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Supplemental Information

PD-1 Regulates GABAergic Neurotransmission and GABA-Mediated Analgesia and Anesthesia

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Supplemental Information

Key Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Animals		
Pd1 knockout mice	Jackson laboratory	21157
Sst-IRES-Cre line	Jackson laboratory	13044
Vglut2- IRES-Cre line	Jackson laboratory	16963
Vgat- IRES-Cre line	Jackson laboratory	16962
Ai32 line	Jackson laboratory	12569
Ai14 line	Jackson laboratory	007909
Antibodies		
Anti PD1 rabbit 1:300	Sigma	PRS4065
anti-NeuN mouse 1:250	Millipore	MAB377
anti-pERK 1:1000	Cell signaling tech	9101
anti-IgG4-FITC 1:200	Abcam	99281
Anti GABAAR α1 subunit rabbit 1:1000	Proteintech	12410-1-AP
Anti GABAAR α2 subunit rabbit 1:1000	Abcam	ab72445
Recombinant Proteins and reagents		
Nivolumab (OPDIVO®)	Bristol-Myers Squibb	
Human IgG4	Abcam	ab183266
SSG	Sigma	567565
U0126	InvivoGen	tlrl-u0126

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ru-Rong Ji (ru-rong.ji@duke.edu).

Transparent Methods

Animals. Adult mice (2-3 months of both sexes) were used for the behavioral tests and young mice (5-8 weeks of both sexes) were used for electrophysiological studies. We purchased *Pd1*$^{-/-}$ mice (#021157), *Sst*-IRES-Cre mice (#013044), *Vglut2*-ires-cre mice (#016963), *Vgat*-ires-cre mice (#016962), and Ai32 (#012569) and Ai14 mice (#007909), all with C57BL/6 background, from the Jackson laboratory and maintained these mouse lines at a Duke University animal facility in a 12h light/dark cycle with access to food and water ad libitum. Cre lines were crossed with Ai32 mice for optogenetic experiments and with Ai14 mice for immunostaining experiments or targeted electrophysiological experiments. All the mouse procedures were approved by the Institutional Animal Care & Use Committee of Duke University. Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Spinal Cord or Brain Slices Preparation. Mice of both sexes (5-7 weeks old) were anesthetized with urethane (1.5-2.0 g/kg, i.p.). The lumbosacral spinal cord or the brain was quickly taken out and placed in ice-cold dissection solution (Sucrose 240 mM, $NaHCO_3$ 25 mM, KCl 2.5 mM, NaH_2PO_4 1.25 mM, $CaCl_2$ 0.5 mM and $MgCl_2$ 3.5 mM), equilibrated with 95% O_2 and 5% CO_2. Transverse spinal slices (400 μm) or brain slices (300 μm) were cut with a vibrating microslicer (VT1200s Leica). The slices were incubated at 34^{0}C for 30 min in ACSF (NaCl 126 mM, KCl 3 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 26 mM, NaH_2PO_4 1.25 mM and glucose 11 mM), equilibrated with 95% O_2 and 5% CO_2, before recording (Wang et al., 2020). For inducing PD-1 blockade the slices were incubated with Nivolumab or IgG before using.

Whole-Cell Patch-Clamp Recording. Spinal cord or brain slices were placed in a recording chamber and perfused at a flow rate of 2-4 ml/min with ACSF which was saturated with 95% O_2 and 5% CO_2 and maintained at room temperature. Whole-cell voltage-clamp recordings were performed with patch-pipettes fabricated from thin-walled, fiber-filled capillaries. Patch-pipette solution used in this study contained: Cs_2SO_4 110, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, Mg-ATP 5, tetraethylammonium (TEA)-Cl 5 (pH 7.3, adjusted with KOH, 300mOsm). The patch-pipettes had a resistance of 8–10 MΩ. The holding potential (V_H) was set to 0 mV when whole-

cell patch-clamp was made. The GABAergic currents, spontaneous or evoked inhibitory postsynaptic currents (IPSCs) were recorded in the presence of strychnine (1 μM), AP-V (50 μM) and CNQX (10 μM).

To analyze the unit current, membrane capacitance (Cm) of recorded neuron was determined through injection of hyperpolarizing current steps (2 s, −5 pA increments, delivered every 10 s). Unit currents (pA/pF) were determined by dividing the peak amplitude of current by the cell membrane capacitance.

Signals were acquired using an Axopatch 700B amplifier. The data were stored and analyzed with a personal computer using pCLAMP 10.3 software. Spontaneous IPSC events were detected and analyzed using Mini Analysis Program ver. 6.0.3. Outward currents were measured by Clampfit. Briefly, the current value was calculated by the peak amplitude value of an outward current subtracted the amplitude value of baseline under voltage-clamp configuration.

Drug Applications. Exogenous GABA, THIP or glycine was applied locally for 500 ms by patch micropipette.

Dorsal Root-Evoked IPSCs. The evoked IPSCs were elicited by stimulating the dorsal root. In brief, the stimulation was performed by using a microelectrode (FHC, #30203). The strength of the stimuli (duration: 0.1 ms) used was 1.2 times the threshold to elicit IPSCs (Kato et al., 2004)

Light-Evoked IPSCs. For optogenetic activation of inhibitory interneurons, vGAT^{Ai32} mice were used. Blue light (473 nm wavelength) illumination (0.1 s) was delivered through a 40X water-immersion microscope (BX51WIF; Olympus) objective to induce a light-evoked response. The interval of the paired pulse of the light was set to 80 ms. Following identification of vGAT-positive or negative neurons, we implemented a recovery period of 10-15 min before recording to allow neurons to recover from blue light exposure (Pagani et al., 2019).

