Activation of central 5-HT2A receptor in brain inhibited 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 with anti-epileptic drug resistance. However, the underlying mechanism of SUDEP remains to be elusive. Our previous study demonstrated that enhancement of serotonin (5-HT) synthesis by intraperitoneal (IP) injection of 5-hydroxytryptophan in brain significantly reduced the incidence of seizure-induced respiratory arrest (S-IRA) in DBA/1 mice SUDEP models. Given that 5-HT2A receptor (5-HT2AR) acts an important role in mediating 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 S-IRA evoked by either acoustic stimulation or PTZ by blocking 5-HT2AR by administration with ketanserin (KET), a selective antagonist of 5HT2AR, in DBA/1 mice SUDEP models to test the role of 5-HT2AR 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 and intracerebroventricularly (ICV) injection of ketanserin in our models. Thus, our data suggested that 5-HT2AR in the brain may be a potential and specific target to prevent SUDEP.

Keywords: Sudden Unexpected Death in Epilepsy; 5-Hydroxytryptophan (5-HTP); ketanserin (KET); 5HT2A receptor.

1. INTRODUCTION

Growing studies have shown that sudden unexpected death in epilepsy (SUDEP) is the most important cause led to the refractory epilepsy patients 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 coustic 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 brain remains to be unclear and need still to further be explored. Given that 5-HT2AR has emerged as a key player in mediating respiratory function in brainstem[11-13], we wonder if the application of 5-HTP reduced the incidence of S-IRA by targeting 5-HT2AR in brain. Therefore, we hypothesized that the suppressant effects of 5-HTP on S-IRA was 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, based on our previous study, we continued to chose acoustic stimulation and PTZ injection DBA/1 mice SUDEP models to test the reversal effects of KET. In two models, the lower incidence of S-IRA by 5-HTP was significantly reversed by intraperitoneal (IP) and intracerebroventricular (ICV) injections 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 by anticonvulsant effects in our models. The anticonvulsant effects of 5-HTP was 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.

2. MATERIALS AND METHODS

2.1 Animals

All experimental procedures were 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 and. The two models were established just as described previously [5-10].

2.2 Seizure induction and resuscitation

S-IRA was evoked by acoustic stimulation and 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 the 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 Effect of IP administration of KET on 5-HTP-mediated suppression of S-IRA evoked by acoustic stimulation

A vehicle and different doses of KET (Cat # 8006; Sigma-Aldrich) were administered to different groups of mice which were then acousticaly 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. In control group, 5-HTP or vehicle (saline) was administered i.p. once a day for 2 days and was performed 30 mins after the second administration and before acoustic stimulation. In experimental group, 5-HTP was administered i.p. once a day for 2 days and induction of S-IRA was performed 1 h after the second administration. KET was administered i.p. 30 mins before acoustic stimulation. The effects of 5-HTP and KET on S-IRA was 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 incidence 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].

2.4 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 120 min 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 120 min 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 120 min 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 above groups was in the manner of administering i.p. once a

day for 2 days.

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. 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 the vehicle group in primed DBA/1 mice, the incidence of S-IRA evoked by acoustic stimulation was significantly reduced by 5-HTP and 25% DMSO ($p < 0.001$). 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) ($p > 0.05$) 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). There were no significant intergroup differences in latencies to AGSZs ($p > 0.05$). However, compared with the group treated with 5-HTP and 25% DMSO, the durations of tonic-clonic seizures were significantly increased in the the vehicle group and groups treated with 5-HTP (200mg/kg, i.p) + KET (5, 20 mg/kg,i.p) ($p < 0.05$). Compared with the vehicle group, seizure scores was significantly reduced in the group treate with 5-HTP and 25% DMSO other than treated groups ($p < 0.05$). (Figure 1)

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

To distinguish whether there is possible differential effects of KET on 5-HTP-mediated suppression of S-IRA evoked by different epileptogenic stimuli, we further chose an established SUDEP model using PTZ to induce S-IRA. 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) ($p < 0.01$). 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) ($p > 0.05$), 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 nmo, i.c.v). There were no significant intergroup differences in latencies to AGSZs and tonic-clonic seizures and seizure scores ($p > 0.05$). Compared with the vehicle control group, The durations of wild running and clonic seizures was significantly reduced in the group treated with 5-HTP and 25% DMSO ($p < 0.05$). (Fig. 2).

