

Original Article

Sleep disturbances promote colorectal cancer perineural invasion via GABA-mediated BDNF secretion

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Received September 26, 2025; Accepted February 9, 2026; Epub September 15, 2026; Published September 30, 2026

Abstract: Perineural invasion (PNI) is a hallmark of aggressive colorectal cancer (CRC) progression, often leading to poor patient outcomes. Emerging evidence suggests a link between sleep disturbances and cancer progression through neural pathways. In this study, we investigated how sleep disturbances contribute to PNI in CRC via γ -aminobutyric acid (GABA) and brain-derived neurotrophic factor (BDNF). Clinical data revealed that CRC patients with poor sleep quality exhibited higher PNI rates and worse prognoses. Sleep-deprived mice showed increased CNS-derived GABA levels and compromised blood-brain barrier integrity, facilitating peripheral GABA exposure. GABA treatment enhanced CRC cell proliferation, migration, and invasion by upregulating FOS expression, which in turn increased BDNF secretion. Elevated BDNF activated Schwann cells through the TrkB receptor, promoting their proliferation, migration, invasion, and angiogenesis. Knockdown of FOS or inhibition of TrkB mitigated these effects. Additionally, GABA was found to promote FOS transcription via upregulation of EGR1. Our findings unveil a novel mechanism whereby sleep disturbances exacerbate CRC progression through the GABA-EGR1-FOS-BDNF axis, highlighting the importance of sleep quality in CRC management and suggesting potential therapeutic targets to inhibit PNI.

Keywords: Colorectal cancer, sleep disturbances, perineural invasion, GABA

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer globally and remains the second leading cause of cancer-related deaths, resulting in nearly 1 million deaths annually and accounting for approximately 10% of all cancer cases and fatalities [1, 2]. Despite substantial advancements in CRC diagnosis and treatment, the incidence continues to rise, particularly among individuals under the age of 50 [3, 4]. Alarming, about 20%-25% of CRC patients present with metastatic disease at the time of diagnosis, significantly worsening their prognosis [1].

Perineural invasion (PNI) represents a distinct and aggressive form of cancer dissemination, characterized by the infiltration and spread of cancer cells along peripheral nerve sheaths [5]. This phenomenon is frequently observed in cancers such as head and neck, pancreatic, and prostate cancers, where interactions between cancer cells and nerve fibers drive both tumor invasion and distant metastasis [6-8]. Importantly, several studies have identified PNI as an independent adverse prognostic factor in CRC, with an incidence rate of approximately 21.5% [9, 10]. The presence of PNI is associated with higher recurrence rates and worse sur-

vival outcomes, underscoring its critical role in CRC progression.

Schwann cells, specialized glial cells in the peripheral nervous system (PNS), are primarily responsible for supporting and nourishing nerve fibers [11]. However, emerging evidence suggests that Schwann cells also facilitate cancer progression by mediating tumor-nerve interactions [12, 13]. Through the secretion of growth factors and modulation of immune responses, Schwann cells foster a tumor-promoting environment, enhancing cancer cell proliferation and invasion [14, 15]. Gaining a deeper understanding of PNI and Schwann cell involvement in the tumor microenvironment is essential for developing effective therapeutic strategies and improving patient outcomes.

Sleep is a vital physiological process integral to human health. Sleep disturbances - disruptions in quality, duration, or timing - can result from stress, environmental noise, lifestyle habits, or health conditions, leading to difficulty sleeping and daytime fatigue [16, 17]. A growing body of research has demonstrated that circadian rhythms and sleep patterns significantly influence cancer progression and patient outcomes. Prolonged sleep disturbances not only increase cancer risk but also exacerbate disease progression in diagnosed patients and reduce the effectiveness of treatments [18, 19]. Conversely, cancer and its treatments frequently disrupt sleep patterns. Psychological factors such as anxiety and depression, along with physical factors like pain and chemotherapy, contribute to poor sleep quality and circadian rhythm disruptions [20]. This complex, bidirectional relationship between sleep and cancer highlights the need for further exploration.

Gamma-aminobutyric acid (GABA), a key inhibitory neurotransmitter in the central nervous system (CNS), plays a critical role in regulating neuronal activity by inhibiting excessive neural signaling and maintaining neurological balance. GABA is also known to influence sleep patterns and quality [21]. Beyond its established functions in the CNS, recent studies have unveiled a more intricate role for GABA in tumor progression [22, 23]. Tumor-derived GABA has been shown to drive cancer cell proliferation via the β -catenin signaling pathway [24], while B cell-derived GABA can help tumor

cells evade immune surveillance by modulating immune responses within the tumor microenvironment [25]. These findings highlight the complex and multifaceted relationship between GABA, sleep regulation, and tumor progression, emphasizing the need for further research into the underlying mechanisms.

Fos, a critical transcription factor, encodes the c-Fos protein, which plays an essential role in regulating cell proliferation, differentiation, apoptosis, and stress responses. Recent studies have linked Fos to the development and progression of several cancers. For instance, in head and neck squamous cell carcinoma, c-Fos enhances tumor proliferation and invasion by promoting epithelial-mesenchymal transition and cancer stem cell reprogramming [26]. In gastric cancer, c-Fos facilitates lymph node metastasis by regulating fatty acid metabolism [27]. Moreover, Fos-mediated signaling pathways, including Ras-ERK, JNK, and p38 MAPK, are crucial in driving the formation of malignant phenotypes in cancer cells [28].

In this study, we aim to investigate how sleep deprivation promotes the synthesis and secretion of CNS-derived GABA, regulates FOS expression in CRC cells, and interacts with Schwann cells to drive CRC perineural invasion. By understanding the role of FOS in GABA-regulated PNI, this research aims to shed light on the mechanisms underlying CRC invasion and metastasis, offering promising avenues for the development of targeted therapeutic interventions.

Material and methods

Patients and clinical specimens

Colon cancer tissues and paired adjacent non-cancerous tissues were collected from patients who underwent radical surgery at Wuhan Union Hospital in 2023 (Table S1). Inclusion criteria included patients diagnosed with colorectal adenocarcinoma who had not received preoperative chemoradiotherapy or radiotherapy. Exclusion criteria encompassed patients with a history of other malignancies or significant comorbidities affecting sleep quality. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Huazhong University of Science

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and Technology. All specimens were immediately frozen in liquid nitrogen and stored at -80°C until use.

Pittsburgh sleep quality index (PSQI) questionnaire

The PSQI was used to assess the sleep quality of CRC patients during the month prior to diagnosis. This validated questionnaire measures seven components of sleep: latency, duration, efficiency, disturbances, subjective quality, medication use, and daytime dysfunction, with each component scored from 0 to 3 (higher scores indicate poorer quality). The cumulative PSQI score ranges from 0 to 21, with scores above 10 indicating poor sleep quality. The questionnaire was administered by trained personnel to minimize bias. For analysis, patients were grouped by PSQI score to examine the association between sleep quality, PNI in CRC, and prognosis ([Table S2](#)).

Animals

Male C57BL/6J mice and BALB/c nude mice (6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed in a pathogen-free environment under controlled conditions of $23\text{--}25^{\circ}\text{C}$ with 50–60% humidity and a 12-hour light/dark cycle. Before the experiment began, the mice were acclimated for seven days. All mice had free access to food and water throughout the experimental period. The care and handling of the mice followed the guidelines of the National Research Council, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

Sleep deprivation mouse model

Mice were randomly assigned to either the sleep deprivation (SD) group ($n = 10$) or the control group ($n = 10$). Sleep deprivation was induced using a rotating rod device (Lafayette Instrument, USA), which operated on a cycle of 20 hours of deprivation followed by 4 hours of rest, preventing mice from sleeping by rotating intermittently under light conditions for 21 consecutive days. The control group was housed under the same conditions but without sleep deprivation. Mice were monitored daily for signs of stress or discomfort, and efforts were made

to minimize suffering. After 21 days, mice were euthanized under deep anesthesia, and tissues were collected for further analysis.

Sciatic nerve injection mouse model

Mice were anesthetized with 2% isoflurane, and ophthalmic ointment was applied to prevent eye dryness. After removing fur from the thigh region, a 1 cm incision was made to expose the sciatic nerve. Using blunt dissection, the gluteus maximus and biceps femoris muscles were gently separated to reveal the nerve. Colon cancer cells (5×10^4) suspended in $2\text{ }\mu\text{L}$ of PBS were injected directly into the sciatic nerve using a Hamilton syringe. The injection was performed over 5 seconds, and the needle was held in place for an additional 3 seconds to minimize backflow. Post-surgery, mice were closely monitored for complications and received appropriate care. Seven days post-injection, PNI was assessed using hematoxylin and eosin (H&E) staining of sciatic nerve sections.

Subcutaneous xenograft model

For the subcutaneous xenograft model, BALB/c nude mice were used. Colon cancer cells (1×10^6), mixed with or without Schwann cells, were suspended in $200\text{ }\mu\text{L}$ of PBS and injected subcutaneously into the right flank of the mice. Mice were randomly assigned to four groups based on different treatments: tumor cells alone with PBS, tumor cells alone with GABA, tumor cells with Schwann cells and PBS, and tumor cells with Schwann cells and GABA. GABA was administered intraperitoneally every other day, while control groups received PBS. After 20 days, the mice were euthanized, and tumors were harvested for further analysis.

Cell culture

Human colorectal cancer cell lines SW480 and LoVo, mouse colon cancer cells MC38, and the human Schwann cell line sNF96.2 were obtained from the American Type Culture Collection (ATCC, USA). All cell lines were cultured in DMEM/high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in a 5% CO_2 atmosphere.

Conditioned medium preparation

To prepare conditioned medium (CM), SW480 and LoVo cells were seeded at a density of $4 \times$

10⁵ cells per well in 6-well plates and cultured in DMEM/high glucose with 10% fetal bovine serum. Depending on experimental requirements, cells were either treated with specific induction conditions or transfected accordingly. After 48 hours, the supernatant was collected and centrifuged at 2000×g for 10 minutes to remove any remaining cells and debris. The resulting CM was used immediately for experiments or stored at -80°C for later use.

RNA sequencing (RNA-seq)

Total RNA was extracted from CRC tissues and cultured cells using Trizol reagent (TaKaRa, Japan) following the manufacturer's protocol. RNA quality was assessed, and mRNA was isolated, fragmented, and converted into cDNA. Libraries were prepared using the TruSeq RNA Sample Prep Kit (Illumina, USA) and sequenced on an Illumina HiSeq platform. Differential gene expression analysis was performed using DESeq2, with criteria of fold change ≥ 2 and adjusted *P*-value < 0.05 . Bioinformatics analysis focused on circadian rhythm-associated genes, such as RORC and CRY2, to elucidate their roles in CRC progression.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from CRC cells and frozen tissues using Trizol reagent (TaKaRa, Japan). RNA was reverse-transcribed into cDNA with the RT Master Mix (TaKaRa, Japan). RT-qPCR was performed on an Applied Biosystems StepOnePlus system (ABI, USA) using SYBR Green Master Mix (TaKaRa, Japan). GAPDH served as an internal control, and relative gene expression was calculated with the 2^{-ΔΔCt} method. Primer sequences are provided in [Table S3](#).

Western blot analysis

Protein lysates were prepared from CRC tissues and cell lines using RIPA buffer with PMSF and phosphatase inhibitors (Sigma, USA). Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk, membranes were incubated overnight at 4°C with primary antibodies ([Table S4](#)), followed by secondary antibodies (Cell Signaling Technology, USA). Protein bands were visualized using enhanced chemiluminescence detection reagent (Pierce, USA) and imaged with the ChemiDoc™

XRS Molecular Imager System (Bio-Rad, USA). Band intensities were quantified using ImageJ software.