Immunohistochemistry. Mice of both sexes (7-8 weeks old) were deeply anesthetized with isoflurane, followed by the perfusion of PBS and in turn 4% paraformaldehyde (PFA)/1.5% picric acid. After the perfusion, lumbar spinal cord (L3-L5) were removed and post-fixed for 2h at room temperature. The tissues were washed several times in PBS, followed by cryopreservation in 20%

sucrose overnight followed by 30% sucrose overnight. The samples were sliced into sections (20 μm) in a cryostat. For the anti-Nivolumab and/or anti-pERK staining after Nivolumab (300 ng/mL or 1000ng/mL) incubation on living spinal cord slices (200 μm), the slices were post-fixeated overnight and then dehydrated in 30% sucrose for 12 h.

The sections or slices were blocked with 10% donkey serum with 0.3% Triton X-100 for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-PD1 antibody (rabbit, 1:300, Sigma, Cat: PRS4065), anti-NeuN antibody (mouse, 1:250, Millipore, Cat: MAB377) and/or anti-pERK (rabbit, 1:1000, Cell signaling tech, Catalog: 9101). After washing, the sections were incubated with Cy3-, Cy5-conjugated secondary antibodies (1:500; Jackson ImmunoResearch) and/or anti-IgG4-FITC (1:200, Abcam, catalog: 99281) at 4℃ overnight. Nissl (1:200, Invitrogen, cat: N21483) was used for 2 hours for visualizing neurons and DAPI was contained in mounting medium (Sigma, Cat: SLCC8848) to stain cell nuclei. Three sections were analyzed in each mouse and 4 mice per group were analyzed.

For the anti-Nivolumab staining after Nivolumab (300 ng/mL) incubation on living cultured cortical neurons, the neurons on coverslips were post-fixed in 4% PFA for 15 min and incubated in 0.3% Triton X-100 containing PBS for 1 hour, followed by incubation with the following primary antibodies: anti-PD1 antibody (rabbit, 1:300, Sigma, Cat: PRS4065) and anti-NeuN antibody (mouse, 1:250, Millipore, Cat: MAB377). After washing, the neurons were incubated with Cy3-, Cy5-conjugated secondary antibodies (1:500; Jackson ImmunoResearch) and/or anti-IgG4-FITC (1:200, Abcam, catalog: 99281) at 4℃ overnight. DAPI was contained in mounting medium (Sigma, Cat: SLCC8848) to stain cell nuclei.

The images were examined with a ZEISS LSM 880 confocal microscope. To confirm the specificity of PD-1 antibody, blocking experiments were conducted in brain sections using a mixture of anti-PD-1 antibody (1:300) and immunizing blocking peptide (1:300, Sigma, Catalog: SBP4065), based on a protocol recommended for blocking with immunizing peptide (www.abcam.com/technical). The specificity of PD-1 antibody was also tested in *Pd1* knockout mice, as we previously reported (Wang et al., 2020).

***In Situ* Hybridization.** Mice were deeply anesthetized with isoflurane and transcardially perfused with PBS followed by 4% PFA/1.5% picric acid. Following perfusion, lumbar spinal cords (L3-L5) were removed and post-fixed for 2h at room temperature. The tissues were washed several

times in PBS, followed by cryopreservation in 20% sucrose overnight followed by 30% sucrose overnight. Spinal cords were then embedded in optimal cutting temperature (OCT) medium (Tissue-Tek) and cryosectioned to produce 14 µm sections which were mounted onto Superfrost Plus charged slides (VWR). Importantly, each slide contained both *Pdcd1*$^{+/+}$ and *Pdcd1*$^{-/-}$ sections to control for possible variability in staining between slides and to control for the specificity of the Pdcd1 probe. *In situ* hybridization was performed using the RNAscope system (Advanced Cell Diagnostics) according to the manufacturer's recommendations using the protocol for the Multiplex Fluorescent Kit version 2. We used probes directed against murine *Pdcd1* (*Pd1*, NM_008798.2, #416781), *Pdl1* (NM_021893.3, #420508-C3) and *Pdl2* (NM_021396.2, 447781-C3), *Slc17a6* (Vglut2, NM_080853.3, #319171-C2) and *Slc32a1* (Vgat, NM_009508.2, #319191-C3). Images were captured using a CCD Spot camera affixed to a Nikon fluorescent microscope (Nikon Eclipse NiE). For higher resolution analysis, images were also captured using a Zeiss LCM 880 confocal microscope with a z-step size of 1 µm and maximum projections were subsequently produced using the Zeiss ZEN software program. Images were taken across animals and genotypes using the same acquisition settings (Wang et al., 2020).

