# Deficiency of Norepinephrine Transmission In Brain Contributed To Seizure-Induced Respiratory Arrest And Is Implicated in ADHD with SUDEP In DBA/1 mice

In the DBA/1 Mouse SUDEP Model

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## Abstracts

Sudden unexpected death in epilepsy (SUDEP) is the key cause resulting in the death of children and adolescents epilepsy patients with attention-deficit hyperactivity disorder (ADHD). The underlying mechanism of SUDEP seems to be elusive to date. Although we had previously reported that seizure-induced respiratory arrest (S-IRA) plays an important role in modulating the occurrence of SUDEP, especially for the epilepsy patients suffering from the ADHD and epilepsy, the exact mechanism of it to decode still needs to be explored. Given

that our previous findings suggested that S-IRA evoked by acoustic stimulation or pentylenetetrazole (PTZ) was markedly reduced by systemic administration of atomoxetine which commonly was used to treat ADHD, a selective norepinephrine reuptake inhibitor (NRI), in DBA/1mice SUDEP model and norepinephrine α-1 receptor (NEα-1R) in brain acts as an important player in mediating the respiration function , we hypothesized that the suppressed effects of S-IRA by atomoxetine was via acting on NEα-1R. To test this hypothesis, we examined whether the suppressant incidence S-IRA evoked by either acoustic stimulation or PTZ by atomoxetine in DBA/1 mice SUDEP model . Our results suggest that the decreased incidence of S-IRA by atomoxetine was significantly reversed by intraperitoneal (IP) and intracerebroventricularly (ICV) injection of prazosin, a selective antagonist of NEα-1R in our models. Furthermore, no obvious changes of electroencephalogram (EEG) data in cerebral cortex between the group with administration with atomoxetine and the group with administration of prazosin in PTZ injection SUDEP model to be observed. In addition, our data showed the repetitive acoustic stimulation or S-IRA didn't result in the reduction of tyrosine hydroxylase (TH)protein, a rate-limiting enzyme for synthesizing norepinephrine, in the brainstem of DBA/1 mice, suggesting that administration of atomoxetine reduced the incidence of S-IRA by enhancing the synaptic space concentration of norepinephrine via bypassing TH. Thus, our data suggest that deficiency of norepinephrine transmission contributed to seizure-induced respiratory arrest and NEα-1R in the brain for children and adolescents epilepsy patients suffering from the co-morbidity of ADHD and epilepsy may be a potential and specific target to prevent SUDEP.

Keywords: SUDEP, prazosin, norepinephrine, norepinephrine α-1receptor, generalized seizures, tyrosine hydroxylase, heart rate, blood pressures, ADHD

Abbreviations:

