Optogenetic activation of the neural circuit between dorsal raphe nucleus and the pre-Bötzinger complex contributes to inhibition of the seizure-induced respiratory arrest in the DBA/1 mouse SUDEP model

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Abstract

Sudden unexpected death in epilepsy (SUDEP) is the fatal cause leading to the death of epilepsy patients, especially those with anti-epileptic drug resistance. However, the underlying mechanism of SUDEP remains elusive. Our previous study demonstrated that enhancement of serotonin (5-HT) synthesis by intraperitoneal (IP) injection of 5-hydroxytryptophan (5-HTP) significantly reduced the incidence of seizure-induced respiratory arrest (S-IRA) in DBA/1 mice SUDEP models. Given that 5-HT2A receptor (5-HT2AR) plays an important role in mediating the respiration system in brain, we

hypothesized that 5-HT2AR is of great significance to modulate S-IRA and SUDEP. To test this hypothesis, we examined whether the decreased incidence of S-IRA evoked by either acoustic stimulation or PTZ injection by administration with 5-HTP will be blocked by administration with ketanserin (KET), a selective antagonist of 5HT2AR, in DBA/1 mice SUDEP models to examine the role of 5-HT2AR in modulating S-IRA. Our results suggested that the decreased incidence of S-IRA by 5-Hydroxytryptophan (5-HTP), a precursor for central nervous system (CNS) serotonin (5-HT) synthesis, was significantly reversed by IP or intracerebroventricularly (ICV) injection of ketanserin in our models. Furthermore, given that 5-HT2AR locating in the pre-Bötzinger complex (PBC) which plays a key role in regulating the respiratory rhythm, we wonder whether the lower incidence of S-IRA by IP with 5-HTP was significantly reversed by KET which inhibits 5-HT2AR in the above models and acts on the 5-HT2AR in pre-Bötzinger complex .To test our hypothesis, we activated the neural circuit between dorsal raphe nucleus and the pre-Bötzinger complex by optogenetics technology. Our results indicated that the lower incidence of S-IRA evoked by PTZ by stimulating the TPH2-ChETA, a variant of channelrhodopsin-2 (ChR2) neurons in the dorsal raphe nucleus (DR) in DBA/1 mice and the suppressant effects was significantly reversed by administration of KET in bilateral pre-Bötzinger complex without changing electroencephalogram (EEG) activity. The neural circuit between DR and the pre-Botzinger complex was confirmed by injection of CTB555, a nerve tracer, in DR or the pre-Botzinger complex, respectively. Taken together, our findings suggested that targeting the neural circuit between the DR and the pre-Bötzinger complex in which 5-HT2AR plays a critical role in regulating this process would be promising to prevent SUDEP.

Keywords: sudden unexpected death in epilepsy; 5-Hydroxytryptophan (5-HTP); ketanserin (KET); pre-Bötzinger complex; dorsal raphe nucleus; 5HT2A receptor; respiration rhythm.

1. INTRODUCTION

Growing studies have shown that sudden unexpected death in epilepsy (SUDEP) is the most important cause leading to the death of epilepsy patients, especially those who suffered from antiepileptic drug resistance [1-4]. The latest advancements of the pathogenesis of SUDEP demonstrated that cardiopulmonary dysfunction plays an important role in the occurrence of SUDEP[1-5]. Our previous findings showed that seizure-induced respiratory arrest (S-IRA) can

lead to the occurrence of SUDEP evoked by either acoustic stimulation or pentylenetetrazole (PTZ) in DBA/1 murine models [5-10], and the incidence of S-IRA by acoustic stimulation or PTZ was significantly reduced by 5-Hydroxytryptophan (5-HTP), a precursor for serotonin synthesis in two models of SUDEP [5]. However, the target of 5-HTP-mediated reduction of S-IRA and SUDEP in the brain remains unclear and still needs to be further explored. Due to that 5-HT2AR has emerged as a key factor in mediating respiratory function in brainstem[11-13], we wonder if the application of 5-HTP could reduce the incidence of S-IRA by targeting 5-HT2AR in the brain. Therefore, we hypothesized that the suppressant effects of 5-HTP on S-IRA would be reversed by ketanserin (KET), a selective antagonist for 5-HT2AR, in the DBA/1 mouse SUDEP models and that it may be a leading therapeutic target to prevent SUDEP.

To investigate the roles of 5-HTP and 5HT2AR in the pathogenesis of S-IRA and SUDEP, we continued to choose acoustic stimulation and PTZ injection DBA/1 mice SUDEP models to test the reversal effects of KET based on our previous study. The lower incidence of S-IRA by 5-HTP was significantly reversed by both intraperitoneal (IP) and intracerebroventricular (ICV) injection of KET in two models, respectively. Different from our previous study that atomoxetine reduced the incidence of S-IRA in DBA/1 mice by acoustic stimulation without affecting seizure behavior [8-9], administration of 5-HTP significantly reduced the incidence of S-IRA through its anticonvulsant effects in our models. The anticonvulsant effects of 5-HTP were basically consistent with other studies that activation of 5-HT neurons can reduce the severity of seizure [14-16]. Meanwhile, administration of KET reversed the lower incidence of S-IRA by 5-HTP with producing only partly anticonvulsant effects, which suggested that 5-HT2AR in brainstem may be a specific target to prevent S-IRA and SUDEP. Furthermore, considering that 5-HT2AR located in the pre-Bötzinger complex which plays a key role in modulating respiration rhythm[12], and that the incidence of S-IRA in DBA/1 mice can be significantly reduced by optogenetic activation of TPH2-ChR2 neurons in DR based on our previous findings, we further activated TPH2-ChETA neurons in the DR of DBA/1 mice to observe whether the suppression of S-IRA in PTZ injection model by photostimulating the DR depends on activating 5-HT2AR located in the pre-Bötzinger complex or not. It turned out that the lower incidence of S-IRA

evoked by PTZ by optogenetic activation of TPH2-ChETA neurons in the DR was remarkably reversed by injection of KET into the pre-Bötzinger complex. Meanwhile, the neural circuit between the DR and the pre-Bötzinger complex was confirmed by injection of tracer CTB-555 into the DR and the pre-Bötzinger complex, respectively. Thus, our findings suggested that activating the neural circuit between the DR and the pre-Bötzinger complex might contribute to preventing.

2. MATERIALS AND METHODS

2.1 Animals

All experimental procedures were in line with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Advisory Committee of Zhejiang University. DBA/1 mice were housed and bred in the Animal Center of Zhejiang University School of Medicine and provided with rodent food and water ad libitum. In the acoustic stimulation murine model, DBA/1 mice were “primed” starting from postnatal day 26-28 to establish consistent susceptibility to audiogenic seizures and S-IRA. PTZ-evoked seizure model, PTZ was administered to non-primed DBA/1 mice of approximately 8 weeks of age. The two models were established just as described previously [5-10]. TPH2-ChETA infected mice was established by viral delivery of pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS into the DR to express for 3 weeks and confirmed the expression of TPH2 -ChETA in DR by immunohistochemistry

2.2 Seizure induction and resuscitation

S-IRA was evoked by acoustic stimulation or intraperitoneal (IP) administration of PTZ, as previously described [5-10]. For the acoustic stimulation model, each mouse was placed in a cylindrical plexiglass chamber in a sound-isolated room, and audiogenic seizures (AGSZs) were evoked by an electric bell (96 dB SPL, Zhejiang People's Electronics, China). Acoustic stimulation was given for a maximum duration of 60 s or until the onset of tonic seizures and S-IRA in most mice in each group. Mice with S-IRA were resuscitated within 5 s post the final respiratory gasp using a rodent respirator (YuYan Instruments, ShangHai, China). S-IRA was also evoked in all non-primed DBA/1 mice by IP administration of a single dose of PTZ (Cat # P6500; Sigma-Aldrich, St. Louis, MO) at a dose of 75 mg/kg. Mice with S-IRA were resuscitated by using a small animal respirator (YuYan Company, ShangHai, China).

2.3. Pharmacology experiment

2.3 .1 Effect of IP administration of KET on 5-HTP-mediated suppression of S-IRA evoked by acoustic stimulation

A vehicle or different doses of KET (Cat # 8006; Sigma-Aldrich) was administered to different groups of mice which were then acoustically stimulated. Susceptibility to S-IRA in primed DBA/1 mice was confirmed 24 h prior to treatment of 5-HTP (Cat # 107751; Sigma-Aldrich) or vehicle, which was followed by KET administration ,respectively. 5-HTP or vehicle (saline) was administered i.p. once a day for 2 days and induction of S-IRA was performed 90 mins after the second administration. KET or vehicle (25% DMSO) was administered i.p. 30 mins before acoustic stimulation. The effects of 5-HTP and KET on S-IRA were examined and digitally recorded for offline analysis, respectively. Additionally, KET was administered by ICV injection 15 mins prior to PTZ injection as the selective antagonist of 5-HT2AR in DBA/1 mice in different groups from the same manner of 5-HTP administration. The incidences of S-IRA latency to AGSZs, duration of wild running, clonic seizures, tonic-clonic seizures, and seizure scores were videotaped for offline analysis [5-10, 17-18].

2.3.2 Effect of ICV administration of KET on 5-HTP-mediated suppression of S-IRA by PTZ

Aiming at the ICV surgery, an ICV guide cannula（O.D.I.41×I.D.O.0.25mm/M3.5，62004，RWD Life Science Inc.,China）was implanted into the right lateral ventricle (AP – 0.45 mm; ML – 1.0 mm; V-2.50 mm) to enable microinjections as previously described [9]. The groups to test as follows: 1) Saline (IP) was administered 75 mins prior to PTZ (75mg/kg, IP) and 25% DMSO at (2 μl, at a rate of 0.5 μl/min ICV) 15 min prior to PTZ injection as controls. 2) 5-HTP (200mg/kg, IP) was administered 75 mins prior to PTZ (75mg/kg, IP) and 25% DMSO (2 μl, at a rate of 0.5 μl/min ICV) 15 min prior to PTZ injection. 3) 5-HTP (200mg/kg, IP) was administered 75 mins prior to PTZ (75mg/kg, IP), and KET (4.764 nmol and 9.528 nmol, dissolved in 2 μl 25% DMSO, at a rate of 0.5 μl/min ICV) 15 min prior to PTZ injection. The timing of 5-HTP administration in the above groups was in the manner of administering i.p. once a day for 2 days.