Primary Cultures of Cortical Neurons. Primary cultures of cortical neurons were prepared from embryonic day 17-19 (E17-19) WT or *Pd1*$^{-/-}$ mice. Embryos were removed from maternal mice anesthetized with isoflurane and euthanized by decapitation. Cortices were dissected and placed in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS, GIBCO) and digested at 37 °C in humidified O^2 incubator for 30 min with collagenase type II (Worthington, 285 units/mg, 12 mg/ml final concentration) and dispase II (Roche, 1 unit/mg, 20 mg/ml) in HBSS (pH 7.3). Digestion was terminated by fetal bovine serum-containing DMEM (GIBCO). Cortex were mechanically dissociated using fire-polished pipettes, filtered through a 100-µm nylon mesh and centrifuged (1,000 g for 5 min). The pellet was resuspended and plated on poly-D-Lysine/Laminin coated glass coverslips (CORNING), and cells were plated in DMEM containing 5% fetal bovine serum, 1% penicillin and streptomycin. After 5-6 h, primary cultures were switched to Neurobasal Plus medium containing 2% B27 supplement, 1% GlutaMAX-I, 1% penicillin and streptomycin (GIBCO). Three days after plating, cytosine arabinoside was added to a final concentration of 10 µM to curb glial proliferation. Whole-cell recoding and immunostaining experiments were conducted on DIV 7-8.

Western Blot. The spinal or brain tissues were homogenized in a lysis buffer containing protease and phosphatase inhibitors at 4°C for 30 min. After centrifugation, the protein concentrations were measured using the BCA protein assay kit (Pierce, Thermo Fisher Scientific). Then protein (50 μg) was loaded for each lane and separated by SDS-PAGE gel (4-20%; Bio-Red). After the transfer, the blots were incubated overnight at 4°C with polyclonal antibodies against $GABA_AR$ α1 subunit (rabbit, 1:1000, Proteintech, Catalog: 12410-1-AP) and polyclonal antibodies against $GABA_AR$ α2 subunit (rabbit, 1:1000, abcam, Catalog: ab72445). For the loading control, the blots were probed with GAPDH antibody (1:5000, mouse, Proteintech, catalog: 60004-1-lg). The blots were then incubated with an HRP-conjugated secondary antibody and developed in ECL solution (Pierce, Thermo Fisher Scientific). Chemiluminescence signal was revealed by Bio-Rad ChemiDoc XRS for 1 to 3 minutes. Specific bands were evaluated according to apparent molecular sizes.

Mouse Model of Inflammatory Pain. Inflammatory pain was induced by complete Freund's adjuvant (CFA, Sigma) via intraplantar injection (20 μL) into a hind paw under brief anesthesia with isoflurane.

Mouse Model of Neuropathic Pain. Neuropathic pain was induced by partial sciatic nerve ligation (pSNL). To produce pSNL, the sciatic nerve was exposed under isoflurane, and a tight ligation of approximately one-third to one-half of the diameter of the left sciatic nerve was performed with 6-0 silk suture.

Von Frey Testing for Mechanical Allodynia. Mice were habituated to the environment for at least 2 days before the testing. All the behaviors were tested blindly. For testing mechanical allodynia, we confined mice in boxes (14 × 18 × 12 cm) placed on an elevated metal mesh floor and stimulated their hind paws with a series of von Frey hairs with logarithmically increasing stiffness (0.16-2.00 g, Stoelting), presented perpendicularly to the central plantar surface. We determined the 50% paw withdrawal threshold by Dixon's up-down method (Dixon, 1980).

Intracerebroventricular injection

For stereotaxic surgery, mouse was anesthetized with isoflurane and the head was fixed in a stereotaxic apparatus (Narishige scientific instrument lab.). A guide cannula (62004, RWD Life Science) was stereotaxically implanted into the left lateral ventricles (AP: -0.3 mm; ML: +1.3 mm; V: -2.0 mm) according to the mouse brain atlas16. After four-day recovery, an infusion needle (62204, RWD Life Science) was inserted into lateral ventricles through the guide cannula to a depth of 2.5 mm for IgG or Nivolumab injection. Following the completion of the infusion, the needle was left for an additional 2 min to limit reflux.

Fraction of Loss of Righting Reflex (LORR). Mice were placed in a cylindrical gas-tight, controlled-environment chamber. After 90 min of habituation with 100% oxygen flowing (at a rate of 5 L/min) each day on two successive days, anesthesia was induced. Anesthetic gas concentrations were determined in triplicate by a gas indicator (Riken FI-8000). The isoflurane concentration was set from 0.55% to 1.2% (an increment of 0.05%) for 15 min, and each animal's righting reflex was assessed by gently rolling the chamber until the mouse was placed on its back during the last 2 min. At each concentration, a mouse was considered to have lost the righting reflex if it did not turn itself prone onto all four limbs within 2 min. To minimize the number of anesthetic exposures, each mouse was only exposed to isoflurane two times and the intervals were more than 2 days. Mouse temperature was maintained between 36.6 ± 0.6°C (McCarren et al., 2013).

Assess the Duration of Induction and Emergence of Anesthesia. For the isoflurane anesthesia, isoflurane was set to a certain concentration in the chamber. The duration of induction (seconds) was determined when the mouse lost its righting reflex from the time it was put into the chamber. The duration of emergence (seconds) was defined from time the isoflurane was turn off until the time mouse regained its righting reflex. For the pentobarbital anesthesia, pentobarbital was injected intraperitoneally (40 mg/kg) (Kelz et al., 2008; McCarren et al., 2013).