SUDEP:sudden unexpected death in epilepsy

S-IRA: seizure-induced respiratory arrest

NE: norepinephrine

NRI: norepinephrine reuptake inhibitor

AGSz: audiogenic seizures

PTZ : pentylenetetrazole

DMSO: dimethyl sulfoxide

NEα-1R: norepinephrine α-1 receptor

IP:intraperitoneal

ICV:intracerebroventricular

EEG: electroencephalogram

TH: tyrosine hydroxylase

ADHD: attention-deficit hyperactivity disorder

## 1. Introduction

Epilepsy is not uncommon in children and adolescents , accounting for 3.2-5.5/1000. About one-third of patients also have attention deficit hyperactivity disorder (ADHD), of which died from complications of epilepsy (Verrotti A, et al., 2018; Socanski D, et al., 2013 ; Donner EJ, et al., 2017; Keller AE, et al., 2018).The most cause for death of epilepsy patients with ADHD was the sudden unexpected death in epilepsy (SUDEP) (Bateman,et al., 2010; Tomson, et al., 2008; Lhatoo , et al., 2015). Latest evidence suggested that the incidence of SUDEP in children is similar to adults including child and adolescent involved in the co-morbidity of ADHD and epilepsy. Although the exact mechanism concerning the occurrence of SUDEP seems still to be unclear, more and more evidence indicate that the dysfunction of cardiopulmonary had been the most cause for it (Devinsky,et al., 2016). However, it needs to figure out whether the SUDEP of child and adolescent involved in the co-morbidity of ADHD and epilepsy was attributed to the dysfunction of cardiopulmonary. Our previous findings demonstrated that seizure-induced respiratory arrest (S-IRA) is the most important cause leading to SUDEP and the incidence of S-IRA in DBA/1 mice evoked by acoustic stimulation or pentylenetetrazole (PTZ) was significantly reduced by systemic administration of atomoxetine for treating ADHD, a selective norepinephrine reuptake inhibitor, (Zhang,et al., 2016; Gordon and George, 2016; Zhang,et al., 2017; Zhao,et al., 2017; Zhang, et al.,2018;). However, the mechanisms underlying atomoxetine suppressing S-IRA are unknown (Devinsky,et al., 2016). In addition, our previous study showed that the excitatory synapses neuro-transmission in brain can promote the epilepsy pathogenesis in different epilepsy models (Zhou,et al., 2009; Shen, et al., 2016). Thus,we need to consider which norepinephrine receptor in the course of norepinephrine transmission acting its important role in our model.

Based on the previous finding that the norepinephrine α-1 receptor ( NEα-1R) in mediating respiration function in brain (Doi and Ramirez, 2010; Zanella, et al., 2014; Schnell, et al., 2016), we think it may be a key target to prevent SUDEP of the co-morbidity of ADHD and epilepsy in our model.

To address the role of NE neurotransmission and the NEα-1R in modulating the pathogenesis of S-IRA in DBA/1 mice from the behavioral and electrophysiological level, we firstly attempted to enhance the concentration of synaptic space in brain in DBA/1 mice by administration with atomoxetine. Thus, we try to work out whether the lower incidence of S-IRA by atomoxetine can be reversed by peripheral and central administration with prazosin. Subsequently, to exclude the the reversed effects by prazosin was via affecting circulation pathway, we choose to measure the changes of mean systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) in DBA/1 mice among different groups. Furthermore, we analyzed and evaluated the specificity of reversed effect on S-IRA in DBA/1 mice by prazosin based on the seizure score and EEG recording analysis. In our study, the lower incidence of S-IRA by atomoxetine in DBA/1 mice evoked by acoustic stimulation and PTZ injection was significantly reversed was reversed by separate administration with intraperitoneal (IP ) and intracerebroventricular (ICV) injection of prazosin in two models without affecting its seizure behavior. Meanwhile, no obvious changes of circulation including the SBP, DBP, MAP and HR. What's more, EEG recording data of DBA/1 mice with IP injection PTZ from different treatment groups with atomoxetine and prazosin showed that the suppressant effects of atomoxetine on the incidence of S-IRA can be significantly as we as specifically reverse by prazosin. Additionally, our results first provided that adminstration of atomoxetine reducing the incidence of S-IRA was mainly due to the enhancement of synaptic space concentration of norepinephrine via bypassing tyrosine hydroxylase (TH). Thus, our present results demonstrated that enhancing the efficiency of the norepinephrine transmission between synaptic space in brain and intervention of NEα-1R in brain may be a potential and specific target to prevent SUDEP of the co-morbidity of ADHD and epilepsy.

## 2. Material and methods

### 2.1 Animals

All experimental procedures were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Advisory Committee at 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 acoustic stimulation DBA/1 mice model, DBA/1 mice were "primed" starting from postnatal day 26-28 to establish the consistent susceptibility to audiogenic seizures (AGSz) and S-IRA. For another model of PTZ injection, DBA/1 mice without being nonprimed at approximately 8 weeks of age were used in the seizure model evoked by PTZ. For acoustic stimulation model in DBA/1 mice, atomoxetine (dissolved in saline, i.p.) was administered 120 mins prior to acoustic stimulation in DBA/1 mice and prazosin 30 mins prior to acoustic stimulation.