2.4 Optogenetics experiments

2.4.1 Stereotactic surgery

DBA/1 mice at 8 weeks of age were anesthetized with 3.5% chloral hydrate and head-fixed in a stereotaxic apparatus, (68018, RWD Life Science Inc.,Shenzhen,China). During the entire process of surgery, the body temperature of anesthetized mice was kept constant at 37 °C using a heating pad. If the DBA/1 mice had a pain reflect in response with a paw pinch, then additional 10% of initial dosage of sodium pentobarbital was given to guarantee a painless state. Other procedures were basically the same with our previous methods concerning the optogenetics experiments. For optogenetic viral delivery of pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS, microinjection (100nl,40nl/min) was performed using the following stereotaxic coordinates for the DR（AP−4.55mm， ML−0.44mm， DV−2.80mm,10°right） based on the mouse brain atlas. Viruses were delivered via a gauge needle by an Ultra Micro Pump (160494 F10E, WPI) over a period of 10 mins; the syringe was not removed until 15–20 min after the end of infusion to allow diffusion of the viruses. Then, the optical fiber (FOC-W-1.25-200-0.37-3.0,Inper,Hangzhou,China) was implanted above the area （AP−4.55mm， ML−0.44mm， DV−2.80mm,10°right） for 0.05 mm （AP−4.55mm，ML−0.44 mm，DV−2.75 mm,10°right). For ICV surgery and an ICV guide cannula implantation was the same to the pharmacology experiment, and was implanted with a headstage for EEG in the same mice as previously described. For the *microinjection of KET* in the bilateral pre-Bötzinger complex, two sides of guide cannula (O.D.0.48×I.D.0.34mm/M3.5，62033，RWD Life Sience Inc.,Shenzhen, China) implantation were performed. CTB-555 100nl (1μg/μL, BrainVTA Technology Co.Ltd, Wuhan, China) was injected in the DR （AP−4.55mm， ML−0.44mm， DV−2.80mm,10°right）or the right side of the pre-Bötzinger complex（AP−6.80mm， ML−1.25mm， DV−4.95mm）at a total content of ~100nL and we waited ~2 weeks to allow the retrograde labeling of projection neurons, respectively.

2.4.2 **Effect of ICV administration of KET on photostimulating of the DR-mediated suppression of S-IRA by PTZ and on EEG changes**

DBA/1 mice undergone by viral delivery of pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS into the DR and implanted an ICV guide cannula with a headstage for EEG for 3 weeks will be used. Those DBA/1 mice were divided into 3 groups. For the control group without

photostimulation of DR (n=7), ICV of the same concentration and volume of vehicle was given at the manner (25% DMSO, 2 μl, at a rate of 0.5 μl/min) prior to IP injection of PTZ (75mg/kg) for 30 mins. For the group treatment by photostimulation of DR without ICV of KET (n=6), ICV of the same concentration and volume of vehicle was given prior to the duration of photostimulation for 15mins and to IP injection of PTZ (75mg/kg) for 30 mins. For another group treatment by photostimulation of DR with ICV of KET(n=7), ICV of KET with the total content of 18.3 nmol was given prior to the duration of photostimulation for 15mins and to IP injection of PTZ (75mg/kg) for 30 mins. The statistical analyses of incidence of S-IRA was performed , respectively. EEG recordings of 3 groups started before PTZ injection for 5 mins and ended post PTZ for 30mins. The statistical analyses of EEG activities were performed as previously described. The parameter of photostimulating of the DR (blue-light, 478 nm, 20 Hz, 10 ms/pulse and 600 pulses, 15 mW ,2omins) was used in the above mice.

2.4.3 Effect of microinjection of KET in the bilateral pre-Bötzinger complex administration of KET on photostimulatiing the DR-mediated suppression of S-IRA by PTZ

DBA/1 mice undergone by viral delivery of pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS into the DR and with the bilateral pre-Bötzinger complex guide cannula implantation for 3 weeks will be used. For the group treated by photostimulation of DR without administration of KET (n=7), microinjection of the same concentration and volume of vehicle into bilateral pre-Bötzinger complex was given in the manner (25% DMSO, 2 μl, at a rate of 0.5 μl/min) prior to IP injection of PTZ (75mg/kg) for 25 mins. For another group treated by photostimulation of DR with administration of KET (n=7), microinjection of KET with 200nl into every unilateralbilateral pre-Bötzinger complex prior to IP injection of PTZ (75mg/kg) for 25 mins. Both groups would be started laser stimulation after microinjection for 10 mins. Total content of KET 400nl the bilateral pre-Bötzinger complex was given for one mouse.The parameter of photostimulating of the DR (blue-light, 478 nm, 20 Hz, 10 ms/pulse and 600 pulses, 15 mW ,20 mins) was used in the above mice. For the laser stimulation, 465-nm blue-light (20Hz, 20ms Pulse Width, 15 mW) was delivered by the laser (B12124,Inper,Hangzhou,China) through a 200-μm optic fiber.

2.4.4 Immunohistochemistry and histology

The placement of the optical fiber cannula tip including ICV and microinjection of KET with bilateral pre-Bötzinger complex in each implanted mouse was verified by histology. After the novelty-seeking test approximately 30mins, DBA/1 mice were sacrificed and perfused with PBS and 4% PFA. After saturated in 30% sucrose (24h), each brain was sectioned into 30μm thickness of coronal slices with a freezing microtome (CM30503, Leica Biosystems, Buffalo Grove, IL,USA), the sections were first washed in PBS 5mins for 3 times and then incubated in blocking solution containing 10% normal donkey serum (017-000-121, Jackson ImmunoResearch, West Grove, PA), 1% bovine serum albumen (A2153, Sigma-Aldrich, St. Louis, MO), 0.3% Triton X-100 in PBS for 1 h at room temperature. Then, for c-fos or TPH2 staining in DR, sections were incubated at 4℃ overnight in rabbit anti-c-fos(1:1000 dilution, 2250T Rabbit mAb /74620 Mouse mAb, Cell Signaling Technolog,Danvers, Massachusetts, USA) primary antibody or mouse anti-TPH2 (1:100 dilution, T0678, Sigma-Aldrich, St. Louis, MO) primary antibody and donkey anti-mouse Alexa 488 secondary antibody (1:1000; A32766, Thermo Fisher Scientific, Waltham, MA, USA), donkey anti-mouse Alexa 546 (1:1000; A10036, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody or goat anti-rabbit cy5 (1:1000; A10523, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody for 2h at room temperature. Similarly, for NK1 or SA-2A staining in PBC, sections were incubated in rabbit anti-NK1(1:1000 dilution, SAB4502913, Sigma-Aldrich, St. Louis, MO) primary antibody or mouse anti-SA-2A (1:100 dilution, sc-166775, Santa Cruz Biotechnology, Dallas,USA) primary antibody at 4℃ overnight and donkey anti-rabbit Alexa 488 secondary antibody (1:1000; A32766, Thermo Fisher Scientific, Waltham, MA, USA), donkey anti-mouse Alexa 546 (1:1000; A10036, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody or goat anti-mouse cy5 (1:400; A10524, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody for 2h at room temperature. After washing with PBS 15mins for 3 times, the sections were mounted onto glass slides and incubated in DAPI (1:4000;Cat# C1002; Beyotime Biotechnology; Shanghai,China) 7mins at room temperature.Finally,the glass slides were sealed sheet by Anti-fluorescence attenuating tablet.All images were taken with Nikon A1 laser-scanning confocal microscope (Nikon, Tokyo, Japan).The numbers of immunopositive cells were

counted and analyzed using ImageJ (NIH, Baltimore, MD). It is worth noting that the mouse of the implantation placement out of the target of brain structure will be abandoned in our experiments. The positive cells co-expression c-fos, CheTA and TPH2 were counted as previously described[7].

2.4.5 Viral vectors

pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS (AVV2/8) (viral titers:6.13×10^13VG/ml,), were purchased from Sheng BO, Co., Ltd (Sheng BO Co. Ltd, Shanghai, China). and the sequence of vectors was designed source by Kazuki Nagayasu (Department of Molecular Pharmacology Graduate School of Pharmaceutical Sciences, Kyoto University). CTB-555(1μg/μL) was purchased from BrainVTA Technology Co.Ltd (Wuhan, China**) .**

2.5 Data analysis

All data are presented as the mean ± SEM. Statistical analyses were performed using SPSS 23 (SPSS Software Inc.,USA). The incidence of S-IRA in different groups was compared using Wilcoxon Signed Rank test. Seizure scores and the latency to AGSZs, the duration of wild running, clonic seizures, tonic-clonic seizures were evaluated using the one-way ANOVA tests or Mann Whitney U test or Kruskal-Wallis H test. ANCOVA was used to compare c-fos-positive cells in the DR of DBA/1 mice with and without photostimulation. Statistical significance was inferred if $p < 0.05$.