Statistical Analyses. All the data were expressed as mean ± SEM. Statistical analyses were completed with Prism GraphPad 8.0. Biochemical, electrophysiology and behavioral data were analyzed using two-tailed unpaired *t*-test (two groups) or Two-Way ANOVA followed by

Bonferroni post-hoc test. The criterion for statistical significance was $P < 0.05$. In all cases of electrophysiology recordings, n refers to the number of the neurons studied.

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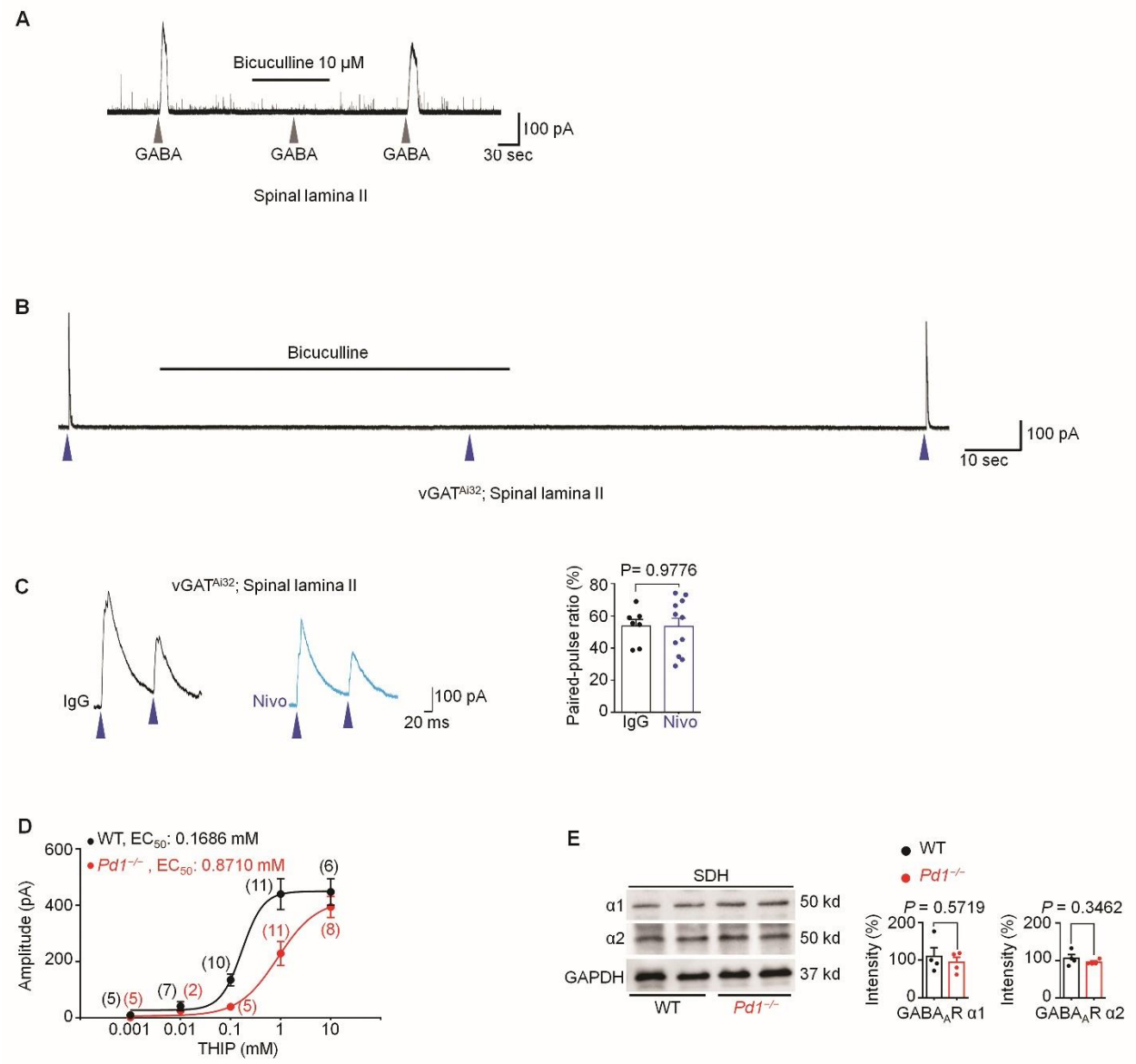

Figure S1. Loss of PD-1 function impairs $GABA_AR$-mediated currents in spinal cord slice. Related to Figure 1.

(A) Blockade of GABA (1 mM) current in spinal lamina II neurons by the $GABA_AR$ antagonist bicuculline.

(B) Blockade of blue light-induced currents by bicuculline in lamina II neurons from $vGAT^{Ai32}$ mice.

(C) Left: example traces of paired pulse blue light-evoked currents in spinal lamina II neurons of $vGAT^{Ai32}$ mice showing the effect of IgG or Nivo (300 ng/mL, 2 h). Right: quantification of the

amplitude of the 2nd currents relative to the 1st currents. n = 7 and 11 neurons from 3 animals per group.

(D) Dose-response curve showing average amplitudes of THIP-induced currents in SDH lamina II neurons of WT and *Pd1*$^{-/-}$ mice. The curves were drawn according to the Hill equation. Values in parentheses denote the number of recorded neurons from 3-4 animals per group.