**2.2 Seizure induction and resuscitation**

**S-**IRA evoked by acoustic stimulation and intraperitoneal (IP) administration of PTZ, as previously described (Zhang,et al., 2016; Zhang,et al., 2017; Zhao,et al., 2017; Zhang, et al.,2018). In brief, aiming at the acoustic stimulation model, each DBA/1 mouse was placed in a cylindrical plexiglass chamber in a sound-isolated room, and audiogenic seizures were evoked by an electric bell (96 dB SPL, Zhejiang People's Electronics, China). The duration of a maximum duration of 60 s for acoustic stimulus was given or until the occurrence of mouse exhibited tonic seizures and S-IRA in most mice. Mice with S-IRA were resuscitated by a little animal respirator (YuYan Company, ShangHai, China). S-IRA induced by PTZ in all nonprimed DBA/1 mice was through IP administration of a single dose of PTZ (Cat # P6500; Sigma-Aldrich, St. Louis, MO) at a dosage of 75 mg/kg.

**2.3 The effect of IP injection of prazosin on the lower incidence of S-IRA by atomoxetine**

A vehicle control group and treatment groups with different dosages of prazosin (Cat # P7791; Sigma-Aldrich**)** were included in acoustic stimulation model of DBA/1 mice undergoing the pre-treatment of atomoxetine( Ca # Y0001586; Sigma-Aldrich**)**. The occurrence of S-IRA was confirmed 24 h prior to administration with atomoxetine or vehicle and prazosin in the acoustic stimulation model. Atomoxetine (15 mg/kg) was given 120 min and prazosin (0.001-1mg/kg) 30 min prior to acoustic stimulation by IP injection in the same mouse, respectively. For vehicle control group, saline was given 120 min and 25% dimethyl sulfoxide

(DMSO) 30 min prior to acoustic stimulation by IP injection in the same mouse, respectively. The incidence of S-IRA and the latency to AGSZs , the duration of wild running, clonic seizures, tonic-clonic and seizure score were videotaped for offline analysis(Zhang,et al., 2016; Zhang,et al., 2017; 2017; Zhang, et al.,2018; Donato, et al., 2004; Kruse, et al., 2019). (n=6-9/per-group)

**2.4 The effect of IP injection of prazosin on S-IRA**

A vehicle control group and treatment groups with different dosages of prazosin (Cat # P7791; Sigma-Aldrich**)** were included in acoustic stimulation model of DBA/1 mice. The occurrence of S-IRA was confirmed 24 h prior to administration with atomoxetine or vehicle and prazosin in the acoustic stimulation model. Saline was administered 120 min and prazosin (0.005-2mg/kg,dissolved in 25% DMSO) 30 min prior to acoustic stimulation by IP injection in the same mouse, respectively. For vehicle control group, saline was given 120 min and 25% DMSO 30 min prior to acoustic stimulation by IP injection in DBA/1 mice. The incidence of S-IRA and the latency to AGSZs ,the duration of wild running plus clonic seizures (W+C), tonic-clonic and seizure score were videotaped for offline analysis. (n = 6-9/per-group)

**2.5 The effect of IP injection of prazosin on blood pressures and heart rate in DBA/1 mice**

Non-invasive measurements of heart rate and blood pressures at 37°C were performed in conscious DBA/1 mice treated with either atomoxetine or vehicle using tail-cuff system (BP-98A, Blood Pressure Analysis System, Softron, Japan). For control group, atomoxetine (15 mg/kg) was given 120 min and 25% DMSO 30 min prior to acoustic stimulation by IP injection in the same mouse. For experimental group, atomoxetine (15 mg/kg) was given 120 min and prazosin (2mg/kg dissolved in 25% DMSO ) 30 min prior to acoustic stimulation by IP injection in the same mouse, respectively. Measuring the changing of SBP, DBP, MAP and HR at the interval of 120 min, 25min prior to acoustic stimulation and 5 min post the acoustic stimulation for one time (60 seconds) in DBA/1 mice in the above two treatment groups, respectively.