Small interfering RNA (siRNA) transfection

FOS and EGR1 knockdown in CRC cells was achieved by transfecting cells with siRNA targeting FOS or EGR1 (RiboBio, China) at a final concentration of 50 nM, using Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Scrambled siRNA (RiboBio, China) was used as a negative control. Cells were harvested 48 hours post-transfection, and knockdown efficiency was verified by RT-qPCR and Western blotting. Functional assays were subsequently performed to assess the effects of FOS or EGR1 knockdown on CRC cell proliferation, migration, and invasion.

Cell counting kit-8 (CCK-8) assay

CRC cell proliferation was assessed using the CCK-8 assay. Cells were seeded in 96-well plates at a density of 3×10^3 cells/well and incubated at 37°C with 5% CO₂ under various treatments. At 24, 48, and 72 hours, CCK-8 reagent was added to each well. After incubating for 1 hour at 37°C, absorbance at 450 nm was measured using a microplate reader (BioTek, USA). Each experiment was conducted with six replicates.

Wound healing assay

CRC cells were grown to 90% confluence in 6-well plates, and a linear scratch was made using a sterile pipette tip. Cells were then cultured in serum-free medium, and images of wound closure were captured at 0 and 48 hours under a microscope. The wound healing rate was calculated based on changes in scratch width over time to quantify cell migration.

Transwell migration and invasion assays

For migration and invasion assays, CRC cells or Schwann cells (5×10^4) were suspended in serum-free medium and seeded into the upper chamber of 8 μm pore Transwell inserts (Corning, USA). For the invasion assay, the inserts were pre-coated with Matrigel (BD Biosciences, USA). The lower chamber was filled with medium containing 10% FBS as a chemoattractant.

After 24 hours (migration) or 30 hours (invasion), cells remaining on the upper side of the membrane were removed, and cells on the lower side were fixed with 5% paraformaldehyde for 20 minutes, stained with 0.1% crystal violet for 15 minutes, and counted under a microscope.

In vitro tube formation assay

Matrigel (BD Biosciences, USA) was thawed on ice, and 50 μ L was added to each well of a 96-well plate, followed by incubation at 37°C for 30 minutes to allow gel formation. Schwann cells were treated under various conditions and suspended in serum-free medium at a density of 2×10^4 cells per well. The cell suspension was then seeded onto the Matrigel-coated wells. Plates were incubated at 37°C with 5% CO₂ for 6 hours. Tube formation was observed using an inverted microscope, and images were captured to assess the formation of capillary-like structures.

Enzyme-linked immunosorbent assay (ELISA)

Serum GABA levels and concentrations of neurotrophic factors in conditioned media from CRC cells were quantified using commercial ELISA kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's instructions.

Hematoxylin and eosin (H&E) staining

Sciatic nerves were embedded in OCT compound, oriented for consistent sectioning, and stored at -80°C if immediate sectioning was not possible. Sections were cut at 5 μ m thickness, mounted on glass slides, and stained with hematoxylin and eosin (H&E) to visualize tissue morphology. The stained slides were digitally scanned, and nerve invasion length and area were quantified. Measurements were taken from multiple sections to ensure accuracy.

Immunohistochemistry (IHC) analysis

Formalin-fixed, paraffin-embedded tumor tissues were sectioned into 5 μ m slices. Sections were incubated with primary antibodies against CD31 (Cell Signaling Technology, USA) overnight at 4°C. The next day, sections were incubated with HRP-conjugated secondary antibodies (Cell Signaling Technology, USA) for 1 hour

at 37°C, followed by washing with PBS. DAB substrate was applied until staining developed, and slides were counterstained with hematoxylin. Staining intensity and the percentage of positive cells were independently evaluated by two experienced pathologists.

Immunofluorescence (IF) analysis

Cryosections of CRC tissues were used for immunofluorescence analysis to detect GFAP-positive Schwann cells. After blocking with 5% donkey serum, sections were incubated with primary antibodies against GFAP (1:500; Abcam, UK) overnight at 4°C. This was followed by incubation with fluorescence-labeled secondary antibodies and counterstaining with DAPI. Fluorescence images were captured using an Olympus epifluorescence microscope (Olympus, Tokyo, Japan).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

To quantify neurotransmitter levels, brain tissues from control and SD mice were homogenized with 1 mL of 0.1% formic acid water solution. Samples were vortexed, sonicated in an ice bath for 20 minutes, and centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was filtered through a 0.22 μ m membrane before analysis. Chromatographic separation was performed using a Thermo Fisher UHPLC system with an Agilent Poroshell 120 SB-C18 column, and detection was conducted on an Orbitrap Q Exactive mass spectrometer, specifically optimized for GABA quantification.

Transmission electron microscopy (TEM)

To observe ultrastructural changes in tight junctions within brain tissue of control and SD mice, brain tissues were fixed in electron microscopy fixative for 8 hours, dehydrated in graded ethanol, and embedded in resin overnight. The resin-embedded tissue was cured for 48 hours. Ultrathin sections (60 nm) were cut using an ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined using a Hitachi HT7700 transmission electron microscope (Hitachi, Japan) to assess blood-brain barrier integrity.

Mouse euthanasia methods

During the animal experiments, mice were anesthetized with isoflurane prior to all proce-

dures. Perineural invasion-related operations were conducted under microscopic guidance to minimize surgical trauma. Throughout the procedures, intervention duration was optimized to balance experimental validity and animal welfare. After collection of the required neural tissue specimens, euthanasia was performed via cervical dislocation under sustained anesthesia.

Statistical analysis

All experiments were performed at least in triplicate, with data expressed as mean $\pm$ standard error of the mean (SEM). Normality of data distribution was assessed using the Shapiro-Wilk test. Group comparisons were conducted using a two-tailed Student's t-test for two-group comparisons or one-way ANOVA with Tukey's post hoc test for multiple group comparisons. For continuous time-series data, such as those obtained in the wound healing assay, we employed repeated measures ANOVA, followed by Tukey's post hoc test. Pearson's correlation coefficient was used to assess correlations between variables. Statistical significance was considered at a *P*-value < 0.05. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, USA).

Results

Sleep disturbances correlate with PNI in CRC patients

We initiated our investigation by collecting tumor specimens from CRC patients who had not received prior radiotherapy or chemotherapy. The specimens were categorized into two groups: those with perineural invasion (PNI) and those without. RNA sequencing revealed a marked downregulation in the expression of the circadian rhythm-associated gene *RORC* (RAR-related orphan receptor C) in the PNI cohort, along with a significant upregulation of *CRY2* (cryptochrome circadian regulator 2), a key negative regulator in the circadian clock (**Figure 1A**). Both genes are closely related to sleep, suggesting a potential link between sleep disturbances and PNI.

To further explore this relationship, we employed the Pittsburgh Sleep Quality Index (PSQI) questionnaire to evaluate the sleep quality of CRC patients during the month preceding their

diagnosis [29]. Postoperative pathological outcomes were assessed and the patients were categorized into PNI and non-PNI groups based on the pathological findings. The results indicated that patients in the PNI group exhibited significantly higher PSQI scores compared to the non-PNI group (**Figure 1B**), indicating a marked decline in sleep quality among those with PNI. Additionally, Kaplan-Meier survival analysis, using data from The Cancer Genome Atlas (TCGA), was performed to investigate the prognostic implications of sleep-related gene expression. Our analysis showed that CRC patients with lower expression of key circadian rhythm genes *CLOCK* (circadian locomotor output cycles kaput) exhibited poorer survival outcomes (**Figure 1C**). Several other sleep-related genes, including *CRY2*, *RORC*, *PER1* (period circadian regulator 1), *PER3* (period circadian regulator 3) and *NR1D1* (nuclear receptor subfamily 1, group D, member 1), were closely associated with the prognosis of CRC patients (**Figures 1D, 1E** and **S1A-C**). Taken together, these findings strongly suggest that sleep disturbances are associated with PNI in CRC patients and may negatively affect their overall prognosis.

Sleep disturbances promote PNI in CRC through CNS-derived GABA

Aiming to investigate whether sleep disturbances contribute to PNI in CRC, we employed a well-established mouse model of sleep deprivation (SD) [30, 31]. Mice underwent 21 days of sleep deprivation, with sciatic nerve injections administered on the 15th day. On day 22, the mice were euthanized, and their sciatic nerves were harvested for further analysis. H&E staining showed a significant increase in nerve invasion in the SD group compared to the control group, characterized by both greater depth and extent (**Figure 2A-D**), indicating that sleep disturbances may promote the progression of PNI.

Given the established link between neurotransmitter dynamics in the central nervous system (CNS) and sleep disturbances [32, 33], we collected brain tissue from SD mice and performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify alterations in neurotransmitter levels. Among the detected neurotransmitters, γ -aminobutyric acid (GABA) was found to be significantly elevated in the SD

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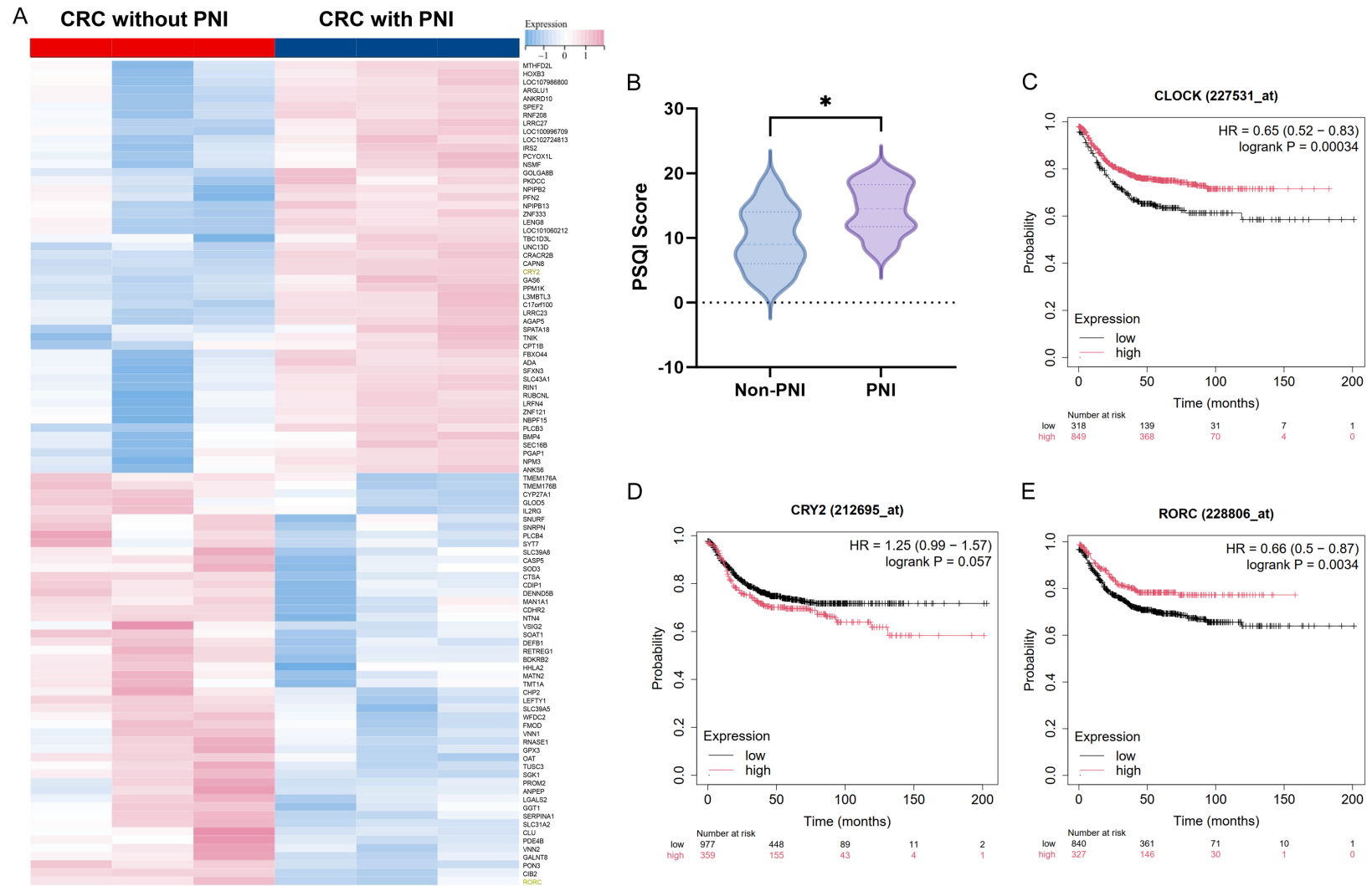