(E) Left: Representative immunoblots showing the expression of α1 and α2 subunits of $GABA_AR$ in the lumber spinal cord from WT and *Pd1*$^{-/-}$ mice. Right: quantification of $GABA_AR$ α1 and α2 immunoblots (normalized to GAPDH) in WT and *Pd1*$^{-/-}$ mice. n = 4 animals/group.

Gray arrowheads indicate the application of GABA. Blue arrowheads indicate the blue light stimulus. V_H = 0 mV. Unpaired two-tailed t-test. All error bars indicate the mean ± SEM.

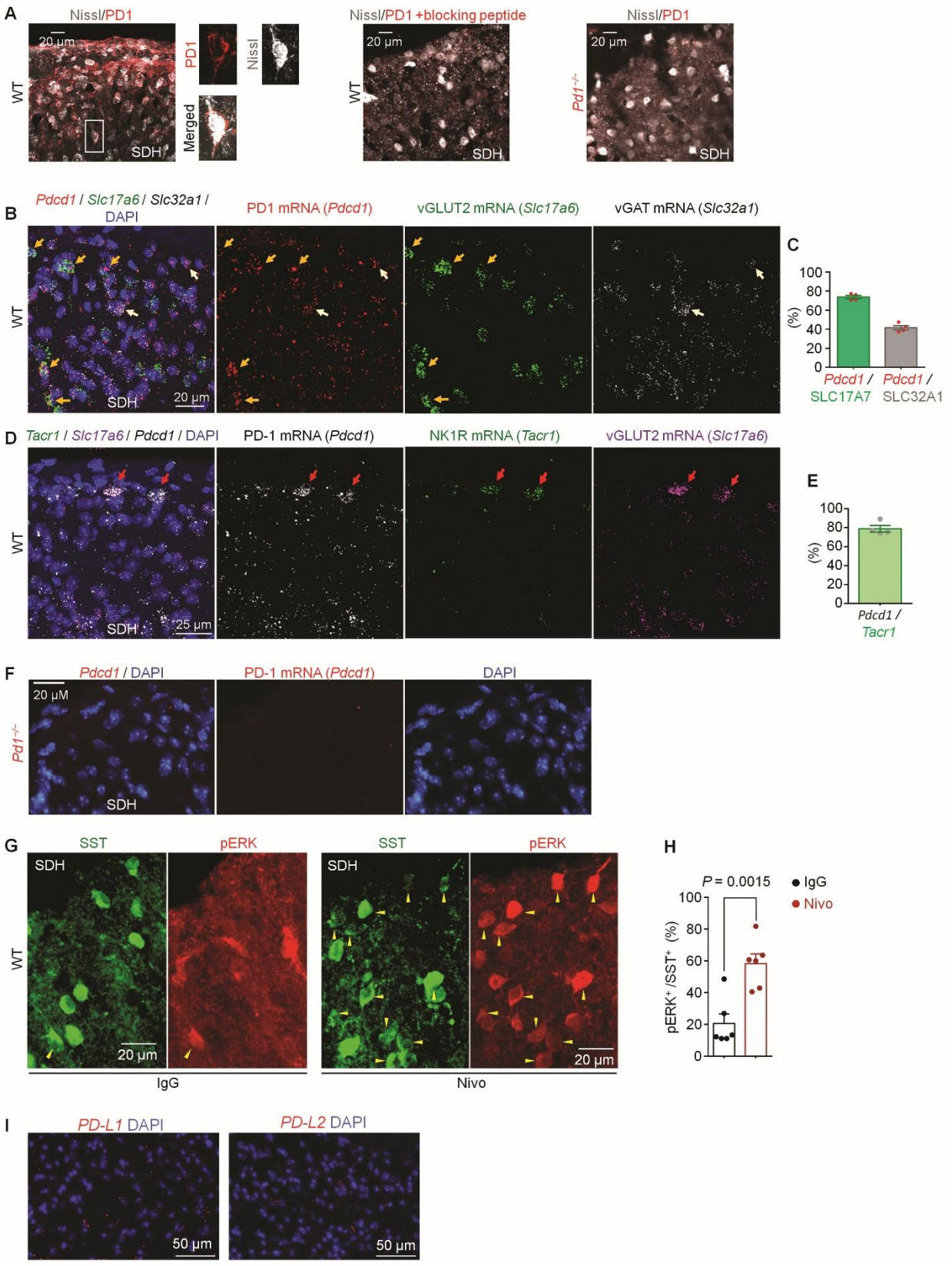
A
Nissl/PD1
20 μm
WT
SDH
PD1
Nissl
Merged
Nissl/PD1 +blocking peptide
Pd1-/-
B
Pdcd1 / Slc17a6 / Slc32a1 / DAPI
PD1 mRNA (Pdcd1)
vGLUT2 mRNA (Slc17a6)
vGAT mRNA (Slc32a1)
25 μm
C
(%)
Pdcd1 / SLC17A7
Pdcd1 / SLC32A1
D
Tacr1 / Slc17a6 / Pdcd1 / DAPI
PD-1 mRNA (Pdcd1)
NK1R mRNA (Tacr1)
vGLUT2 mRNA (Slc17a6)
E
Pdcd1 / Tacr1
F
Pdcd1 / DAPI
PD-1 mRNA (Pdcd1)
DAPI
20 μM
G
SST
pERK
IgG
Nivo
H
P = 0.0015
IgG
Nivo
pERK+ /SST+ (%)
I
PD-L1 DAPI
PD-L2 DAPI
50 μm

Figure S2. Characterization of *Pd1* mRNA and PD-1 protein expression in SDH neurons. Related to Figure 2.