4. DISCUSSION

SUDEP is a fatal complication for epilepsy patients. Although initial advancements about 5-HT nerve system has been made to identify the causes of SUDEP, the pathogenesis of SUDEP seems still to be elusive [1-4]. Currently, to our certain knowledge, 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 l-tryptophan from dietary sources is converted to 5-HTP by the enzyme tryptophan hydroxylase-2 (TPH2) in the brain, and 5-HTP is then converted to 5-HT by aromatic L-amino acid decarboxylase [19-21]. 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 a study had tested 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 make endogenous 5-HTP covert into serotonin synthesis to reduce the incidence of S-IRA and prevent SUDEP. However, it is unknown how 5-HT to mediate 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 which 5-HT2AR plays a key role in modulating the pathogenesis of S-IRA in our models. In our modes, the lower incidence of S-IRA by 5-HTP was significantly reversed by IP and 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 the manner of dose-dependence in PTZ injection model, there was a ceiling effects of KET on reversing the lower incidence of S-IRA by 5-HTP in acoustic stimulation SUDEP model .

As our previous study showed that administration of 5-HTP reduced significantly the incidence of S-IRA by anti-convulsant effects, indicating 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 of acoustic stimulation and PTZ injection models with producing partly wild running, clonic and/or tonic - clonic seizures, which demonstrated administration 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 administration of KET on the incidence of S-IRA

by acting on the central nucleus in 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 mediating the reversal effects of the S-IRA incidence by IP injection of 5-HTP were in line with ICV injection pathway, suggesting that the reversal effects by KET in our models is independent of SUDEP models and specific .

Meanwhile, S-IRA in most of DBA/1 mice recover at 24-72 hours between different treatment groups, which showed the recover interval of S-IRA depended on the concentration of 5-HT in brain. In addition, We found that there is a dose-dependent effects on reversing the incidence of S-IRA by 5-HTP and a ceiling effect with KET 25mg/kg (i.p). Indeed, our previous study that 5-HT3 receptor in brain plays a key role in mediating S-IRA and the lower incidence of S-IRA by activating 5-HT neurons in the dorsal raphe (DR) by optogenetics and the lower incidence of S-IRA 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 brain. Of course, it need 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 prevent 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 of 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, our findings of the role of 5-HT2AR in modulating S-IRA supported by those from the MES model will further strengthen understanding the pathogenesis of S-IRA and SUDEP in the 5-HT nerve system, which will help design therapeutic strategies to prevent SUDEP. In addition, the limitation existing in our study is that we

failed to identify the 5-HT2AR-mediated neural circuits and characterized the precise target involved in S-IRA in brain. However, in the following experiments, we will further explore the 5-HT2AR-mediated neural circuits using optogenetic technology.

5. **Conclusion**

Our current data suggested that the lower incidence of S-IRA by administration of 5-HTP can be significantly reversed by KET and enhancing central the synthesis of 5-HT and serotonergic neurotransmission, and targeting the central 5HT2AR may provide the rational therapeutic strategies to prevent SUDEP.

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7. 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.

8. **Figure legends**

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

B. Compared with the control group treated with vehicle, the incidence of S-IRA was significantly reduced in the group treated with 5-HTP and 25% DMSO ($p < 0.001$). Compared with the control group treated with vehicle, the incidence of S-IRA was significantly reduced in the groups treated with 5-HTP and 25% KET (5,10,25 mg/kg) ($p < 0.01$, $p < 0.05$, $p < 0.05$). However, there was no difference between the control group and the group treated with 5-HTP and KET (20 mg/kg) ($p > 0.05$). 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 significantly increased ($p < 0.05$). C and D. There were no intergroup differences in latencies to AGSZs, durations of wild running plus clonic seizures (W+C) ($p > 0.05$) . E. Compared with the group treated with 5-HTP and 25% DMSO, the durations of tonic-clonic seizures were significantly increased in the the vehicle group and groups treated with 5-HTP (200mg/kg, i.p) + KET (5, 20 mg/kg,i.p) ($p < 0.05$). F. Compared with the vehicle group, seizure scores was significantly reduced in the group treate with 5-HTP and 25% DMSO other than treated groups ($p < 0.05$).