Figure 1. Sleep disturbances correlate with PNI in CRC patients. (A) Heatmap showing the gene expression profiles from colorectal cancer (CRC) patients with ($n = 3$) or without ($n = 3$) perineural invasion (PNI) based on RNA sequencing. The heatmap highlights significant downregulation of RORC and upregulation of CRY2 in the PNI group, both of which are associated with circadian rhythm and sleep regulation. (B) Violin plot comparing Pittsburgh Sleep Quality Index (PSQI) scores between CRC patients with and without PNI. Patients in the PNI group exhibited significantly higher PSQI scores, indicating poorer sleep quality. (C-E) Kaplan-Meier survival curves for CRC patients stratified by expression levels of circadian rhythm genes. Patients with low expression of *CLOCK* (C) and *RORC* (E) had significantly worse survival, while high expression of *CRY2* (D) was associated with shorter survival. $*P < 0.05$.

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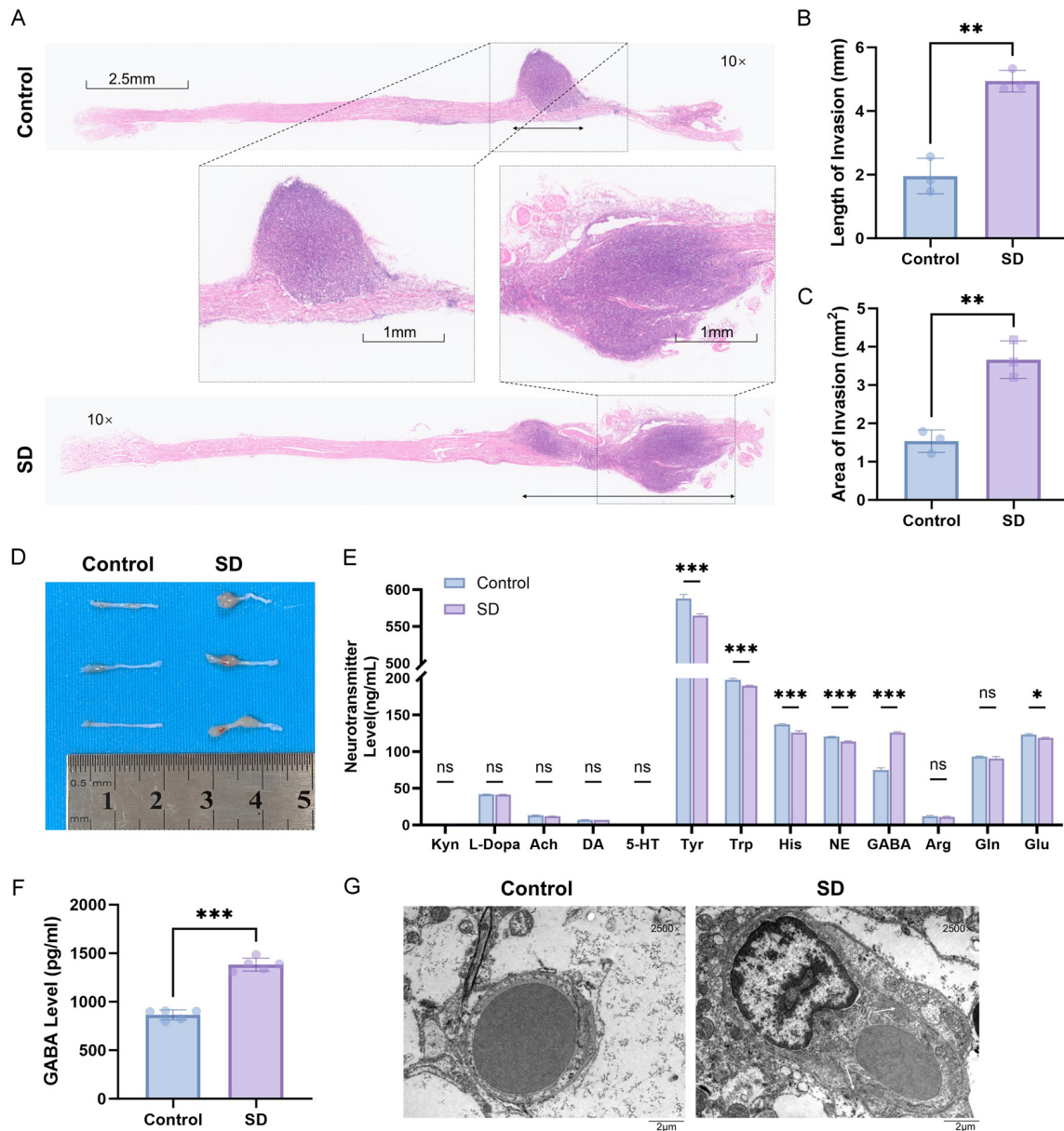


Figure 2. Sleep disturbances promote PNI in CRC through CNS-Derived GABA. A-C. H&E staining of sciatic nerve sections from control and sleep-deprived (SD) mice reveals a significant increase in the length and area of nerve invasion in the SD group, with quantification showing marked differences. D. Representative images of dissected sciatic nerves. E. LC-MS/MS analysis of neurotransmitters in brain tissue shows significantly increased GABA levels in SD mice. F. ELISA measurement confirms a significant elevation of GABA levels in peripheral blood of SD mice. G. TEM images of the blood-brain barrier (BBB) illustrate structural damage in SD mice, including widened tight junctions and endothelial cell damage. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

group (Figure 2E). Subsequently, serum GABA concentrations, measured using enzyme-linked immunosorbent assay (ELISA), confirmed a substantial increase in GABA levels in SD mice compared to controls (Figure 2F). Transmission electron microscopy (TEM) further highlighted structural damage to the blood-brain barrier, including widened tight junctions and endothe-

lial cell damage (Figure 2G). These findings indicated that sleep disturbances increase GABA synthesis in the brain, compromise blood-brain barrier integrity, and facilitate the entry of GABA into the peripheral circulation.

To clarify whether GABA promotes the progression of CRC, we re-established the sciatic nerve

injection model and administered intraperitoneal injections of either GABA or phosphate-buffered saline (PBS) as a control. H&E staining revealed a significant increase in both the area and depth of nerve invasion in GABA-treated mice compared to controls ([Figure S2A-D](#)), confirming GABA a critical mediator in PNI progression. Based on these results, we propose that sleep disturbances may induce the production and peripheral secretion of CNS-derived GABA, thereby promoting PNI in CRC.

GABA-induced FOS upregulation drives PNI in CRC

To investigate the impact of GABA on PNI in CRC, we treated SW480 cells with GABA and conducted RNA sequencing to identify gene expression changes (**Figure 3A, 3B**). Among the five most significantly altered genes, *FOS* (FBJ osteosarcoma oncogene) was selected for further analysis due to the consistent elevation across multiple CRC cell lines, validated using RT-qPCR (**Figure 3C**). *FOS* is a crucial oncogene that encodes the C-FOS protein, essential for processes such as cell proliferation, differentiation, and transformation. Western blot analysis further validated these observations, revealing a significant increase in C-FOS protein levels in GABA-treated CRC cells (**Figure 3D**). Next, to verify whether GABA influences CRC progression through the regulation of *FOS*, we knocked down *FOS* expression in GABA-treated CRC cells using siRNA. Functional assays, including CCK-8, wound healing, and Transwell assays, indicated that the knockdown of *FOS* significantly attenuated the GABA-induced promotion of CRC cell proliferation, migration, and invasion (**Figure 3E-G**). Supporting these observations, western blot analysis showed increased levels of CyclinD1, CyclinE, and N-cadherin, alongside a reduction in E-cadherin expression in the GABA-treated group. All of these changes were reversed following *FOS* knockdown (**Figure 3H**), highlighting the critical role of *FOS* in GABA-mediated CRC progression.

In line with our *in vitro* findings, knockdown of *Fos* also suppressed tumor growth *in vivo*. We administered intraperitoneal injections of GABA and injected CRC cells into the sciatic nerve of mice, either with *Fos* knockdown or unmodified *Fos* expression. H&E staining revealed that *Fos* knockdown significantly reduced

the severity of PNI in CRC cells ([Figure S3A-C](#)). Collectively, these findings suggest that GABA enhances CRC cell proliferation, migration, and invasion by upregulating *FOS* expression, which ultimately drives the progression of PNI.

GABA-induced BDNF secretion from CRC cells activates schwann cells

Schwann cells have gained increasing attention for their involvement in the initiation and progression of multiple cancers, particularly in PNI. Recent studies indicate that in the tumor microenvironment, Schwann cells become activated and reprogrammed, shifting toward an immature, repair-like phenotype. This reprogramming involves upregulation of immature markers and downregulation of differentiated ones [34]. Notably, these reprogrammed Schwann cells express high levels of glial fibrillary acidic protein (GFAP) [35]. To investigate whether sleep disturbance affect Schwann cell activity in CRC, we categorized patients into two groups based on their PSQI scores: a good sleep quality group (scores 0-10) and a poor sleep quality group (scores 11-21). Immunofluorescence staining of CRC tissue samples revealed a significantly higher infiltration of GFAP-positive Schwann cells in the poor sleep quality group, compared to the good sleep quality group and normal colorectal tissues (**Figure 4A, 4B**). Kaplan-Meier survival analysis showed that CRC patients with elevated GFAP expression exhibited a worse prognosis (**Figure 4C**), supporting the notion that Schwann cells contribute to PNI progression under the influence of sleep disturbances.