(A) Transverse sections of SDH showing double staining of PD-1 (red) and Nissl (gray) in WT mice with the box enlarged (left), in WT mice in the presence of a blocking peptide (middle) and in $Pd1^{-/-}$ mice (right). Note that PD-1 immunostaining is lost after treatment of the blocking peptide and in KO mice.

(B,C) ISH shows mRNA expression of *Pdcd1 (=pd1, red)* in excitatory neurons (*Slc17a6* for vGLUT2, green) and inhibitory neurons (*Slc32a1* for vGAT, white) of SDH. (B) Typical images. DAPI staining labels nuclei in SDH. (C) Percentage expression of *Pd1* in SDH excitatory and inhibitory neurons. n = 4 mice/group. Note there is more PD-1 expression in excitatory neurons (orange arrows) than in inhibitory neurons (yellow arrows) in SDH.

(D,E) ISH shows *Pdcd1* mRNA expression in SDH projection neurons (*Tacr1* for NK-1, green) and excitatory neurons (*Slc17a6* for vGLUT2, purple). (D) Typical images. Red arrows indicate projection neurons with triple staining. (E) Percentage expression of *Pd1* in SDH projection neurons. n = 4 mice/group.

(F) ISH shows absence of *Pdcd1* mRNA expression in SDH of $Pd1^{-/-}$ mice.

(G,H) Nivolumab-induced pERK expression in SDH neurons of SST reporter mice. (H) Double staining of pERK (red) and SST (green). (I) Quantification of pERK in SST^{+} neurons. n = 6 slices from 3-4 mice per group. Spinal cord slices were prepared from $SST^{tdTomato}$ mice, incubated with IgG or Nivo (300 ng/mL) for 2 h, and then fixed for immunostaining.

(I) ISH shows PD-L1 and PD-L2 mRNA expression in SDH.

All error bars indicate the mean ± SEM.

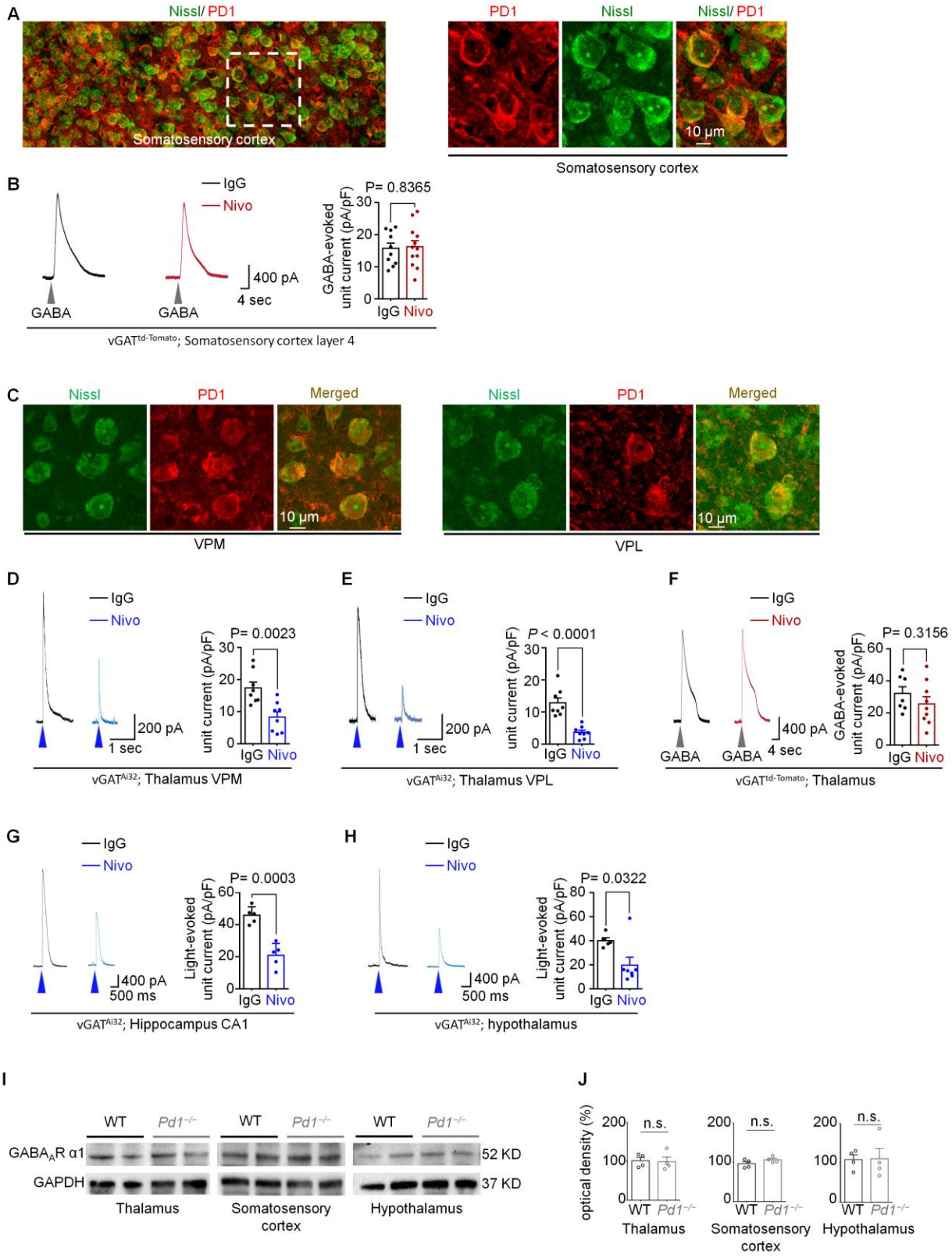
A
Nissl/ PD1
Somatosensory cortex
PD1
Nissl
Nissl/ PD1
10 μm
Somatosensory cortex
B
IgG
Nivo
400 pA
4 sec
GABA
GABA
P= 0.8365
GABA-evoked unit current (pA/pF)
IgG Nivo
vGAT^td-Tomato; Somatosensory cortex layer 4
C
Nissl
PD1
Merged
10 μm
VPM
Nissl
PD1
Merged
10 μm
VPL
D
IgG
Nivo
200 pA
1 sec
P= 0.0023
unit current (pA/pF)
IgG Nivo
vGAT^Ai32; Thalamus VPM
E
IgG
Nivo
200 pA
1 sec
P < 0.0001
unit current (pA/pF)
IgG Nivo
vGAT^Ai32; Thalamus VPL
F
IgG
Nivo
400 pA
4 sec
GABA
GABA
P= 0.3156
GABA-evoked unit current (pA/pF)
IgG Nivo
vGAT^td-Tomato; Thalamus
G
IgG
Nivo
400 pA
500 ms
P= 0.0003
Light-evoked unit current (pA/pF)
IgG Nivo
vGAT^Ai32; Hippocampus CA1
H
IgG
Nivo
400 pA
500 ms
P= 0.0322
Light-evoked unit current (pA/pF)
IgG Nivo
vGAT^Ai32; hypothalamus
I
WT
Pd1^-/-
WT
Pd1^-/-
WT
Pd1^-/-
GABA_AR α1
GAPDH
52 KD
37 KD
Thalamus
Somatosensory cortex
Hypothalamus
J
optical density (%)
n.s.
n.s.
n.s.
WT Pd1^-/-
Thalamus
Somatosensory cortex
Hypothalamus