3. Results

3.1 5-HTP-mediated suppression of S-IRA evoked by acoustic stimulation was reversed by IP with KET

Compared with vehicle group in primed DBA/1 mice, the incidence of S-IRA evoked by acoustic stimulation was significantly reduced by 5-HTP with the dosage of 200 mg/kg ,i.p ($p < 0.001$). Compared with vehicle group, the incidence of S-IRA in the group pre-treated with 5-HTP (200mg/kg, i.p) + KET (5,10, 25 mg/kg,i.p) was significantly decreased ($p < 0.01$, $p < 0.05$), instead, which indicated the these dosages of KET didn't significantly reversed the lower incidence of S-IRA by 5-HTP. By contrast, there was no difference between the vehicle group and the group with treatment with 5-HTP (200mg/kg, i.p) + KET (20 mg/kg,i.p) ($p > 0.005$) and the incidence of S-IRA was significantly reduced in the group (5-HTP +25% DMSO) compared with the group (KET, 20 mg/kg,i.p) ($p < 0.05$), on the contrary, the

suppressant effects by 5-HTP was markedly reversed administration with KET (20 mg/kg, i.p). (Figure 1)

3.2 5-HTP-mediated suppression of S-IRA evoked by PTZ was reversed by ICV with KET

Compared with vehicle control group, the incidence of S-IRA by PTZ was significantly reduced in the group treatment with 5-HTP and 25% DMSO (i.c.v) ($p < 0.05$). Compared with vehicle control group, the incidence of S-IRA by PTZ was not significantly reduced in the group treatment with 5-HTP and the KET (9.15 nmol, i.c.v) ($p > 0.05$), and compared with the group treated with 5-HTP and 25% DMSO (i.c.v), the incidence of -S-IRA in the group treated with 5-HTP and the KET (9.15 nmol, i.c.v) was significantly increased ($p < 0.05$), which suggested that the suppressant effects of S-IRA by 5-HTP was significantly reversed by KET with the dosage of 9.15 nmol (i.c.v). Furthermore, compared with vehicle control group, the incidence of S-IRA in the group with 5-HTP + KET (18.30 nmol, i.c.v) was not significantly reduced ($p > 0.05$) and the incidence of S-IRA by PTZ in the group treatment with 5-HTP and 25% DMSO (i.c.v) was significantly reduced compared with the group with 5-HTP and KET (18.30 nmol,i.c.v) ($p < 0.05$), instead, the suppressant effects of S-IRA by 5-HTP can be significantly reversed by the dosage of KET (18.30 nmol, i.c.v). There were no significant intergroup differences in latencies to AGSZs, durations of wild running, clonic seizures, tonic-clonic seizures and seizure scores (AGSZs latencies: $p = 0.763$, $F= 6$; durations of wild running: $p = 0.14$, $F= 6$; duration of tonic-clonic seizures: $p = 0.119$, $F= 6$; seizure scores: $p = 0.304$, $F= 6$) (Fig. 1). The seizure score of two models from different groups was no significantly difference ($p > 0.05$), no obvious intervention in seizure behavior of the revered effects by KET was to be fund. (Figure 2)

3.3 Selective expression of ChETA on 5-HT neurons in the DR of DBA/1 mice

The viral of pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS, under the control of promoter of TPH2, was delivered into the DR of wild DBA/1 mice with 50 days to express for 3 weeks to create theTPH2-ChETA mice. We first examined the expression of ChETA and TPH2 in 5-HT neurons in the DR of DBA/1 mice using immunohistochemistry (n = 3 mice). The expression of GFP, a surrogate marker for ChETA, was predominantly localized on the membrane of cell body and axons of 5-HT neurons of the DR. TPH2 was predominantly confined to the cytosol

of 5-HT neurons in the DR of DBA/1 mice. The merged expression ratio of ChETA and TPH2 was basically consistent with our previous data that the co-expression of ChR2 and TPH2 was located on the walls of 5-HT neurons in the DR of DBA/1 mice[7]. (Figure 3, Figure 4)

3.4 Activation of 5-HT neurons in the DR reduces S-IRA evoked by PTZ injection in DBA/1 mice and the the lower incidence of S-IRA by photostimulating the DRwas significantly reversed by ICV of KET

We examined the effect of selective enhancement of 5-HT neurotransmission on S-IRA by PTZ by applying photostimulation (blue light, 20 ms pulse duration, 20 Hz, 20 mins) to 5-HT neurons in the DR of noprimed DBA/1 mice. Comapred with the incidence of S-IRA by PTZ of DBA/1 mice without photostimulation (n=7), photostimulation of the DR for 20 mins significantly decreased the incidence of S-IRA in DBA/1 mice (n=6, $p < 0.05$). However, the incidence of S-IRA evoked by PTZ by photostimulation of the DR with the same parameter by ICV administration of KET was significantly (n=7, $p < 0.05$), which indicated the lower incidence of S-IRA by PTZ via photostimulating the DR was significantly reversed by blocked 5HT2A receptor in tthe brain. (Figure 5)

3.5 Activating 5-HT neurons in the DR to reduce S-IRA evoked by PTZ and the reverse effects to be given for KET produced obvious effects on EEG activity at the different stages

Based on the above findings in the same groups, we further examined the effect of activating 5-HT neurons in the DR to reduce incidence of S-IRA and the reversal effect by KET on EEG activity. Compared with the group without light, the EEG activity was significantly reduced. Analyzing the EEG wave, Delta wave of EEG was significantly reduced by light and reversed by KET ($p < 0.05$) . There is no obvious changes of Theta, Alpha, Beta and Gamma waves in different treatment groups. The findings of EEG activity may reflect the specificity of 5-HTA receptor in brain modulating S-IRA and SUDEP. (Figure 6)

3.6 The reduction of S-IRA by photostimulating the DR is dependent on 5-HT2A receptor locating the pre-Bötzinger complex

Although the incidence of S-IRA can be reduced by 5-HTP and by photostimulating DR and was significantly reversed by both P and ICV injection of KET, it still was to determine whether 5-HT2A receptor in the pre-Bötzinger complex mediated the process of S-IRA and

SUDEP. The incidence of S-IRA evoked by PTZ by photostimulating the DR was 14.28%. However, the incidence of S-IRA evoked by PTZ by photostimulating the DR with microinjection of KET into bilateral pre-Bötzinger complex with total volume of 400 nl was 85.71% . Compared with the group by photostimulating DR without microinjection of KET, the incidence of S-IRA in the group by photostimulating DR with microinjection of KET was significantly reduced ($p < 0.01$). Subsequently, the existing of neural circuit between DR and the pre-Bötzinger complex was verified by using a nerve tracer named as CTB-555. Therefore, in the present study, optogenetic activation of the neural circuit between DR and the pre-Bötzinger complex contributes to inhibit the S-IRA and 5-HT2A receptor in the pre-Bötzinger complex may be a specific target to intervene for preventing SUDEP. (Figure 7-9)

3.7 Photostimulation increases c-fos expression in the 5-HT neurons in the DR

To investigate whether photostimulation increased the excitability of 5-HTneurons, we examined the neuronal expression of c-fos, an immediate early gene that is widely accepted as a marker for neuronal activity in optogenetics studies in the DR of DBA/1 mice with photostimulation and those without photostimulation. Compared with the c-fos expression in 5-HT neurons in the DR of DBA/1 mice without photostimulation (n=2) , the expression of c-fos in 5-HT neurons in the DR was significantly increased in the group with photostimulation (20 ms pulse duration, 20 Hz) at 15 mW for 20 min ($n=2$, $p < 0.05$), which showed the reduction of S-IRA by photostimulation via activating 5-HT neurons. (Figure 10)

4. DISCUSSION

SUDEP is a fatal complication for epilepsy patients. Although initial advancements about 5-HT nerve system have been made to identify the causes of SUDEP, the pathogenesis of SUDEP still seems elusive [1-4]. Currently, respiratory dysfunction during seizures has been regarded as a leading mechanism of SUDEP. Some studies indicated that several selective serotonin (5-HT) reuptake inhibitors (SSRIs) can prevent S-IRA in DBA/1 mice evoked by generalized audiogenic seizures (AGSz) by elevating 5-HT levels in the synaptic cleft [13-16]. However, due to the limitation of animal SUDEP models, the role of 5-HT synthesis and targeting 5-HT receptors in brain in modulating S-IRA and SUDEP remains unclear and still

needs to be further explored.

It had been accepted that tryptophan hydroxylase-2 (TPH2) in brain could convert l-tryptophan to 5-HTP, which can be further converted to 5-HT by aromatic L-amino acid decarboxylase [19-21]. A previous study showed that TPH2 is the rate-limiting enzyme in 5-HT synthesis in brain [19]. Although we only tested the protein changes of TPH2 in our previous study, another study had tested the correlation between TPH2 protein and activity which showed that TPH2 protein varied consistently with TPH2 activity in the same model [22]. Thus, it is vital for TPH2 to make endogenous 5-HTP convert into serotonin synthesis to reduce the incidence of S-IRA and prevent SUDEP. However, it is unknown how 5-HT mediated S-IRA and SUDEP in our model.

Based on our previous study that administration of 5-HTP significantly reduced the incidence of -S-IRA in AGSz and PTZ SUDEP models, it is essential for us to further identify in which 5-HT2AR in the brain plays a key role in modulating the pathogenesis of S-IRA in our models. In our models, the lower incidence of S-IRA by 5-HTP was significantly reversed by IP or ICV injection of KET in the SUDEP models evoked by acoustic stimulation and PTZ administration, a chemoconvulsant that is widely used to model human generalized seizures. While KET reversed the suppressant effects of S-IRA by administration of 5-HTP in a dose-dependent manner in PTZ injection model, there was a ceiling effect of KET on reversing the lower incidence of S-IRA by 5-HTP in the acoustic stimulation SUDEP model.

Our previous study showed that administration of 5-HTP significantly reduced the incidence of S-IRA by anti-convulsant effects. This indicated that most of DBA/1 mice avoided S-IRA without producing tonic-clonic seizures other than wild running and generalized clonic seizures, which remained the sensitivity for seizures. However, administration of KET increased the lower incidence of S-IRA by 5-HTP in both acoustic stimulation and PTZ injection models with producing partly wild running, clonic and/or tonic -clonic seizures, which demonstrated administration of IP of KET reversed the lower incidence of S-IRA by 5-HTP with only partly affecting seizure behaviors by acting on the neuron nucleus functioning in respiratory activity.

To further determine the effects of KET on the incidence of S-IRA by acting on the central nucleus in the brain, we administrated KET with ICV pathway and measured its effect on

S-IRA in DBA/1 mice in both SUDEP models, respectively. The results of KET mediated the reversal effects of the S-IRA incidence by 5-HTP via IP injection in line with via ICV injection, suggesting that the reversal effects by KET in our models are independent of SUDEP models .

Meanwhile, S-IRA in most of DBA/1 mice recovered at 24-72 hours between different treatment groups, which showed the recovery interval of S-IRA depended on the concentration of 5-HT in brain. In addition, we find that there is a dose-dependent effect on reversing the incidence of S-IRA by 5-HTP and a ceiling effect with KET 25mg/kg (i.p). Indeed, our previous study showed that the lower incidence of S-IRA by activating 5-HT neurons in the DR by optogenetics was significantly reversed by ondansetron, a specific antagonist for 5-HT3 receptor[7]. However, it can't rule out that 5-HT2A receptor mediated the pathogenesis of S-IRA and SUDEP by closely interacting with 5-HT3 receptor in the brain. Of course, it needs to be further tested in the future experiments.