Since GABA, as a neurotransmitter, may also affect Schwann cells, we sought to investigate its potential role in this process. Schwann cells treated directly with GABA exhibited no significant changes in their proliferation ([Figure S4A](#)). However, when co-cultured Schwann cells with conditioned medium (CM) from GABA-treated CRC cells, their proliferation, migration, and invasion were markedly enhanced, as confirmed by CCK8, wound healing, and Transwell assays (**Figure 4D-F**). Notably, these effects were significantly attenuated upon *FOS* knockdown in CRC cells, underscoring the role of *FOS* in mediating the GABA-induced activation of Schwann cells. Additionally, KEGG pathway analysis of Schwann cells co-cultured with CM

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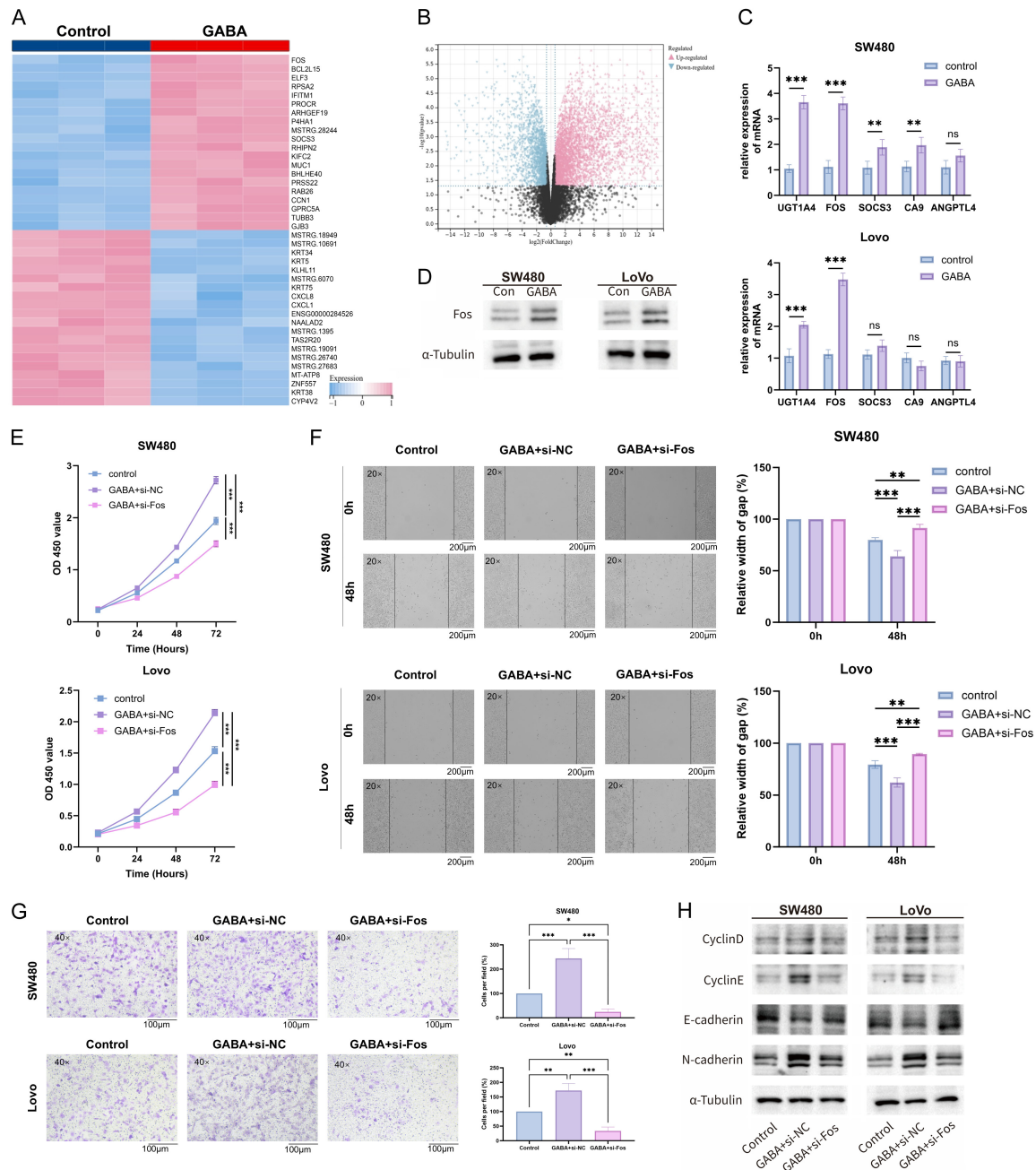


Figure 3. GABA-induced *FOS* upregulation drives PNI in CRC. (A, B) Heatmap (A) and volcano plot (B) showing differentially expressed genes in SW480 cells treated with GABA. (C) RT-qPCR analysis of gene expression levels in SW480 and Lovo cells treated with GABA. *FOS* showed significant upregulation in both cell lines. (D) Analysis confirming elevated C-FOS protein levels in GABA-treated SW480 cells. (E-G) Functional assays showing the effects of *FOS* knockdown on GABA-treated CRC cells. CCK-8 assays (E) demonstrate reduced cell proliferation, wound healing assays (F) show impaired migration, and Transwell assays (G) reveal decreased invasion in SW480 and Lovo cells after *FOS* knockdown. (H) WB analysis of key proliferation and migration markers (CyclinD, CyclinE, N-cadherin) and reduced E-cadherin levels in GABA-treated cells, with changes reversed upon *FOS* knockdown. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

from GABA-treated CRC cells exhibited a significant upregulation of genes related to inflammation and angiogenesis (Figure S4B).

Given that Schwann cells are modulated by various neurotrophic factors [12, 36], we collected the CM from GABA-treated CRC cells

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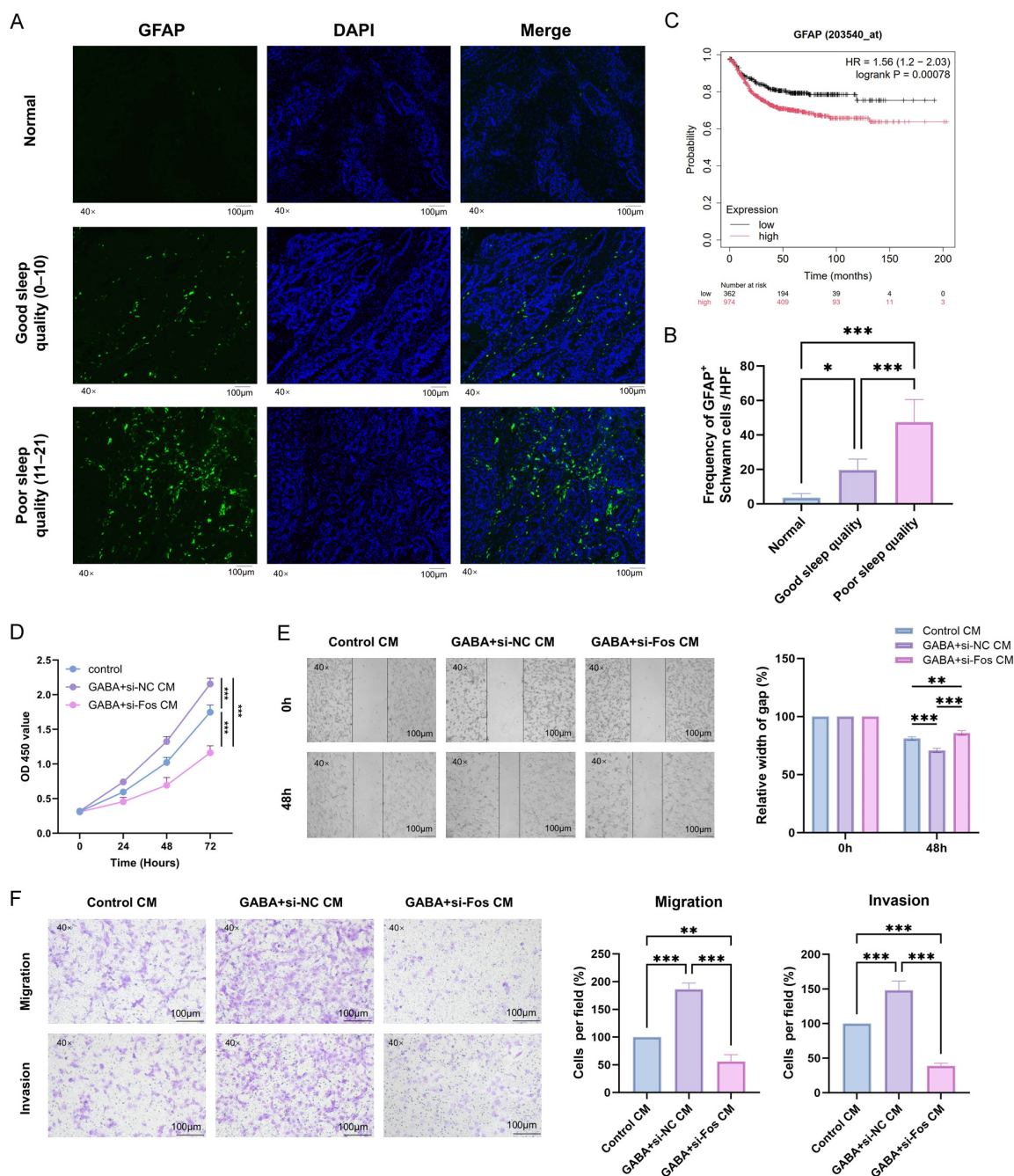


Figure 4. GABA-induced BDNF secretion from CRC cells activates schwann cells. (A) Representative immunofluorescence images showing GFAP-positive Schwann cells in normal colorectal tissues and CRC tissues from patients with good or poor sleep quality. Schwann cell infiltration was higher in CRC tissues, particularly in the poor sleep quality group. (B) Quantification of GFAP-positive Schwann cells per HPF, showing significantly more Schwann cells in the poor sleep quality group compared to the good sleep quality group and normal tissues. (C) Kaplan-Meier survival analysis of CRC patients stratified by GFAP expression, with higher GFAP levels associated with worse prognosis. (D-F) CCK8 (D), wound healing (E), and Transwell assays (F) show that conditioned medium (CM) from GABA-treated CRC cells significantly enhanced Schwann cell proliferation, migration, and invasion, effects that were reduced by FOS knockdown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

and quantified the concentrations of several neurotrophic factors using ELISA. The results

revealed a marked elevation in brain-derived neurotrophic factor (BDNF) levels following