Figure S3. Characterization of PD-1 expression, $GABA_AR$-mediated currents, and $GABA_AR$ expression in different brain regions. Related to Figure 3.

(A) Double staining of PD-1 (red) and Nissl (green) in S1 somatosensory cortex. Right: higher magnification images of the dashed box. Note PD-1 is present on the surface of cortical neurons.

(B) GABA (1 mM) induced currents from $vGAT^+$ neurons in S1-layer IV of Vgat reporter mice following incubation with IgG or Nivo (300 ng/mL, 2 h). Right: unit currents of GABA. n = 10 and 12 neurons from 3 animals/group.

(C) Double staining of PD-1 (red) and Nissl (green) in S1, thalamus VPM (left) and VPL (right).

(D-H) Effects of Nivolumab and IgG (300 ng/ml, 2 h) on light-evoked and GABA-evoked (F) currents and in vGAT-negative neurons of $vGAT^{Ai32}$ mice or $vGAT^{tdTomato}$ mice (F).

(D,E) Light-evoked GABA currents in VPM neurons (D) and VPL neurons (E). Left: Traces of light-induced currents. Right: unit currents. n =7 and 9 neurons from 3 animals/group (D). n = 9 and 7 neurons from 3 animals/group (E).

(F) GABA (1 mM) induced currents in thalamic neurons from $vGAT^{tdTomato}$ mice. Left, traces of currents. Right: unit currents. n =7 and 9 from 3 animals/group.

(G,H) Light-evoked GABA currents from hippocampal CA1 neuronal (G) and hypothalamic neurons (H). n = 5 neurons from 3 animals/group (G). n = 5 and 7 neurons from 3 animals/group (H).

(I,J) Western blot shows $GABA_AR$ expression in different brain regions of WT and $Pd1^{-/-}$ mice.

(I) Western gels showing the expression of α1 subunits of $GABA_AR$ in the thalamus, somatosensory cortex and hypothalamus. (J) Quantification of band density of α1-$GABA_AR$ (normalized to GAPDH). n = 4 mice/group.

Unpaired two-tailed t-test. All error bars indicate the mean ± SEM.