Although an SSRI is also found effective in reducing S-IRA evoked by maximal electroshock (MES) in Lmx1b(f/f) mice on a primarily C57BL/6J background, a strain that is resistant to AGSz and depleting 5-HT neurons enhanced the seizure severity, which led to the S-IRA and can be prevented by 5-HT2AR activation through IP with (2,5-dimethoxy-4-iodophenylpropane hydrochloride, DOI), a selective agonist for 5-HT2AR, in MES model [15], it was unclear whether depleting 5-HT neurons itself led to S-IRA and the reversal effects by DOI targeted the peripheral or central 5-HT2AR. By contrast, based on our previous findings, we further explored the role of 5-HT neurotransmission and 5-HT2AR by peripheral and central intervention approaches with KET, a selective antagonist for 5-HT2AR, in different SUDEP models. Thus, according to our findings, the role of 5-HT2AR in modulating S-IRA supported by those from the MES model will further strengthen the understanding of the pathogenesis of S-IRA and SUDEP in the 5-HT nerve system, which will help design therapeutic strategies to prevent SUDEP.

What's more, based on 5-HT2AR located in the pre-Bötzinger complex which plays an important role in modulating respiration rhythm and on our previous finding that the incidence of S-IRA can be significantly reduced by activation of TPH2-ChR2 neurons in the DR, to further test the above pharmacology experiment concerning the exact mechanisms of

the interaction between 5-HT and 5-HT2AR mediating S-IRA and SUDEP in the same models, we used the optogenetics methods to test whether activation of TPH2-ChR2 neurons in the DR significantly reduced the incidence of S-IRA evoked by PTZ via activating 5-HT2AR in the pre-Bötzinger complex or not. In the present study, the lower incidence of S-IRA evoked by PTZ via optogenetic activation of TPH2-ChETA neurons in the DR was remarkably reversed by ICV injection of KET. Subsequently, the lower incidence of S-IRA evoked by PTZ by photostimulating the DR was significantly reversed by microinjection of KET into bilateral pre-Bötzinger complex, in turn, suggesting that photostimulating the DR remarkably reduced the incidence of S-IRA by activating 5-HT2AR in the pre-Bötzinger complex in our model. To further test the bridge between DR and the pre-Bötzinger complex to modulate S-IRA in our study, CTB-555, a nerve tracer, was used in our study to confirm and establish the neural circuit between the DR and the pre-Bötzinger complex. Thus, the lower incidence of S-IRA in PTZ evoked models by photostimulating DR stem from the neural circuit between the DR and the pre-Bötzinger complex. However, we only tested it using photostimulating the DR and direct inhibiting 5-HT2AR in the bilateral pre-Bötzinger complex in our study. Whether activating TPH2-neurons in the pre-Bötzinger complex could reduce the incidence of S-IRA still needs to be verified in the following experiments. Of course, other neural circuits between dorsal raphe nucleus and other brain structures involved in modulating S-IRA and SUDEP can't be excluded in our models. Nevertheless, pharmacologic and optogenetic findings suggested that the neural circuit between the DR and the pre-Bötzinger complex plays a key role in modulating S-IRA and SUDEP.

In addition, different from the pharmacology experiments, although S-IRA of DBA/1 mice was blocked in optogenetic experiments, most of DBA/1 mice remained clonic and tonic seizures. Analyzing of the cause for difference, the specificity of optogenetics and the specific injection of KET by both of ICV and pre-Bötzinger complex may be a cause. Meanwhile, the incidence of S-IRA evoked by PTZ was significantly reduced by activating the neural circuit between DR and the pre-Bötzinger complex without changing EEG activities, which contributed to reflecting the specificity of optogenetic experiments to inhibit S-IRA from another level as well.

5. Conclusion

Our current data suggested that the lower incidence of S-IRA by administration of 5-HTP can be significantly reversed by KET. Furthermore, the suppressant effects of optogenetic activating the neural circuit between DR and the pre-Bötzinger complex on S-IRA were obviously reversed by KET in the DBA/1 mice as well. Therefore, 5-HT2AR in the pre-Bötzinger complex may be a specific and key target to intervene for preventing SUDEP.

6. Funding

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7. ACKNOWLEDGEMENTS

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8. DISCLOSURE

All authors declare no competing interests. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this study is in accordance with those guidelines.

9. Figure legends

Figure 1 5-HTP-mediated reduction of S-IRA evoked by acoustic stimulation was significantly reversed by prazosin.

A. evoked by acoustic stimulation was markedly lower in groups treated with 5-HTP and DMSO,* $p = 0.031$, $p < 0.05$). Compared to the control group treated with vehicle, the incidence of S-IRA was significantly reduced in the group treated with 5-HTP and KET (5-10 mg/kg). However, there was no difference between the control group and the group treated with 5-HTP and KET (20 mg/kg). Furthermore, compared with the group treated with 5-HTP and DMSO, the incidence of S-IRA in the group treated with 5-HTP and KET (20 mg/kg) was increased. B. There were no intergroup differences in latencies to AGSZs, durations of wild

running plus clonic seizures (W+C), tonic-clonic seizures, and seizure scores ($p > 0.05$).

Figure 2 5-HTP-mediated reduction of S-IRA evoked by PTZ was significantly reversed by KET

A. Compared to the control group, S-IRA evoked by PTZ was markedly lower in groups treated with 5-HTP and DMSO. However, there is no difference between the control group and the groups treated with 5-HTP and KET (9.15 and 18.30 nmol). By contrast, the incidence of S-IRA was significantly reduced in the group treated with 5-HTP and i.c.v DMSO as compared with both groups treated with 5-HTP and KET (9.15 and 18.30 nmol).

B. C-F. There were no intergroup differences in latencies to AGSZs, durations of wild running plus clonic seizures (W+C), tonic-clonic seizures, and seizure scores ($p > 0.05$). S-IRA, seizure-induced respiratory arrest; AGSZs, audiogenic seizures; i.p., intraperitoneal injection; DMSO, dimethyl sulfoxide. (mean ± SEM) .

Figure 3 Selective expression of ChETA on 5-HT neurons in the DR of DBA/1 mice

a1, a2 and a3, neuronal immunostaining of GFP, a surrogate marker for ChETA, on 5-HT neurons in the DRof a coronal brain slice. b1, b2 and b3, immunostaining of TPH2, a key enzyme for 5-HT synthesis in the central nervous system (CNS). a1, b1, c3 and d1, merged images, showing the co-expression of TPH2 and GFP in 5-HT neurons. These data demonstrate that ChETA is restrictively expressed on the surface of 5-HT neurons in the DR(n = 3 mice). Confocal image magnifications: a1–d1, 10x; a2–d2, 20x; a3–d3, 40x.

Figure 4 Placement of fiberoptic cannula tips in the DR

A. An example of coronal brain slice, showing the location of a optic fiber cannula tip and the expression of ChETA in the DR of a DBA/1 mouse, according to the mouse atlas of Paxinos and Franklin (4th Edition, Paxinos and Franklin,2013). B, the neuronal immunostaining of co-expression of ChETA and TPH2 in the DR of a DBA/1 mouse. No thermal injury due to photostimulation was observed in the area around the fiberoptic tips.

Figure 5 Optogenetic activation of TPH2-ChETA neurons in DRN-mediated reduction of S-IRA evoked by PTZ was significantly reversed by ICV injection of KET without changing seizures behavior

A. Compared with the control group by treatment PTZ without photostimulation, the incidence of S-IRA by PTZ was significantly reduced by photostimulation (n=7, n=6, p <

0.05). However, the lower incidence of S-IRA by photostimulation was remarkably reversed by ICV injection of KET with the total content of 18.3 nmol (n=6, n=7, $p < 0.05$). No obvious difference between treatment groups in the analysis of seizure score, duration of wild ruing and clonic seizure, GSzs latency and duration of tonic seizure was to be found.

Figure 6 The changing of EEG activity between optogenetic activation of TPH2-ChETA neurons in DRN-mediated reduction of S-IRA by PTZ and it was significantly reversed by ICV injection of KET

A. Compared with the control group by treatment PTZ without photostimulation, the EEG activity of duration clonic seizure and tonic seizure was significantly suppressed in the group treatment PTZ with photostimulation. B. However, the suppression of EEG activity was significantly increased in the group in the group treatment PTZ with photostimulation by microinjection ICV of KET. C. Data wave of EEG activities was significantly reduced by light and reversed by KET.

Figure 7 Optogenetic activation of TPH2-ChETA neurons in DRN-mediated reduction of S-IRA evoked by PTZ was significantly reversed by injection of KET into the pre-Bötzinger complex

Compared with the control group by treatment PTZ with photostimulation, the incidence of S-IRA by PTZ was significantly reduced by photostimulation by microinjection of KET into the bilateral pre-Bötzinger complex with the total volume of 400 nl . No obvious difference between treatment groups in the analysis of seizure score, duration of wild ruing and clonic seizure, GSzs latency and duration of tonic seizure was to be found.

Figure 8 The neural projection from DRto the pre-Bötzinger complex was established by application of a nerve tracer of CTB555

A. An example of coronal brain slice, showing the location of a injection of CTB555 with the co-expression with TPH2 in DRN. B. The projection to pre-Bötzinger complex with the co-expression between NK1 and 5HT2AR .

Figure 9 The neural projection from pre-Bötzinger complex to the DRwas established by application of a nerve tracer of CTB555

B. An example of coronal brain slice, showing the location of a injection of CTB555 with the co-expression with NK1 and 5HT2AR in DRN. B. The projection to DRwith the

co-expression between TPH2 and CTB555 .