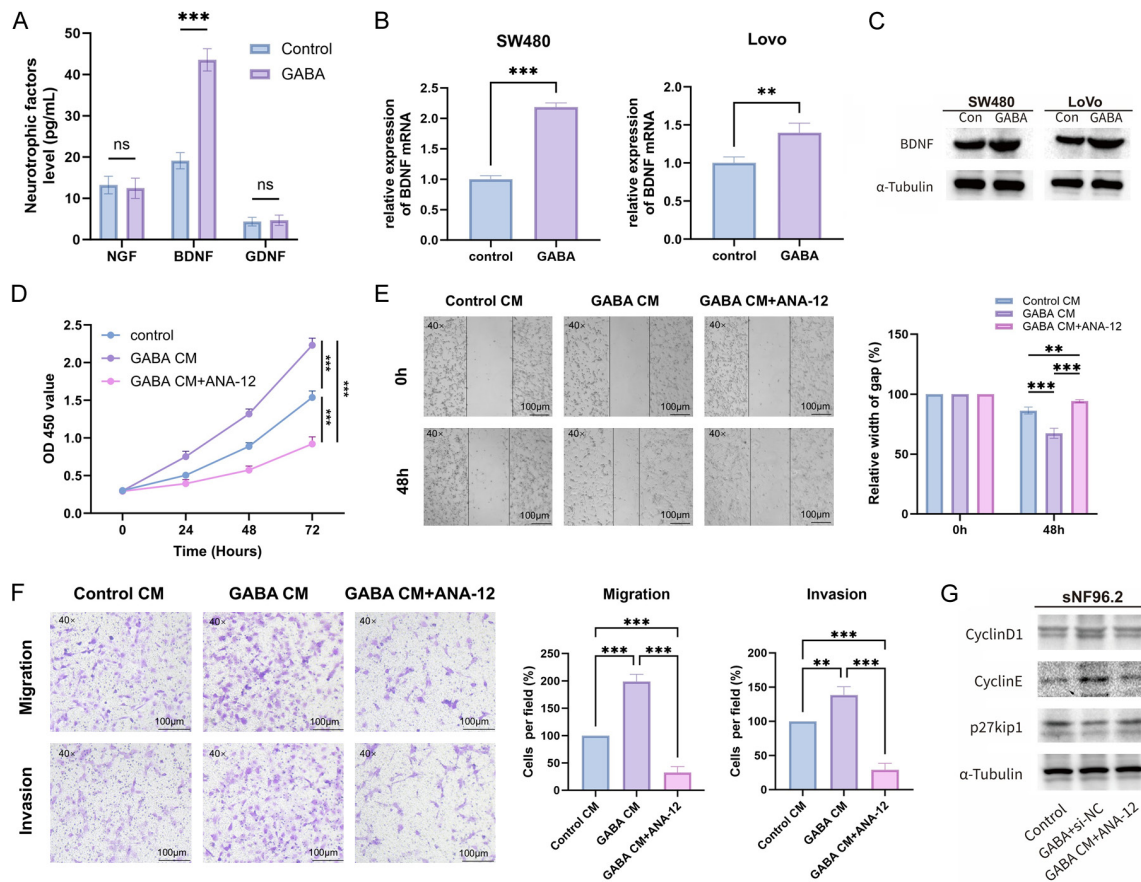


Figure 5. GABA-induced BDNF secretion from CRC cells activates Schwann cells. (A) ELISA of neurotrophic factor levels in CM from GABA-treated CRC cells, showing a significant increase in BDNF compared to controls. (B) RT-qPCR analysis of BDNF mRNA expression in SW480 and Lovo cells treated with GABA, showing significant upregulation. (C) WB analysis confirms elevated BDNF protein levels in GABA-treated CRC cells. (D-F) CCK8 (D), wound healing (E), and Transwell assays (F) demonstrate enhanced Schwann cell proliferation, migration, and invasion following treatment with CM from GABA-treated CRC cells, effects significantly reduced by the TrkB inhibitor ANA-12. (G) WB analysis of key proliferation markers shows increased CyclinD1, CyclinE, and reduced p27kip1 expression in Schwann cells treated with GABA CM. $^{**}P < 0.01$, and $^{***}P < 0.001$.

GABA treatment (Figure 5A). We then analyzed the expression of BDNF in CRC cells and observed a significant upregulation at both RNA and protein levels following GABA treatment (Figure 5B, 5C). Previous studies have demonstrated that BDNF exerts its effects primarily through its high-affinity receptor, TrkB [37]. To confirm the impact of BDNF on Schwann cell behavior, we used the TrkB-specific inhibitor ANA-12. Inhibition of TrkB markedly diminished the GABA-induced enhancement of Schwann cell proliferation, migration, and invasion stimulated by CM derived from CRC cells (Figure 5D-F). Western blot analysis further supported these findings, showing increased expression of CyclinD1 and CyclinE and decreased expression of p27kip1 in the presence of

GABA, which were reversed upon TrkB inhibition (Figure 5G). Moreover, the addition of recombinant human BDNF, at concentrations comparable to those secreted by CRC cells, significantly promoted Schwann cell proliferation, migration, and invasion (Figure S4C-E). To determine the role of *FOS* in GABA-induced BDNF secretion in CRC cells, we treated CRC cell lines with GABA and subsequently knocked down *FOS* expression. Following *FOS* knockdown, we observed a significant reduction in both BDNF mRNA and protein levels (Figure S4F, S4G), accompanied by a corresponding decrease in BDNF secretion (Figure S4H). These findings are consistent with the previous results, where *FOS* knockdown inhibited Schwann cell proliferation, migration, and inva-

sion. Taken together, our results strongly demonstrate that GABA stimulates Schwann cell activation by inducing BDNF secretion from CRC cells through the upregulation of *FOS* expression, thereby enhancing Schwann cell proliferation, migration, and invasion, which contribute to PNI progression.

GABA promotes FOS transcription through EGR1 upregulation

Based on the above findings, GABA was shown to significantly upregulate *FOS* expression in CRC cells, leading to enhanced proliferation, migration, and invasion, as well as increased synthesis and secretion of BDNF, which ultimately activates Schwann cells. Despite these insights, the precise mechanism through which GABA regulates *FOS* transcription has remained unclear. To address this, we used the JASPAR database to predict potential transcription factors involved in *FOS* regulation and integrated with transcriptome sequencing data, identifying *EGR1* as the key regulator (**Figure 6A**). *EGR1*, a zinc finger transcription factor, plays a pivotal role in key biological processes such as cell proliferation, differentiation, and apoptosis, and is known to be induced by various stimuli, including growth factors, cytokines, stress signals, and pharmacological agents [38, 39]. We first validated *EGR1* expression in various CRC cell lines using RT-qPCR and Western blot analysis. Since GABA typically exerts its regulatory effects via GABA B receptors [40], we also used the GABA B receptor antagonist SCH 50911 to assess GABA's effect on *EGR1* expression. Our results revealed that GABA treatment consistently elevated both mRNA and protein levels of *EGR1* in CRC cells (**Figure 6B, 6C**). Furthermore, knocking down *EGR1* resulted in a marked reduction in both mRNA and protein levels of *FOS* (**Figure 6D, 6E**), accompanied by a decrease in BDNF secretion (**Figure 6F**). Together, these findings suggest that GABA facilitates PNI in CRC by upregulating *EGR1*, which in turn activates *FOS* transcription.

BDNF promotes schwann cell angiogenesis

Building on our previous sequencing results, we observed that GABA-treated tumor CM significantly amplified the angiogenic potential of Schwann cells. Given this observation, we hypothesized that BDNF might accelerate

tumor progression by stimulating angiogenesis in Schwann cells. According to the results of the in vitro tube formation assay, GABA-induced CM significantly increased Schwann cell angiogenesis compared to the control group (**Figure 7A**), with a corresponding increase in VEGF expression (**Figure 7B**). As expected, this effect was markedly diminished following TrkB inhibition. Furthermore, the application of recombinant human BDNF further stimulated Schwann cell angiogenesis and elevated VEGF expression levels (**Figure 7C, 7D**). We then performed subcutaneous injections of Schwann cells mixed with tumor cells into mice, followed by intraperitoneal administration of either GABA or PBS as a control. In PBS-treated mice, there were no significant differences in CD31 staining between the single-injection and mixed-injection groups. In contrast, GABA-treated mice exhibited a significant increase in CD31 staining in the mixed-injection group (**Figure 7E**). These results indicate that GABA promotes the secretion of BDNF by CRC cells, which in turn enhances Schwann cell-mediated angiogenesis and drives further tumor progression.

Discussion

In this study, we uncovered several key findings that enhance our understanding of how sleep disturbances promote perineural invasion (PNI) in colorectal cancer (CRC) through neural signaling pathways. First, we observed that sleep disturbances lead to elevated levels of GABA in the central nervous system (CNS). This CNS-derived GABA crosses the compromised blood-brain barrier into the peripheral circulation and significantly promotes the secretion of brain-derived neurotrophic factor (BDNF) in CRC cells. This establishes a direct link between poor sleep quality and a neurochemical cascade that enhances tumor cell behavior.

Second, our data demonstrated that GABA-induced BDNF secretion plays a crucial role in reprogramming Schwann cells, the glial cells that support and insulate peripheral nerves. Once activated, these Schwann cells exhibit increased proliferation, migration, and angiogenic activity, facilitating the spread of cancer cells along nerve pathways and promoting PNI. Finally, we identified the upregulation of *FOS* in CRC cells as a key downstream mechanism in GABA-mediated progression. *FOS*, a well-known oncogene, amplifies CRC cell aggressiveness and indirectly influences Schwann cell activa-

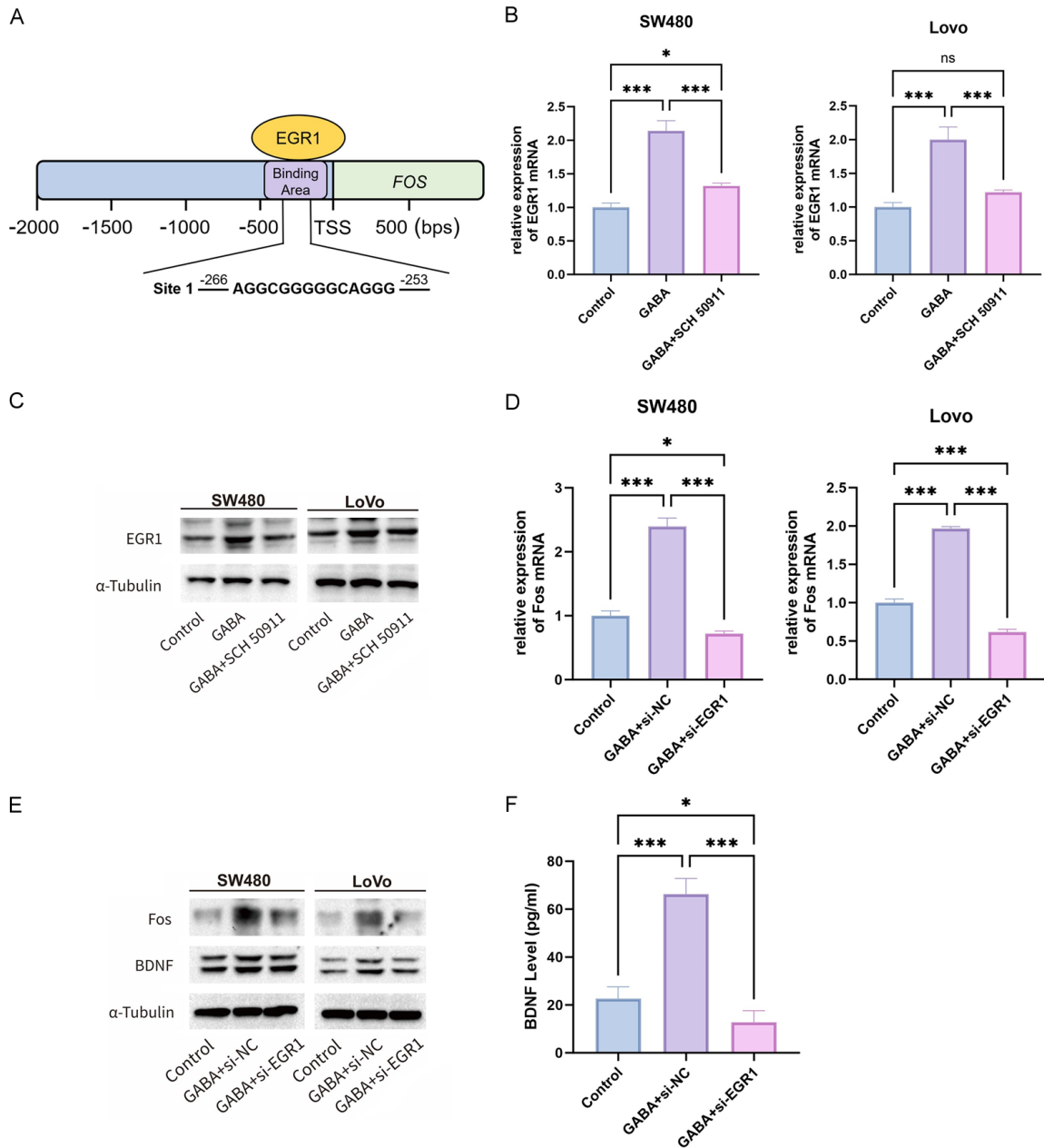


Figure 6. GABA promotes fos transcription through EGR1 Upregulation. A. Diagram showing the predicted EGR1 binding site in the FOS promoter, identified using the JASPAR database. B. RT-qPCR analysis showing GABA-induced upregulation of EGR1 mRNA levels in SW480 and Lovo CRC cells. C. WB confirming increased EGR1 protein expression following GABA treatment. D. Knockdown of EGR1 reduces FOS mRNA expression in SW480 and Lovo cells, as shown by RT-qPCR. E. WB analysis of C-FOS and BDNF protein levels, showing decreased expression upon EGR1 knockdown. F. ELISA results showing reduced BDNF secretion in CRC cells after EGR1 knockdown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

tion through enhanced BDNF signaling, creating a supportive microenvironment for PNI. Collectively, these findings highlight a novel pathway through which sleep disturbances may enhance CRC aggressiveness and invasiveness by activating neural dynamics within the tumor microenvironment (**Figure 8**).