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

B. Compared to the control group, the incidence of S-IRA evoked by PTZ was markedly lower in groups treated with 5-HTP and 25% DMSO ($p < 0.01$). 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 25% DMSO as compared with both groups treated with 5-HTP and KET (18.30 nmol)($p < 0.05$). However, compared to the control group, the incidence of S-IRA in the groups treated with 5-HTP and KET (9.15 and 18.30 nmol) was not significantly reduced ($p > 0.05$). C. There were no intergroup differences in latencies to AGSZs ($p > 0.05$). D. Compared with control group, durations of wild running plus clonic seizures (W+C) was significantly reduced ($p < 0.05$). E and F. There were no intergroup differences in tonic-clonic seizures, and seizure scores ($p > 0.05$). F. A representative illustration for the implantation of ICV cannula in DBA/1 mice.

Figure 3 Mechanism of 5-HTP-mediated reduction of S-IRA was reversed by KET in the synaptic space of central nervous system.

Sudden unexpected death in epilepsy (SUDEP); 5-Hydroxytryptophan (5-HTP); 5-Hydroxytryptamine (5-HT), ketanserin (KET); 5-HT2A receptor (5-HT2AR) .

9. References

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Table1. Summary of experimental groups of DBA/1 mice.

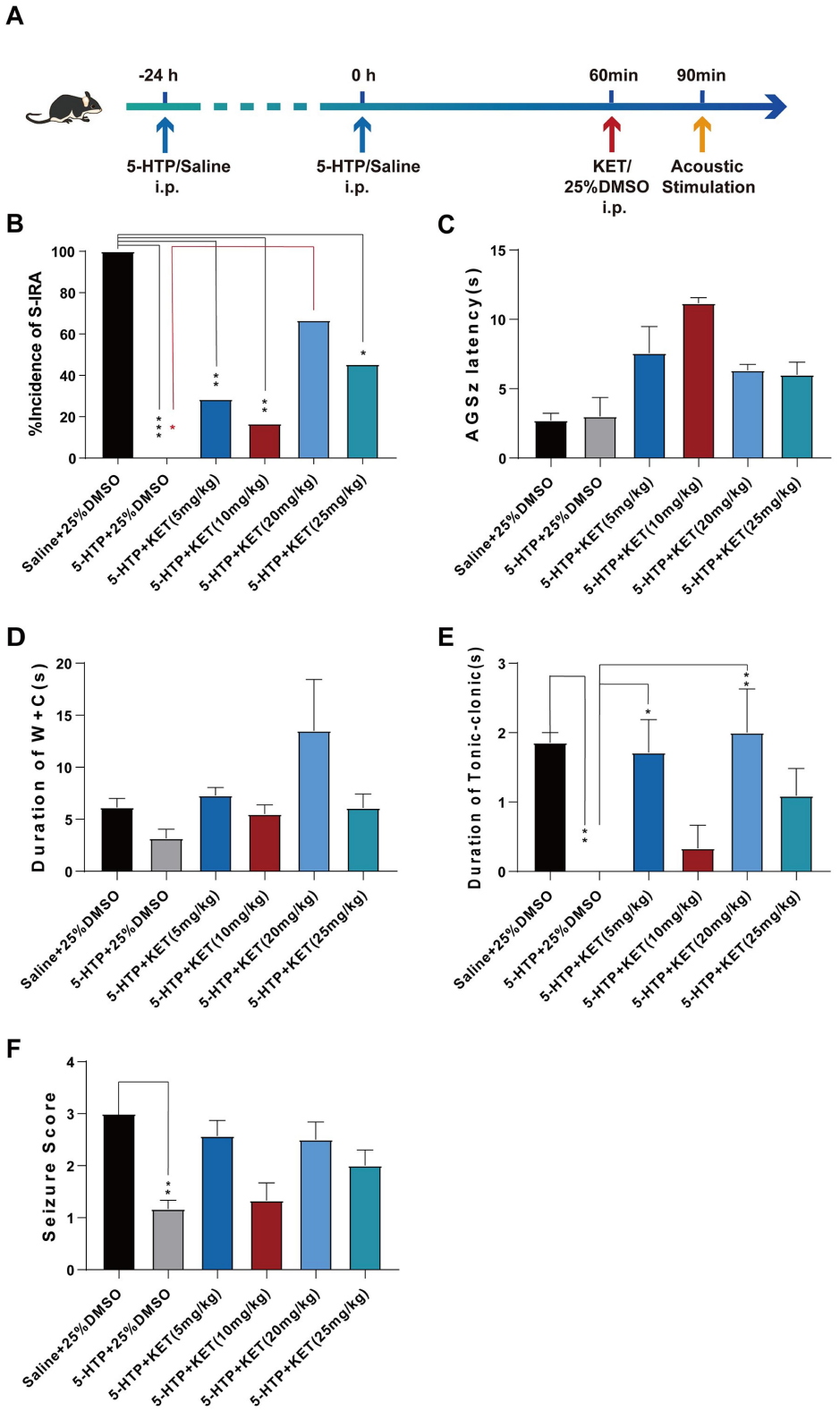
Figure	Experimental groups	Numbers of mice
Fig.1	Vehicle+25%DMSO	7
	5-HTP+25%DMSO	6
	5-HTP+KET(5mg/kg)	7
	5-HTP+KET(10mg/kg)	6
	5-HTP+KET(20mg/kg)	6
	5-HTP+KET(25mg/kg)	11
Fig.2	Vehicle+25%DMSO	7
	5-HTP+25%DMSO	7
	5-HTP+KET(9.15nmol)	5
	5-HTP+KET(18.30nmol)	7