Figure 10 C-fos expression was significantly increased in DRby photostimulation of TPH2-ChETA neurons in DRin DBA/1 mice

A, neuronal immunostaining of c-fos , TPH2, GFP and co-localization c-fos,TPH2 and GFP in a DBA/1 mouse that was implanted with fibe roptic cannula without photostimulation (n = 2 mice). B, immunostaining of c-fos-, TPH2 GFP and co-localization c-fos, TPH2 and GFP, in an implanted DBA/1 mouse with photostimulation at 15 mW for 20 min (n = 2 mice). C, quantification of c-fos(+)/GFP(+)/TPH2(+) cells in implanted DBA/1 mice without photostimulation and those in implanted DBA/1 mice with photostimulation. No PS = no photostimulation; PS = photostimulation

Figure 11 Mechanism of 5-HT neurons in brain modulates the S-IRA and SUDEP via the neural circuit between DRand the pre-Bötzinger complex

SUDEP: sudden unexpected death in epilepsy; S-IRA; seizure-induced respiratory arrest; KET: ketanserin; PBC; pre-Bötzinger complex; DR; dorsal raphe nucleus; 5HT2AR; 5HT2A receptor

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Figure1

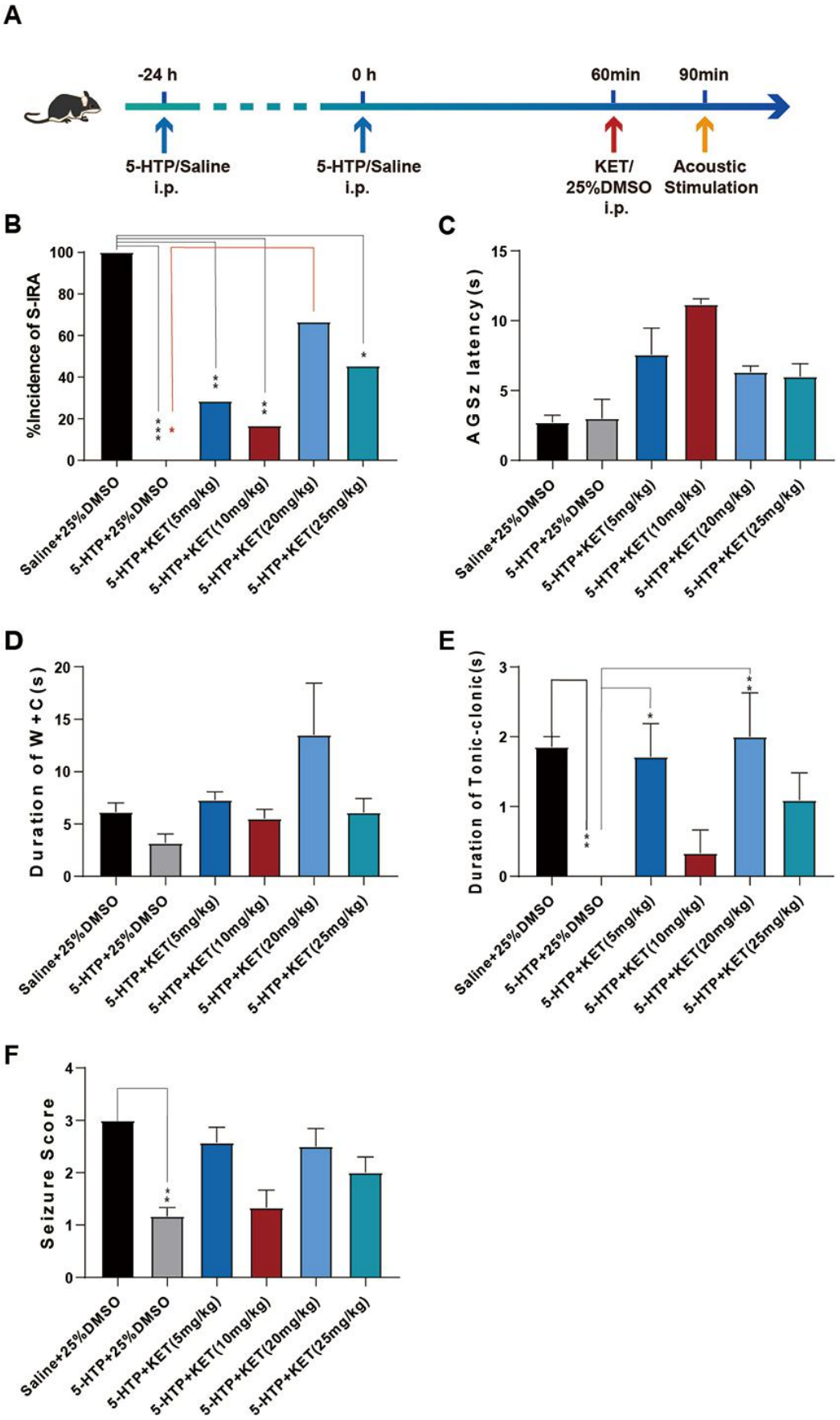
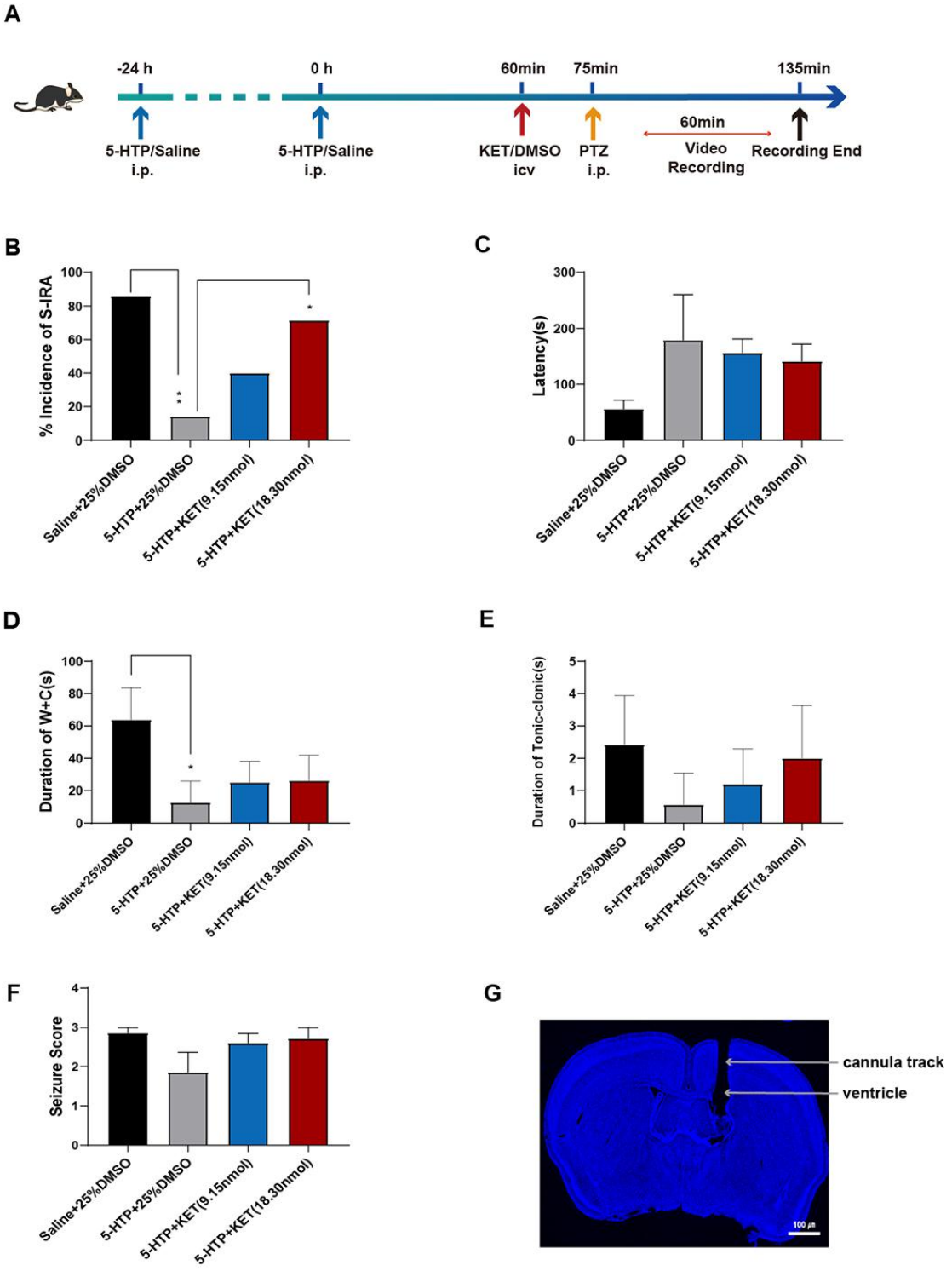

A
-24 h
0 h
60min
90min
5-HTP/Saline i.p.
5-HTP/Saline i.p.
KET/ 25%DMSO i.p.
Acoustic Stimulation
B
%Incidence of S-IRA
C
AGSz latency(s)
D
Duration of W+C(s)
E
Duration of Tonic-clonic(s)
F
Seizure Score
Saline+25%DMSO
5-HTP+25%DMSO
5-HTP+KET(5mg/kg)
5-HTP+KET(10mg/kg)
5-HTP+KET(20mg/kg)
5-HTP+KET(25mg/kg)