Our findings align with existing literature on the roles of neurotransmitters like GABA in cancer progression and immune modulation but also expand upon it. Previous studies have shown that GABA influences tumor growth through mechanisms such as the β -catenin signaling pathway, which is involved in immune evasion

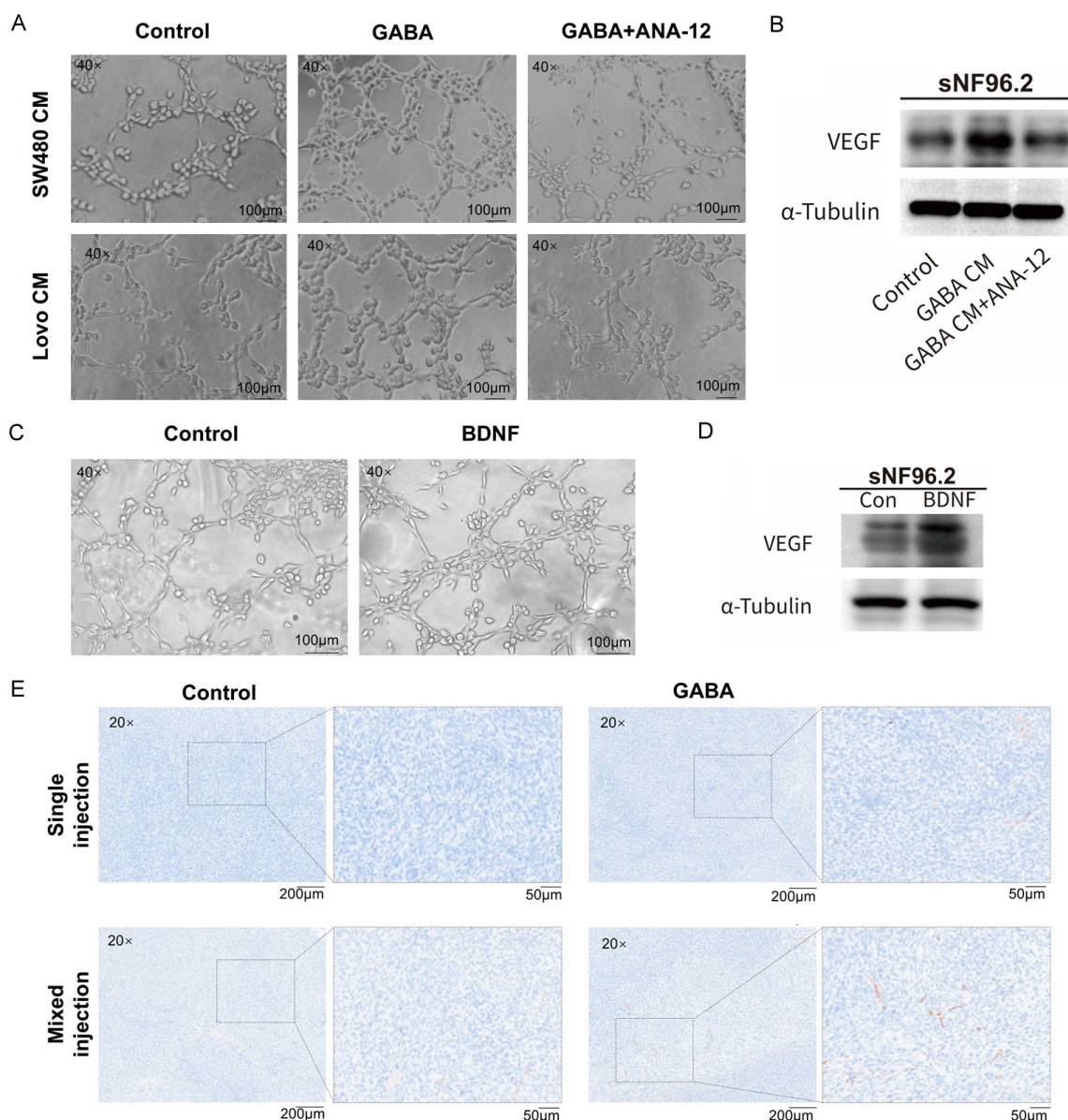


Figure 7. BDNF promotes Schwann cell angiogenesis. A. Schwann cell angiogenesis is enhanced by GABA-treated CRC cell CM, with reduced effects after TrkB inhibition. B. WB analysis shows increased VEGF expression in Schwann cells treated with GABA CM. C. Recombinant BDNF promotes Schwann cell angiogenesis. D. WB confirms increased VEGF expression in Schwann cells treated with BDNF. E. CD31 staining shows increased angiogenesis in GABA-treated mice with mixed Schwann cell-tumor cell injections.

and cellular proliferation [24, 25]. While prior research has linked GABA to immune modulation and tumor cell behavior, our study demonstrates that GABA also acts as a potent modulator of Schwann cells and stimulates BDNF secretion, unveiling a new mechanism of tumor-nerve interaction. This suggests that GABA's role in cancer progression extends beyond direct effects within the CNS to influencing the surrounding neural microenviron-

ment and facilitating PNI. This challenges the traditional view of GABA as a CNS-limited factor in cancer progression, proposing instead that it acts systemically to link sleep disturbances with CRC aggressiveness through both direct and indirect mechanisms.

Of course, sleep disturbances induces a stress response in the body, with stress hormones such as cortisol and norepinephrine typically

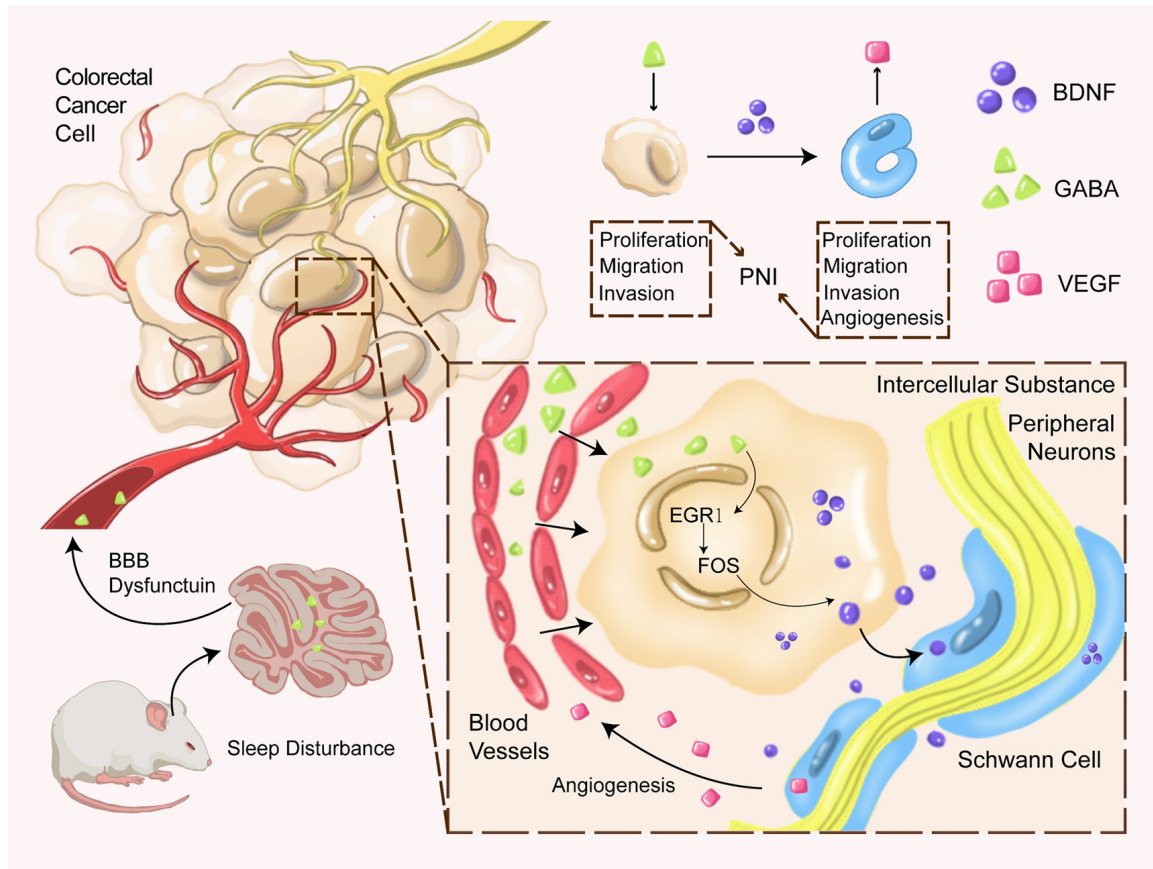


Figure 8. Proposed mechanism by which sleep disturbances promote colorectal cancer perineural invasion via GABA-mediated BDNF secretion. Sleep deprivation leads to an increase in GABA synthesis in the central nervous system and disruption of the blood-brain barrier (BBB), allowing GABA to enter the peripheral circulation. Elevated peripheral GABA levels stimulate CRC cells, leading to upregulation of EGR1 expression and increased transcription of FOS. This results in enhanced tumor cell proliferation, migration, and invasion. Additionally, GABA-treated CRC cells secrete higher levels of BDNF, which activates Schwann cells by promoting their proliferation, migration, and angiogenesis. Activated Schwann cells further facilitate tumor progression by enhancing angiogenesis within the tumor microenvironment. Collectively, this pathway illustrates how sleep disturbances can exacerbate CRC perineural invasion through a GABA-FOS-BDNF axis involving Schwann cell activation.

rising as a result. Our team's previous research indicates that alterations in stress hormones and cytokines under such conditions further affect intestinal metabolism and disrupt the stability of the gut microbiota [41]. These changes may also serve as confounding factors that influence tumor progression to some extent. This represents one of the limitations in the mechanistic discussion of this study. Moreover, regarding the origin of GABA, based on our earlier experiments, we observed changes in the levels of various neurotransmitters - including GABA, norepinephrine, epinephrine, and 5-HT - in the peripheral blood of sleep-deprived mice, among which GABA showed a significant increase. Concurrently, we detected markedly elevated expression in the brain of

glutamate decarboxylase-1, which is involved in GABA synthesis, as well as GABA vesicular transporters responsible for its release [42]. This collectively contributes to maintaining elevated GABA levels in the peripheral circulation. Therefore, we hypothesize that the substantially increased GABA in peripheral blood is primarily of central nervous system origin and plays a pivotal role in tumor progression.

Theoretically, this research broadens our understanding of neuroendocrine regulation in cancer by highlighting the complex role neural signals play in CRC progression. By demonstrating how sleep disturbances lead to neural interactions that enhance tumor aggressiveness, our study lays a foundation for advancing

new models within cancer neuroscience. Traditionally, neural factors have been recognized primarily in terms of local nerve-tumor interactions. However, our findings indicate a broader, systemic impact - specifically, that CNS-derived GABA acts beyond the brain to modulate cancer cell behavior and the tumor microenvironment. This underscores the importance of the GABA-FOS-BDNF axis as a novel pathway connecting neural regulation to cancer cell proliferation, migration, and Schwann cell activation. Identifying FOS and BDNF as downstream mediators of GABA in CRC provides a deeper theoretical framework for understanding CRC progression at the intersection of neuroscience and oncology. It suggests that the CNS may indirectly influence cancer progression through neurochemical cascades triggered by behavioral factors like sleep quality, potentially reshaping our neurobiological approach to CRC.