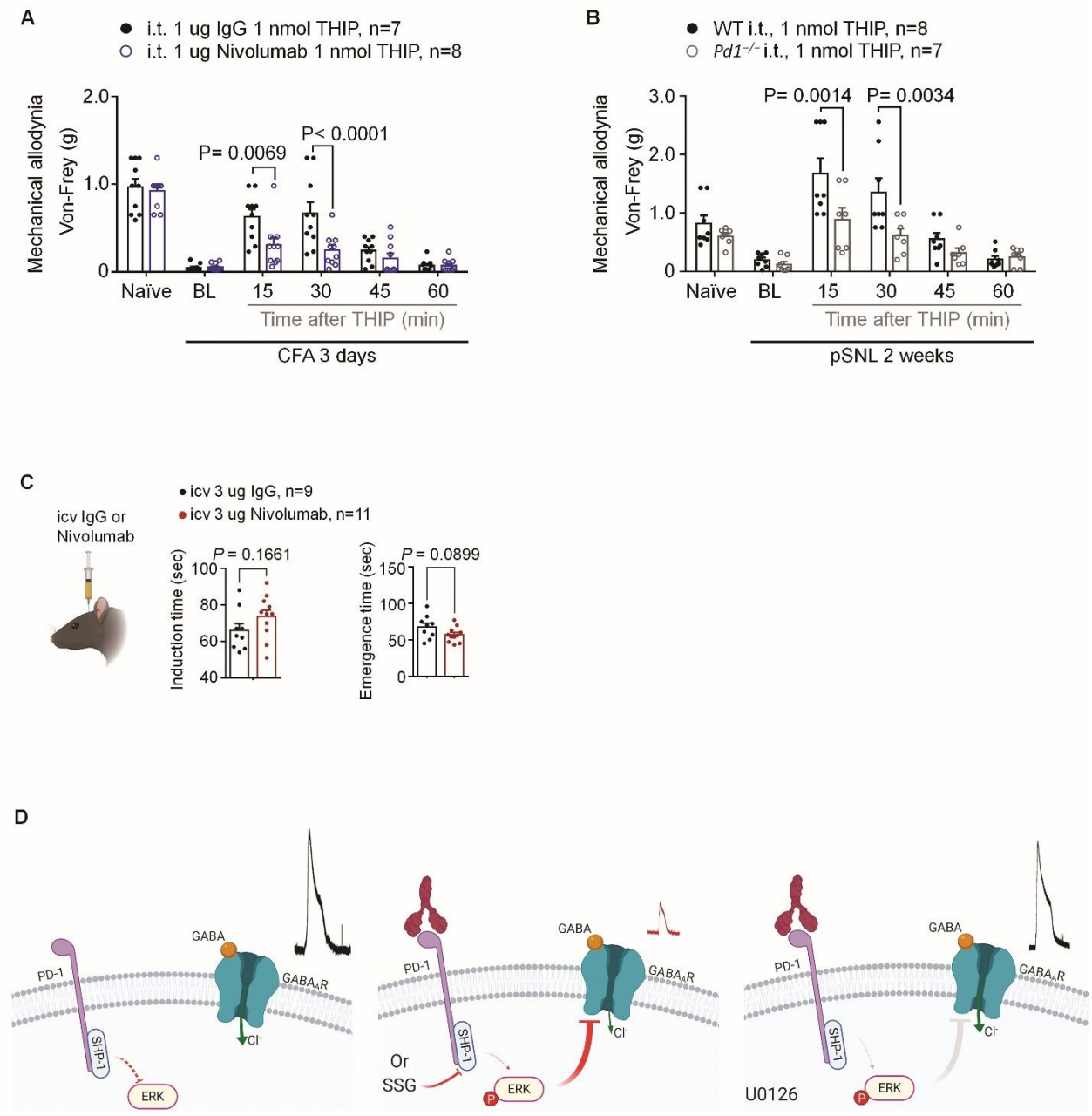

Figure S4. Further characterization of GABA-mediated analgesia after PD-1 blockade and the schematic of PD-1 regulation of GABAergic transmission. Related to Figure 4.

(A) Von Frey testing showing the effect of IgG or Nivo pre-treatment (1 μg, i.t.) on intrathecal THIP (1 nmol) induced inhibition of inflammatory pain in CFA-inflamed mice. $P < 0.0001$, $P = 0.0069$, IgG4 + THIP versus nivolumab + THIP, two-way ANOVA, followed by Bonferroni's post hoc test.

(B) Von Frey testing showing the effect of intrathecal THIP (1 nmol) on neuropathic pain in the sciatic nerve ligation (pSNL) model in WT and *Pd1*$^{-/-}$ mice. $P = 0.0014$, $P = 0.0034$, WT versus *Pd1*$^{-/-}$, two-way ANOVA, followed by Bonferroni's post hoc test.

(C) Left: a schematic diagram of intracerebroventricular injection. Middle and Right: the effects of 1.5% isoflurane on the induction time (middle) and emergence time (right) in IgG- and Nivolumab-treated mice (n = 9-11 animals in each group).

(D) Schematic illustration of the proposed mechanism by which PD-1 potentiates GABAergic neurotransmission in SDH. Left, under the normal conditions, PD-1 suppresses ERK activation via SHP-1, thereby enabling $GABA_AR$-mediated synaptic transmission. Middle, inhibition of PD-1 signaling using anti-PD1 neutralizing antibodies (e.g. Nivolumab) or SHP-1 inhibitors (SSG) leads to activation of ERK (pERK), which in turn suppresses $GABA_AR$-mediated currents. Right, inhibition of the pERK pathway with U0126, in turn, relieves pERK suppression of $GABA_AR$ function, and thus, restores the normal GABAergic neurotransmission.

All error bars indicate the mean ± SEM.