Figure2


A
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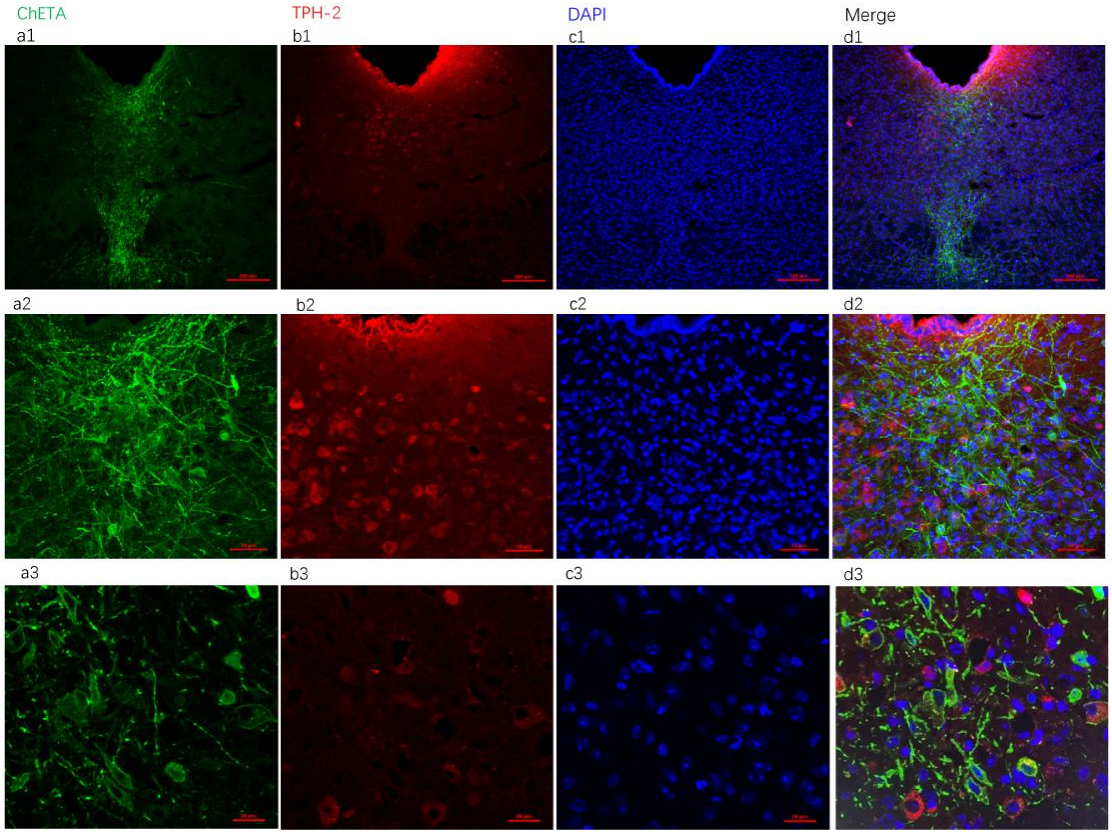
0 h
60min
75min
135min
5-HTP/Saline i.p.
5-HTP/Saline i.p.
KET/DMSO icv
PTZ i.p.
60min
Video Recording
Recording End
B
% Incidence of S-IRA
C
Latency(s)
D
Duration of W+C(s)
E
Duration of Tonic-clonic(s)
F
Seizure Score
Saline+25%DMSO
5-HTP+25%DMSO
5-HTP+KET(9.15nmol)
5-HTP+KET(18.30nmol)
G
cannula track
ventricle
100 μm

1

Figure 3



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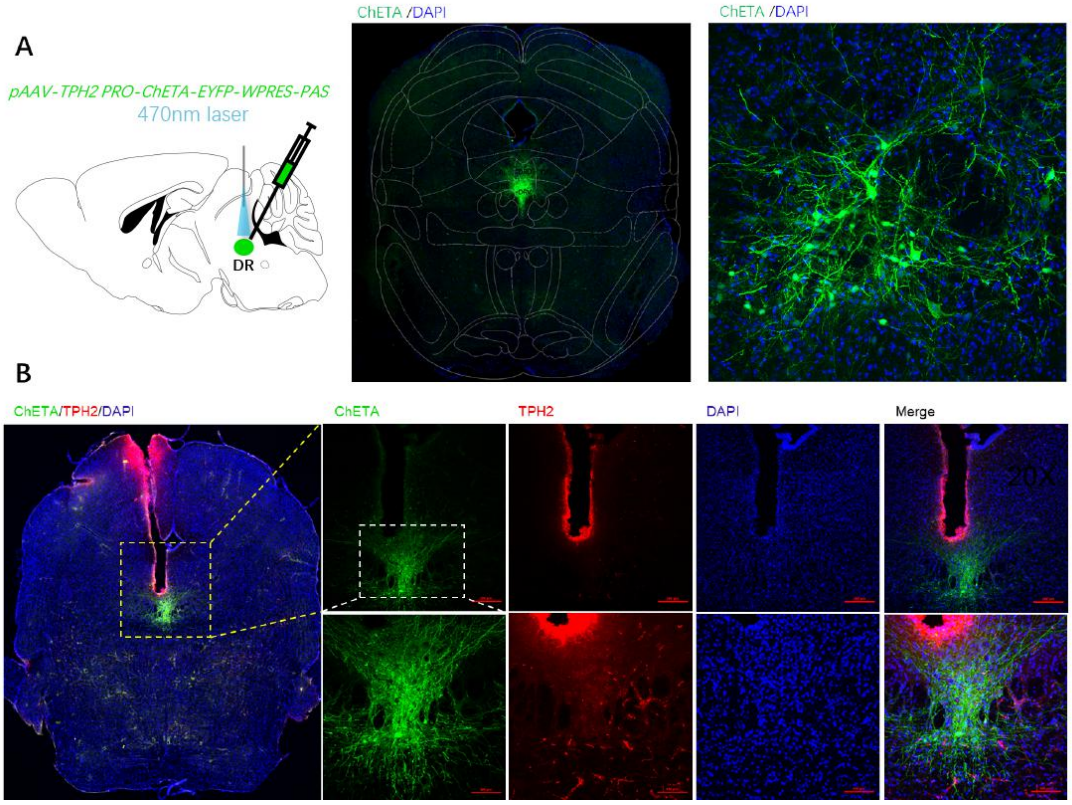

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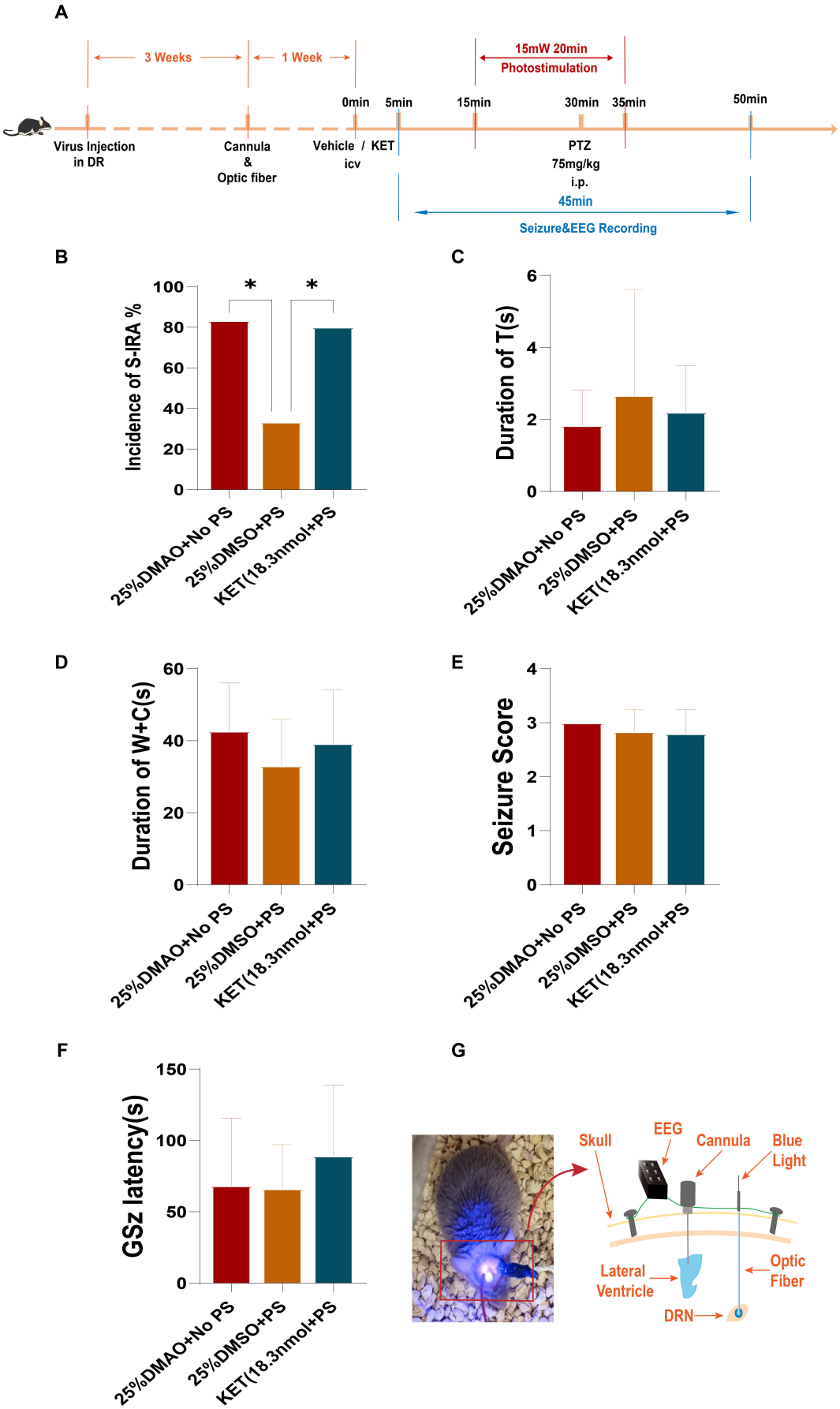

A
3 Weeks
1 Week
15mW 20min
Photostimulation
0min
5min
15min
30min
35min
50min
Virus Injection
in DR
Cannula
&
Optic fiber
Vehicle / KET
icv
PTZ
75mg/kg
i.p.
45min
Seizure&EEG Recording
B
Incidence of S-IRA %
100
80
60
40
20
0
*
*
25%DMAO+No PS
25%DMSO+PS
KET(18.3nmol+PS
C
Duration of T(s)
6
4
2
0
25%DMAO+No PS
25%DMSO+PS
KET(18.3nmol+PS
D
Duration of W+C(s)
60
40
20
0
25%DMAO+No PS
25%DMSO+PS
KET(18.3nmol+PS
E
Seizure Score
4
3
2
1
0
25%DMAO+No PS
25%DMSO+PS
KET(18.3nmol+PS
F
GSz latency(s)
150
100
50
0
25%DMAO+No PS
25%DMSO+PS
KET(18.3nmol+PS
G
Skull
EEG
Cannula
Blue
Light
Lateral
Ventricle
Optic
Fiber
DRN