Moreover, our insights into the interaction between neural signals and tumor progression contribute to the emerging concept of “neuro-immuno-oncology [43]”. This field focuses on how neural signals shape processes within tumors, which has significant implications for understanding the tumor microenvironment and metastasis. The role of GABA as both a sleep regulator and a modulator of tumor dynamics in CRC reinforces the potential for integrating cancer biology with sleep neuroscience. This integration creates a more cohesive theoretical model for the tumor microenvironment. Overall, our findings could pave the way for future theoretical exploration into the neurobiological determinants of cancer, especially as researchers seek to unravel how CNS-derived signals might affect the behavior of other tumor-associated cell types, such as macrophages, fibroblasts, and immune cells.

From a practical standpoint, our findings underscore the critical importance of sleep management in CRC patient care. Poor sleep quality, by increasing GABA levels and activating Schwann cells, could potentially accelerate CRC progression, particularly in cases involving PNI. Integrating sleep quality assessments into routine care for CRC patients could serve as an early intervention strategy to mitigate risks associated with aggressive disease. Monitoring biomarkers like GABA and BDNF might also help clinicians identify patients at higher risk for PNI

and other invasive phenotypes, enabling more personalized and targeted treatments. This approach offers a novel, non-invasive diagnostic tool that aligns with a more holistic view of cancer management, considering behavioral and lifestyle factors alongside traditional clinical markers.

These insights also have implications for public health policies. Given the established link between sleep quality and cancer progression, campaigns promoting better sleep hygiene could be valuable preventive measures - not only for CRC but potentially for other cancers where neural interactions contribute to tumor aggressiveness. Public health initiatives could educate at-risk populations about the importance of sleep for overall health, encouraging lifestyle adjustments to support both general well-being and cancer prevention. Additionally, these findings may inspire further research into sleep-modifying interventions for CRC patients, such as cognitive-behavioral therapy for insomnia or pharmacological aids that regulate GABA signaling pathways. By emphasizing sleep as a modifiable factor in cancer progression, this study opens pathways for healthcare providers to advocate for sleep-focused interventions as part of a comprehensive cancer care strategy, potentially improving outcomes for CRC patients.

However, this study has limitations. The use of animal models, while insightful for mechanistic exploration, raises questions about the direct applicability of these findings to human CRC. The specific conditions under which GABA influences Schwann cell behavior may vary across different CRC subtypes, limiting the generalizability of our conclusions. We also did not examine potential interactions with other neurotransmitters that could impact CRC and PNI similarly. Additionally, the precise conditions under which GABA promotes BDNF secretion and FOS activity in CRC remain unclear, especially in diverse environmental and genetic contexts.

Future studies should investigate these pathways further by exploring the role of other neurotransmitters in CRC-related PNI [5], identifying molecular inhibitors targeting GABA or FOS in CRC, and examining the effects of sleep modulation therapies on CRC progression. Employing advanced techniques like spatial tran-

scriptomics could provide more detailed insights into the molecular dynamics of Schwann cell-tumor interactions.

Conclusion

In conclusion, our study elucidates a novel mechanistic pathway linking sleep disturbances to the progression of CRC through the facilitation of PNI. We demonstrate that sleep disruption elevates central nervous system-derived GABA, which, upon reaching the peripheral circulation due to a compromised blood-brain barrier, up-regulates FOS expression in CRC cells via EGR1. This in turn enhances BDNF secretion. The increased BDNF activates Schwann cells through the TrkB receptor, promoting their proliferation, migration, and pro-angiogenic activity, thereby creating a neural microenvironment conducive to PNI and tumor aggressiveness. These findings establish the GABA-EGR1-FOS-BDNF axis as a critical link between behavioral factors and cancer biology, highlighting the importance of sleep quality in CRC management. They not only advance the theoretical framework of cancer neuroscience but also offer practical implications, suggesting that sleep assessment and modulation, alongside targeting this neurochemical axis, could serve as promising strategies for risk stratification and therapeutic intervention in CRC patients. Future research should validate these mechanisms in human contexts and explore integrated therapeutic approaches.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (Grant number: 82072744; 822074874) and Hubei Key Laboratory of Regenerative Medicine and Multi-disciplinary Translational Research (Grant number: 2025zsyx11).

Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

None.

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Table S1. Information of CRC patients for transcriptome sequencing

characteristic	CRC patients without PNI	CRC patients with PNI
No.of patients	3	3
Age (average)	67.33	71
Gender (male/female)	2/1	1/2
Pathologic T stage	T3	T3
Pathologic N stage	N0	N0
Pathologic M stage	M0	M0
Tumor differentiation	moderate	moderate

Table S2. Information of CRC patients for PSQI test

PSQI score	Pathologic T stage	PNI	Lymph Node Metastasis	Distant Metastasis
3	T3	-	-	-
8	T3	+	-	-
20	T3	+	-	-
18	T4	+	+	+
9	T3	+	-	-
6	T4	-	-	-
12	T3	+	-	-
4	T3	-	-	-
19	T4	+	-	-
11	T3	+	+	-
19	T3	+	-	-
16	T3	+	-	-
13	T3	-	+	-
12	T3	+	-	-
8	T3	-	-	-
15	T3	+	+	-
12	T3	+	+	-
14	T3	-	-	-
9	T2	-	-	-
17	T3	+	+	-
14	T3	+	+	-
18	T4	-	+	-
7	T3	-	-	-
15	T3	-	+	-
11	T3	-	+	-

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Table S3. Primers of genes in this research for qRT-PCR

Primer set	Primers	Sequence (5'-3')
GAPDH	Forward	GGGGAGCCAAAAGGGTCATCATCT
	Reverse	GACGCCTGCTTCACCACCTTCTTG
UGT1A4	Forward	GAGCAGAGCTACAGCAACAG
	Reverse	AGGTCAGGATGCTGAGGAAC
FOS	Forward	AGCTGACTGATACCTAGCTGCA
	Reverse	AAAGGAAGACGTGTAGGCTTGG
SOCS3	Forward	AGCTTGTGCCTCAAGACCTTC
	Reverse	ATCTTGTGCTTGTGCCATGTG
CA9	Forward	TGCACTGTCACCAAGGAGAT
	Reverse	ATGTGCCCTCAGGCTTGAAG
ANGPTL4	Forward	GTGCGACTCAGACACGGAA
	Reverse	TGGCAGGGAACAGGAGACT
BDNF	Forward	GAGCGTGTGTGACAGTATTAGCG
	Reverse	GGAACATTGTGGCTTTGACG
EGR1	Forward	GAGCGAACAACCCTATGAGC
	Reverse	GTGAGGAAGACGATGAAGCC

These primers sequences had been validated in the NCBI database.

Table S4. Information of primary antibodies applied in this study

Gene Specificity	Manufacture	Catalogue number	Dilution rate			specificity
			WB	IHC	IF	
α-tubulin	Abclonal	AC007	1:500	-	-	Rabbit
E-cadherin	Proteintech	60335-1-Ig	1:1000	-	-	Mouse
N-cadherin	Proteintech	22018-1-AP	1:1000	-	-	Rabbit
Cyclin E	Proteintech	11554-1-AP	1:1000	-	-	Rabbit
Cyclin D1	Proteintech	60186-1-Ig	1:1000	-	-	Mouse
BDNF	Abclonal	A1307	1:500	-	-	Rabbit
EGR1	Abclonal	A23424	1:500	-	-	Rabbit
FOS	Abclonal	A0236	1:500	-	-	Rabbit
VEGF	Abclonal	A1199	1:500	-	-	Rabbit
p27kip1	Abclonal	A16633	1:500	-	-	Rabbit
CD31	Abclonal	A19014	-	1:500	-	Rabbit
GFAP	Abclonal	A26778	-	-	1:200	Rabbit

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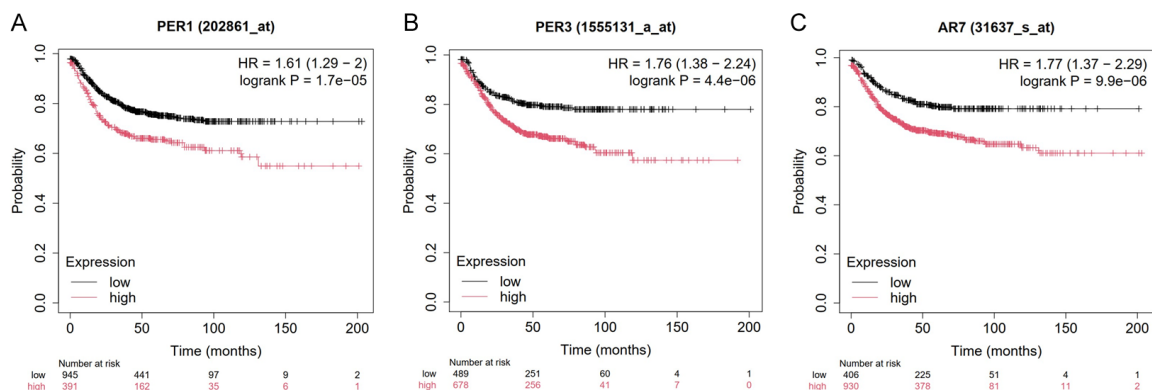


Figure S1. Sleep disturbances correlate with PNI in CRC patients. (A-C) Kaplan-Meier survival curves for CRC patients stratified by gene expression levels of PER1 (A), PER3 (B), and NR1D1 (C). Higher expression of these genes was associated with significantly shorter overall survival. *P* values and hazard ratios are indicated in each panel.

