAs, morphine impairs neurogenesis and subsequent behavioral alterations related to hippocampal functions (Fig. 7).

The post-transcriptional mechanism that modulates miRNA processing and maturation is supposed to show a universal effect on all members of the microRNA family, as Dicer and TRBP are in the microprocessing complex of all miRNAs. However, this study shows the specific regulation of morphine on

Figure 6. TRBP phosphorylation modulates morphine-induced astrocyte-preferential differentiation of neural progenitor cells (NPCs). (A): NPCs transfected wild-type, SΔA, or SΔD TRBP were cultured in complete differentiation medium for 4 days, with or without the treatment of 1 μM morphine. Cells were stained with markers for neurons (Tuj1), astrocytes (GFAP), and with DAPI. Scale bar = 25 μm. Images are representative of four independent experiments with similar results. (B): Quantification of cells stained with each marker, calculated as the percentage of the total number of cells stained with DAPI. Red: Tuj1; Green: GFAP. *, *p < .05 compared to wild-type groups with the same treatment. #, #, p < .05 compared to the wild-type control. (C): The mRNA levels of βIII-tubulin and GFAP were determined by qRT-PCR after 4 days of differentiation with indicated treatments. The results were normalized against GAPDH levels. *, p < .05; **, p < .01, compared to wild-type groups with the same treatment. #, p < .05 compared to the wild-type control. All data represent mean ± SEM of four independent experiments. Abbreviations: GFAP, glial fibrillary acidic protein; TRBP, TAR RNA-binding protein; WT, wild type.
miR-181a. The specificity of such TRBP-mediated regulation has been demonstrated in the preferential upregulation of the progrowth miRNAs such as miR-17, miR-20a, and miR-92a, but downregulation of let-7 [37]. The inhibition of miR-103 maturation induced by apigenin is also related to decrease phosphorylation of ERK and TRBP [38]. It is likely that certain RNA-binding proteins might participate in the processing of pre-miRNAs. For example, the KH-type splicing regulatory protein facilitates Dicer-mediated processing of a series of pre-miRNAs through its interaction with the terminal loop [44]. Lin28 was shown to modulate pri-let-7 and pre-let-7 processing [45]. Dead End 1 was demonstrated to modulate interactions between the miRNA silencing machinery and target mRNAs [46]. RNA methylation mediated by RNA methyltransferase BCDIN3D was also shown to interfere with Dicer-mediated pre-miRNA processing [47]. Whether such proteins are involved in the morphine-induced TRBP-mediated modulation of pre-miRNA-181a processing remains to be demonstrated.

In spite of the specificity of TRBP-mediated regulation of pre-miRNA processing shown by previous studies, it is nevertheless probable that morphine modulates some other miRNAs besides miR-181a via this pathway. In a microarray screen, we have previously shown that morphine upregulates the expression of several miRNAs including miR-224, 331, 365, and 20a [21]. Others have reported that morphine modulates miR-103, 107 [48], and 133b [49]. Because ERKs activated by morphine do not translocate into the nucleus, it is likely that morphine modulates these miRNAs via TRBP phosphorylation in the cytosol, rather than at the transcription level. Hence, the multiple miRNAs that are regulated by morphine could modulate many aspects of adult neurogenesis via TRBP phosphorylation and miRNA processing.

**CONCLUSIONS**

Morphine and fentanyl activate ERK via differential pathways. Morphine-induced ERK phosphorylation is mediated by PKCε and localizes in the cytosol, and is therefore capable of inducing TRBP phosphorylation. Phosphorylated TRBP promotes Dicer expression by stabilizing the miRNA-generating complex and thus facilitates miR-181a maturation, which finally results in astrocyte-preferential differentiation of NPCs.

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**AUTHOR CONTRIBUTIONS**

C.X.: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; H.Z. and H.H.L.: conception and design; P.-Y.L.: conception and design, financial support, provision of study material, data analysis and interpretation, manuscript writing, and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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