Table2. Statistical Analysis

Figure	Comparisons	Test
Fig.1	Incidence of S-IRA	Wilcoxon Signed Rank Test
	AGSz latency(s)	Kruskal-Wallis H Test
	Duration of W+C	Kruskal-Wallis H Test
	Duration of Tonic-clonic(s)	Kruskal-Wallis H Test
	Seizure Score	Kruskal-Wallis H Test

	Incidence of S-IRA	Wilcoxon Signed Rank Test
	AGSz Latency(s)	Kruskal-Wallis H Test
Fig.2	Duration of Tonic-clonic(s)	Kruskal-Wallis H Test
	Seizure Score	Kruskal-Wallis H Test
	Duration of W+C	Kruskal-Wallis H Test

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)

1

Figure2

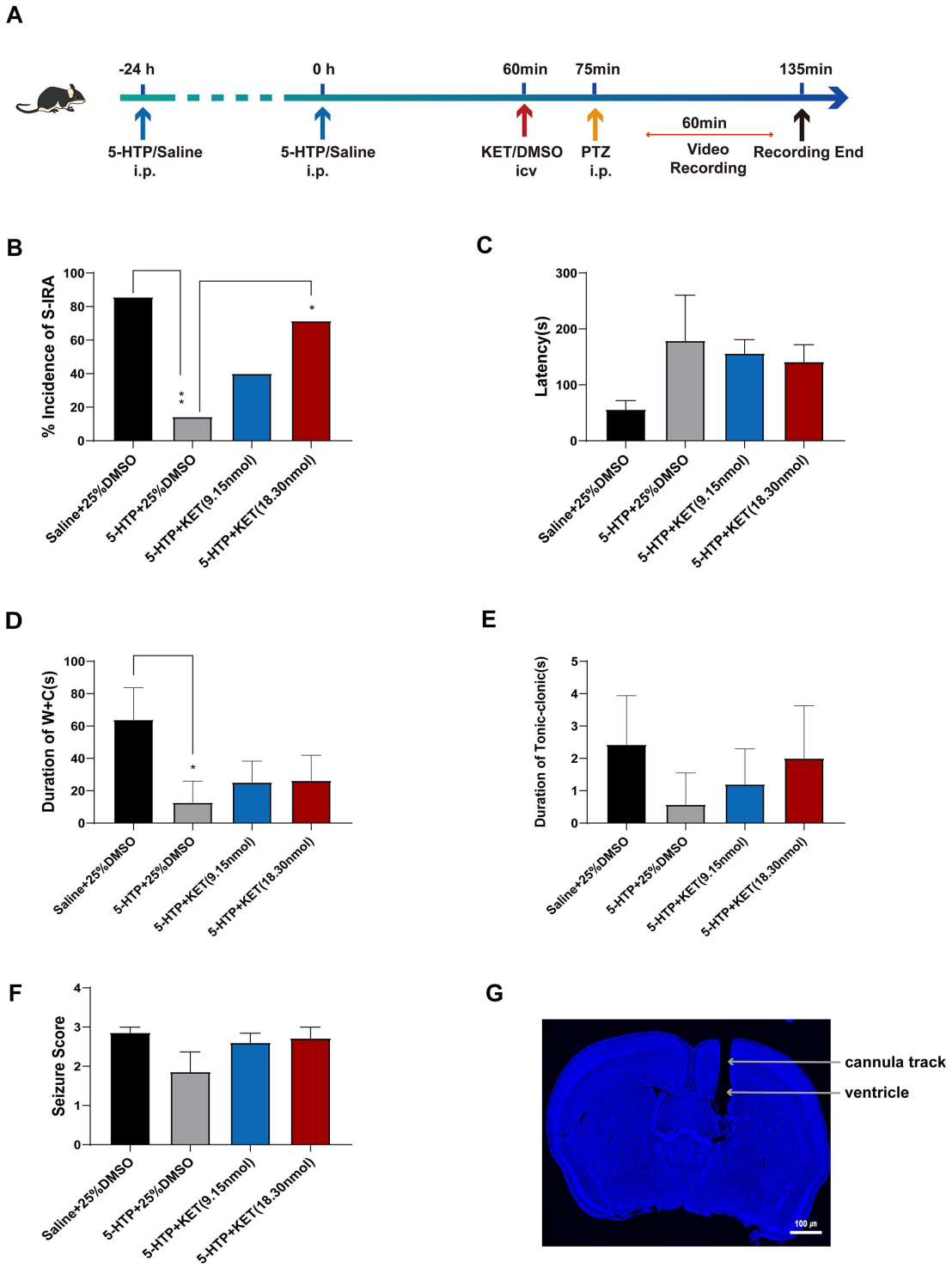
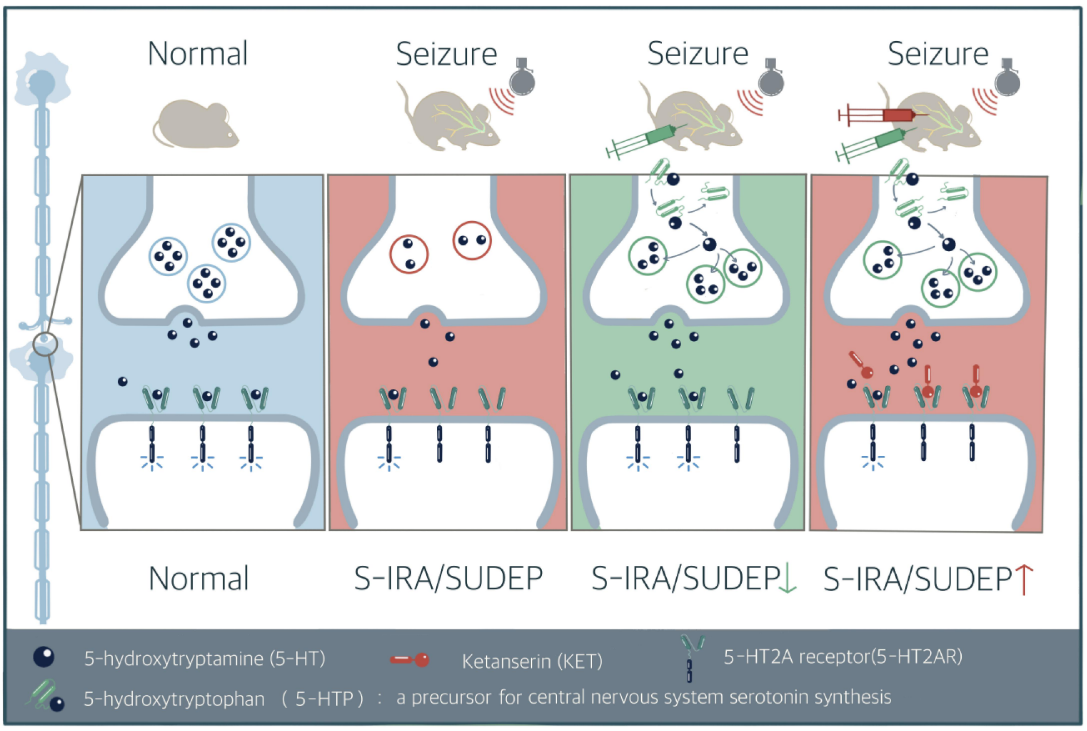


FIG3


Normal
Seizure
Seizure
Seizure
Normal
S-IRA/SUDEP
S-IRA/SUDEP↓
S-IRA/SUDEP↑
5-hydroxytryptamine (5-HT)
Ketanserin (KET)
5-HT2A receptor(5-HT2AR)
5-hydroxytryptophan （5-HTP）：a precursor for central nervous system serotonin synthesis

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