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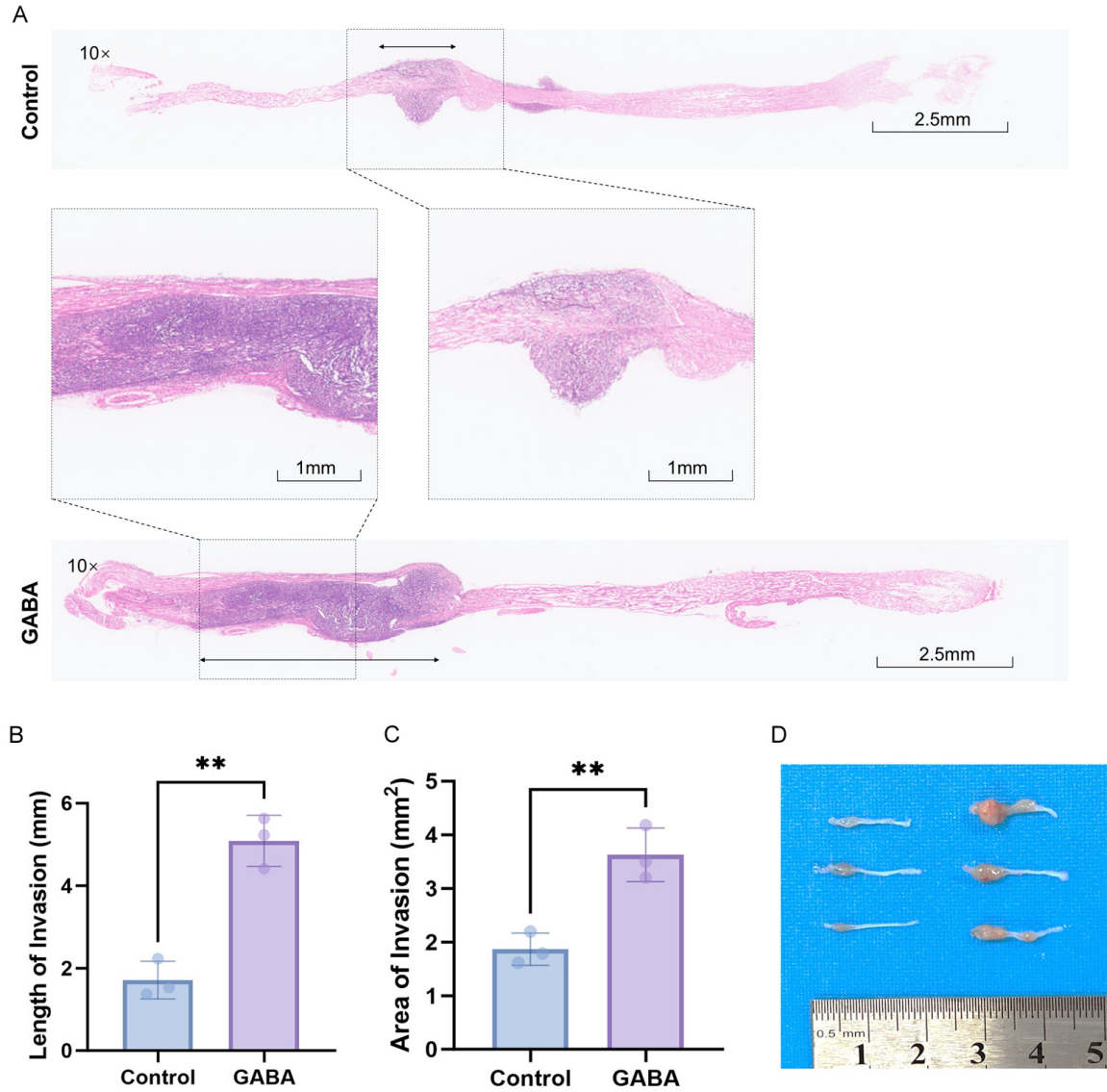


Figure S2. Sleep disturbances promote PNI in CRC through CNS-Derived GABA. (A) H&E staining of sciatic nerves from control and GABA-treated mice shows a significant increase in the length and area of tumor invasion in the GABA group. Insets provide higher magnification of the invasion sites. (B, C) Quantification of tumor invasion length (B) and area (C) in sciatic nerves of control and GABA-treated mice. GABA treatment significantly increased both parameters. (D) Representative images of dissected sciatic nerves from control and GABA-treated mice. $**P < 0.01$.

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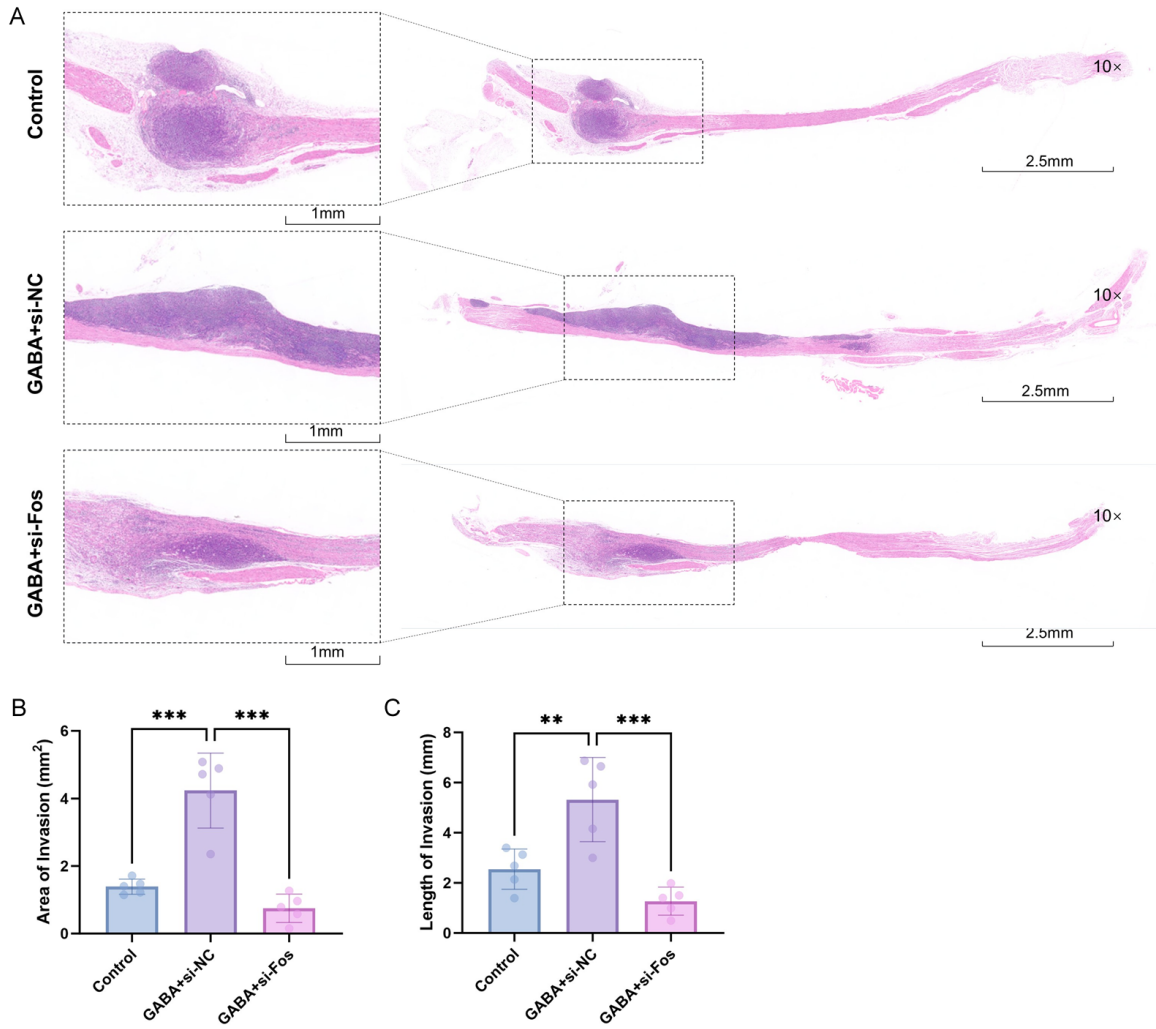


Figure S3. GABA-Induced *FOS* Upregulation Drives PNI in CRC. (A) H&E staining of sciatic nerves from control, GABA+si-NC, and GABA+si-Fos groups shows a significant reduction in the area and length of tumor invasion in the GABA+si-Fos group compared to controls and GABA+si-NC groups. (B, C) Quantification of tumor invasion area (B) and length (C) in sciatic nerves of mice. GABA treatment significantly increased tumor invasion, which was attenuated by *FOS* knockdown. ** $P < 0.01$, and *** $P < 0.001$.

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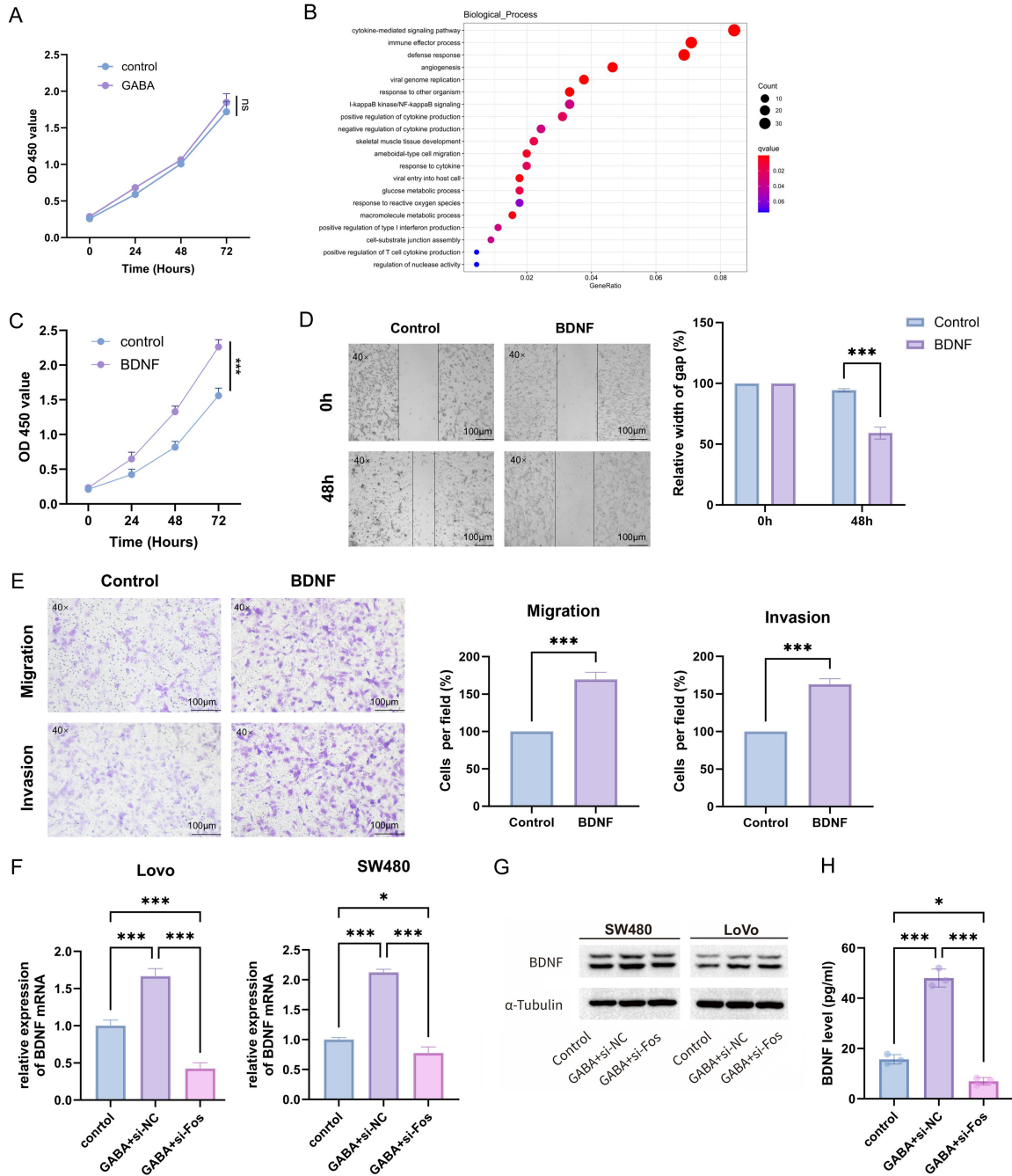


Figure S4. GABA-induced BDNF secretion from CRC cells activates Schwann cells. (A) CCK8 assay shows no significant change in Schwann cell proliferation with direct GABA treatment. (B) KEGG pathway analysis of Schwann cells co-cultured with CM from GABA-treated CRC cells shows upregulation of pathways related to angiogenesis. (C-E) CCK8 (C), wound healing (D), and Transwell assays (E) confirm that recombinant human BDNF at comparable levels to GABA CM promotes Schwann cell proliferation, migration, and invasion. (F-H) FOS knockdown in CRC cells treated with GABA reduces BDNF mRNA (F) and protein levels (G), along with a corresponding decrease in BDNF secretion (H). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.