Figure6

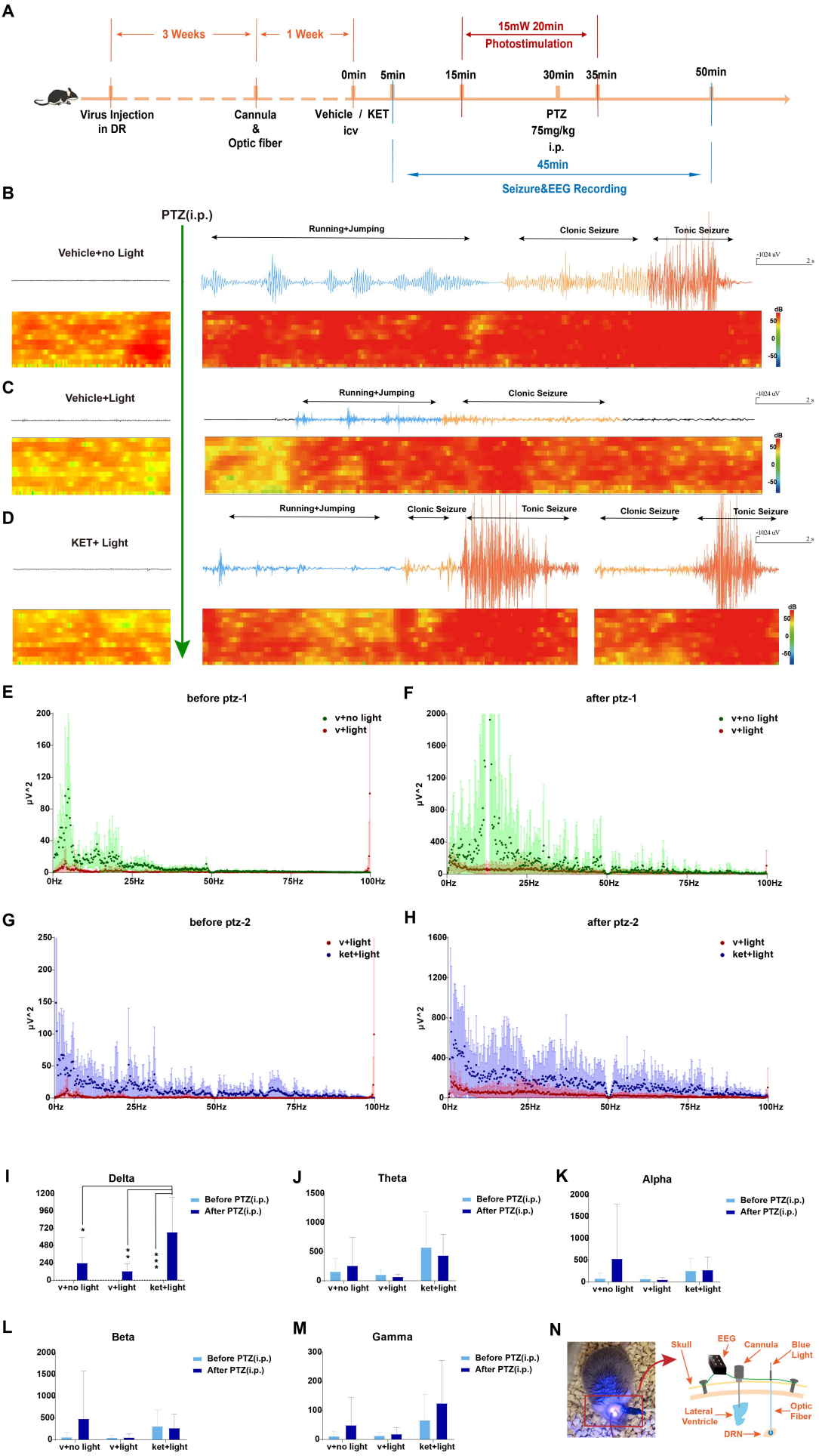

A
3 Weeks
1 Week
15mW 20min
Photostimulation
0min
5min
15min
30min
35min
50min
Virus Injection in DR
Cannula & Optic fiber
Vehicle / KET icv
PTZ 75mg/kg i.p.
45min
Seizure&EEG Recording
B
PTZ(i.p.)
Vehicle+no Light
Running+Jumping
Clonic Seizure
Tonic Seizure
C
Vehicle+Light
D
KET+ Light
E
before ptz-1
v+no light
v+light
F
after ptz-1
G
before ptz-2
ket+light
H
after ptz-2
μV^2
0Hz
25Hz
50Hz
75Hz
100Hz
I
Delta
J
Theta
K
Alpha
L
Beta
M
Gamma
Before PTZ(i.p.)
After PTZ(i.p.)
N
Skull
EEG
Cannula
Blue Light
Lateral Ventricle
Optic Fiber
DRN

Figure7

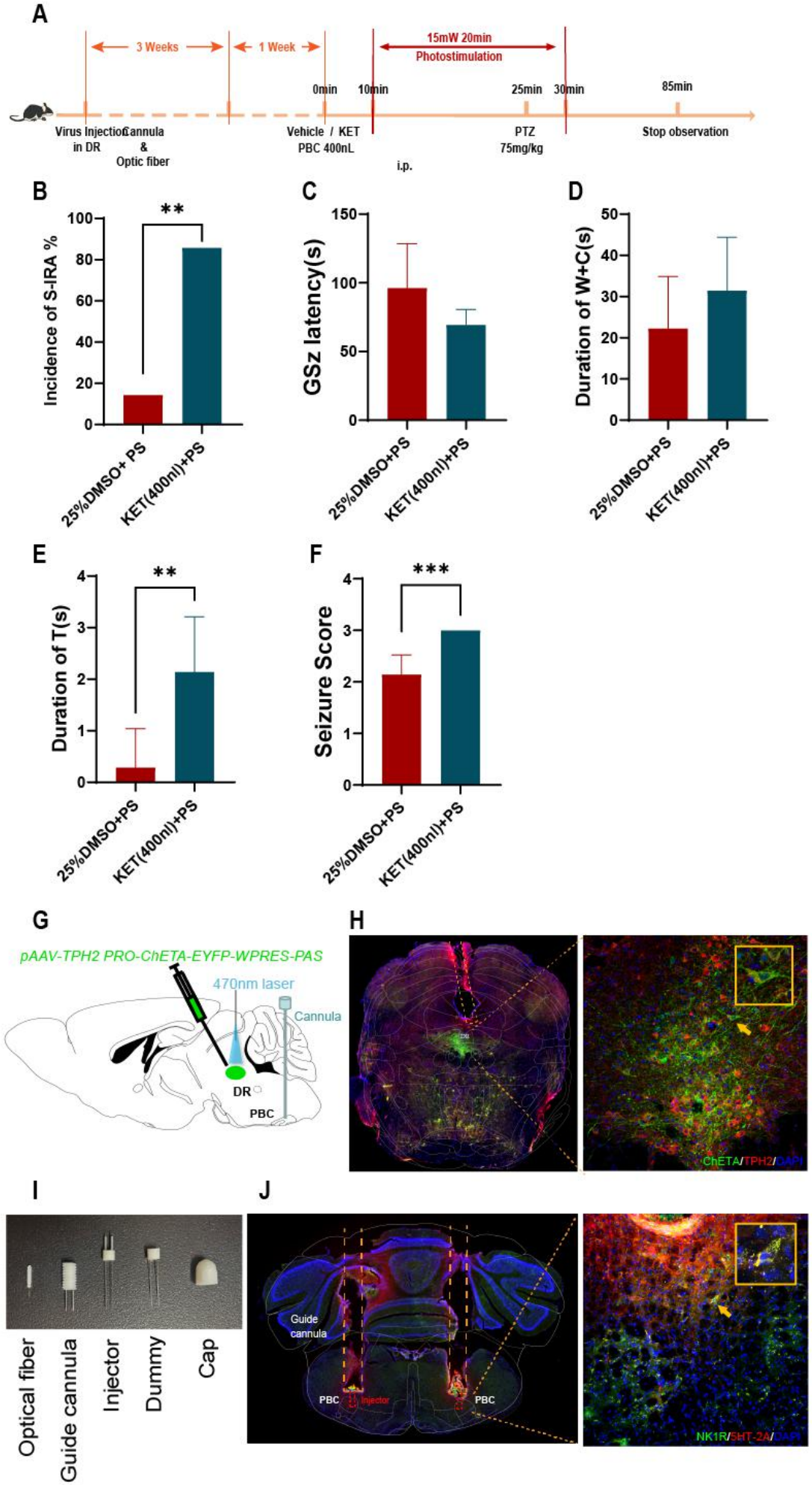

A
3 Weeks
1 Week
15mW 20min
Photostimulation
0min
10min
25min
30min
85min
Virus Injection in DR
Cannula & Optic fiber
Vehicle / KET PBC 400nL
i.p.
PTZ 75mg/kg
Stop observation
B
Incidence of S-IRA %
**
25%DMSO+ PS
KET(400nl)+PS
C
GSz latency(s)
25%DMSO+PS
KET(400nl)+PS
D
Duration of W+C(s)
25%DMSO+PS
KET(400nl)+PS
E
Duration of T(s)
**
25%DMSO+PS
KET(400nl)+PS
F
Seizure Score
***
25%DMSO+PS
KET(400nl)+PS
G
pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS
470nm laser
Cannula
DR
PBC
H
ChETA/TPH2/DAPI
I
Optical fiber
Guide cannula
Injector
Dummy
Cap
J
Guide cannula
PBC
Injector
PBC
NK1R/5HT-2A/DAPI

Figure 8

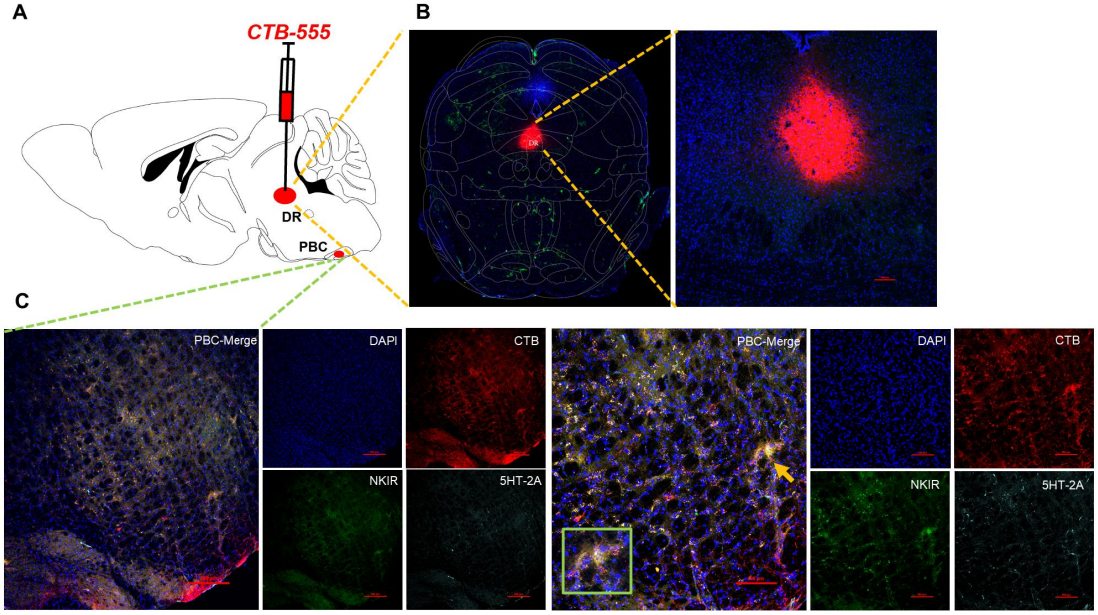


Figure9

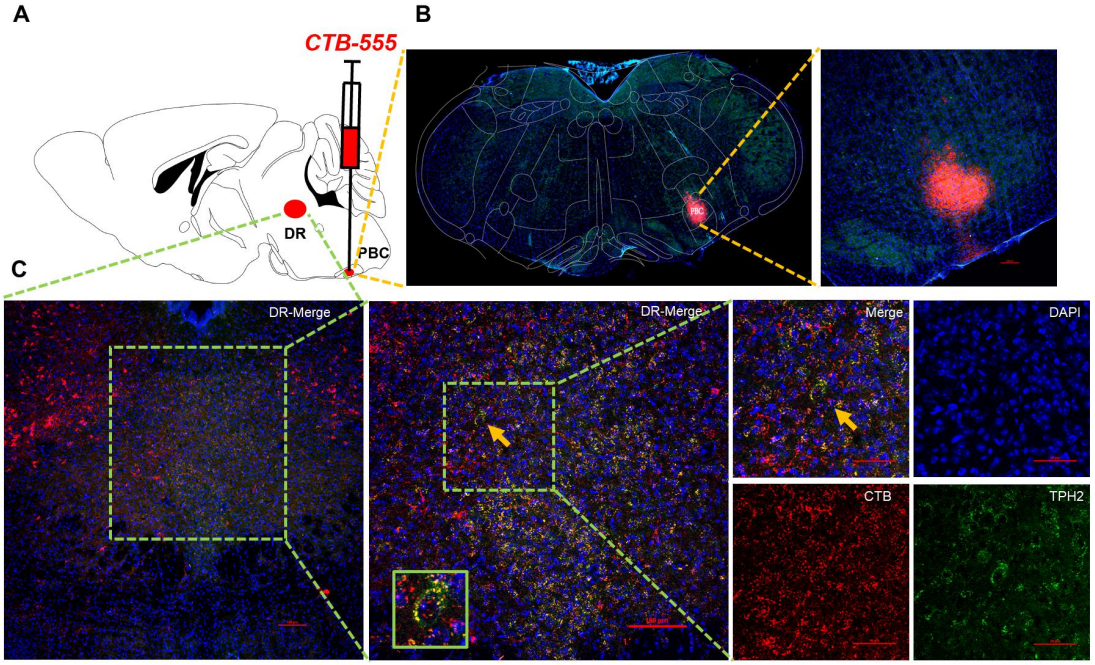
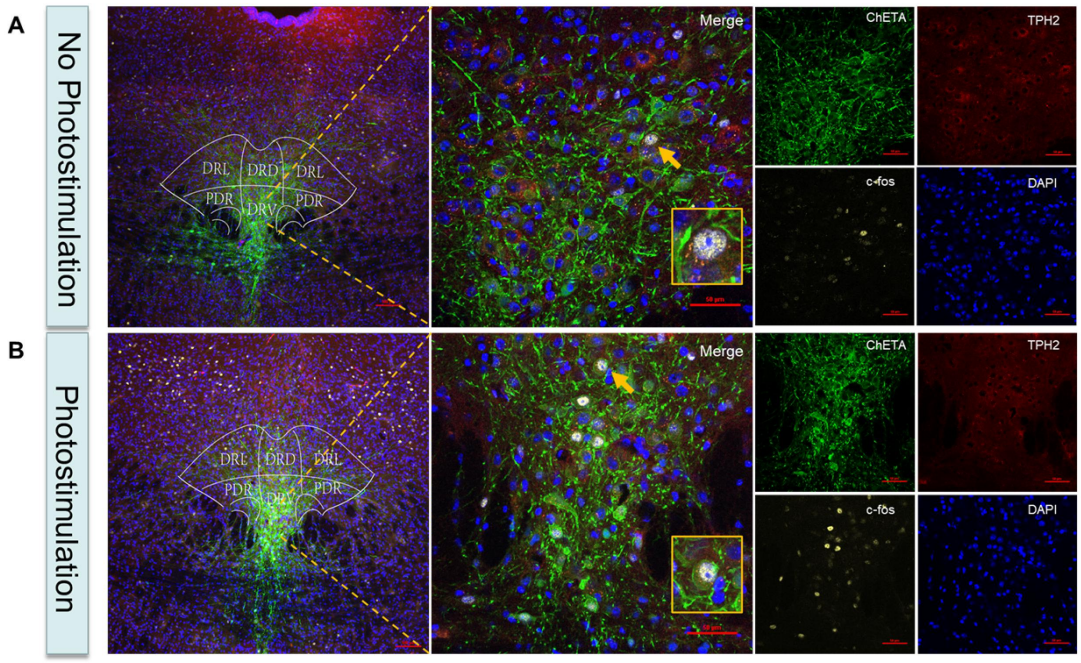
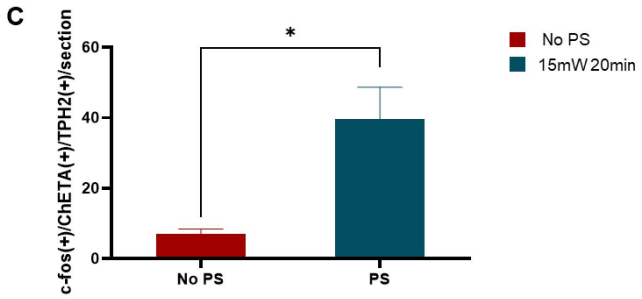
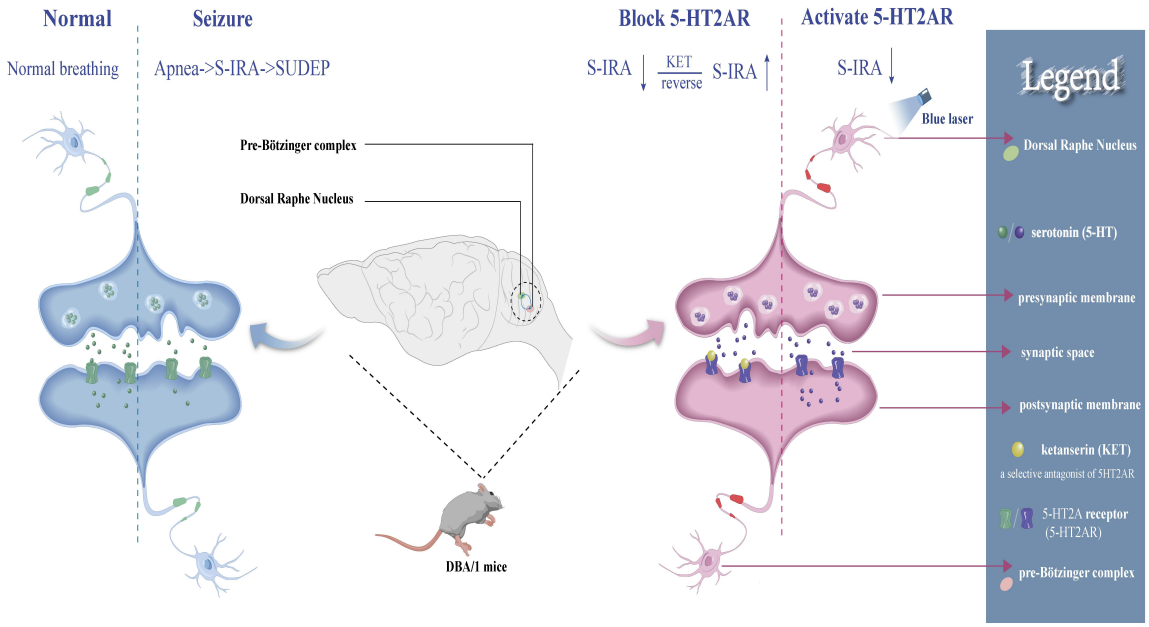


Figure10


A
No Photostimulation
B
Photostimulation
DRL
DRD
DRL
PDR
DRV
PDR
Merge
ChETA
TPH2
c-fos
DAPI


C
c-fos(+)/ChETA(+)/TPH2(+)/section
60
40
20
0
*
No PS
PS
No PS
15mW 20min

1


Normal
Seizure
Normal breathing
Apnea->S-IRA->SUDEP
Block 5-HT2AR
Activate 5-HT2AR
S-IRA
KET
reverse
S-IRA
S-IRA
Blue laser
Pre-Bötzinger complex
Dorsal Raphe Nucleus
DBA/1 mice
Legend
Dorsal Raphe Nucleus
serotonin (5-HT)
presynaptic membrane
synaptic space
postsynaptic membrane
ketanserin (KET)
a selective antagonist of 5HT2AR
5-HT2A receptor
(5-HT2AR)
pre-Bötzinger complex

2