**2.6 The effect of ICV injection of prazosin on S-IRA**

The guide cannula was implanted for lateral cerebro-ventricle (ICV) as previously described

(Zhao,et al., 2017). In brief, a DBA/1 mouse was anesthetized using 3.5% chloral hydrate (0.525mg/kg, i.p.), and a guide cannula (22G, RWD, ShenZhen, China) was stereotaxically implanted into the right lateral ventricle (AP – 0.45 mm; ML – 1.0 mm; V– 2.50 mm) (Paxinos and Franklin, 2013). After the end of surgery for 7 days, the microinjection was performed by a minipump and a Hamilton syringe connected to the infusion cannula (33G, Plastics One) by a polyethylene tubing (PE10, ShenZhen, China). Microinjection was performed using a minipump (ZheJiang University) and a Hamilton syringe connected to the infusion cannula (33G, Plastics One) by a polyethylene tubing. The experimental grouping as follows: 1) Saline (i.p.)120 min prior to PTZ (75mg/kg, i.p.) was administered and 25% DMSO at 2 μl volume was administered ICV at a rate of 0.5 μl/min 15 min prior to PTZ injection in DBA/1 mice as control. 2) Atomoxetine (15mg/kg, i.p.)120 min prior to PTZ (75mg/kg, i.p.) was administered and 25% DMSO at 2 μl volume was administered ICV at the same rate to control group in DBA/1 mice 15 min prior to PTZ injection, respectively. 3) Atomoxetine (15mg/kg, i.p.)120 min prior to PTZ (75mg/kg, i.p.) was administered and prazosin ( 4.764 nmol and 9.528 nmol, dissolved in 25% DMSO) at 2 μl volume was administered ICV at the same rate with the above groups in DBA/1 mice 15 min prior to PTZ injection, respectively,as well(n=6-9/per-group).

**2.7 The effect of ICV injection of prazosin on EEG**

After finishing the implantation of ICV guide cannula justly described above , the same DBA/1mice implant a headstage for recording the electroencephalogram (EEG). The headstage consisted of a 6-pin connector soldered to 2 EEG screws. The EEG electrode was inserted into the surface of cerebral cortex fixed by screws inserting the skull with a layer of dental cement mixed with cyanoacrylate glue. After one week for surgery, the EEG recording of DBA/1 mice experiment will be performed. The experimental grouping for EEG recording (Neuroscan, Australia) as follows: 1) Saline (i.p)120 min prior to PTZ (75mg/kg, i.p.) was administered and 25% DMSO at 2 μl volume was administered ICV at a rate of 0.5 μl/min 15 min prior to PTZ injection in DBA/1 mice as control. 2) Atomoxetine (15mg/kg ,i.p)120 min prior to PTZ (75mg/kg, i.p.) was administered and 25% DMSO at 2 μl volume was administered ICV at the same rate to control group in DBA/1 mice 15 min prior to PTZ injection, respectively. 3) Atomoxetine (15mg/kg, i.p.)120 min prior to PTZ (75mg/kg, i.p.)

was administered and prasion ( 9.528 nmol dissolved in 25% DMSO) at 2 μl volume was administered ICV at the same rate with the above groups in DBA/1 mice 15 min prior to PTZ injection. The EEG recording for each mouse in the above 3 groups is immediately to start after finishing ICV injection. (n=6-9/per-group)

**2.8 The measurement of content of tyrosine hydroxylase by ELISA**

DBA/1mice from 3 groups including naive group without acoustic stimulation, S-IRA group by acoustic stimulation without resuscitation by animal respirator, S-IRA by acoustic stimulation with resuscitation group by animal respirator were decapitated under anaesthetization an overdose of chloral hydrate (n=6/group). As we described previously (zhang, et.al, 2016), the whole brainstems between bregma −3 mm and bregma −9 mm and the rest of brain were collected after removing excess blood in cold PBS and then immediately frozen in nitrogen tank and transferred to −80°C refrigerator. Tissue pieces from different treatment groups were weighed and then homogenized in ice-cold PBS.The homogenates were then centrifuged for 5 minutes at 5000g to obtain the supernatant to test. The content of tyrosine hydroxylase (TH) in three groups of mice were examined by ELISA. All the procedures by ELISA were performed following the manufacturer's instructions in the kits (Mouse TH, ELISA Kit, YanSheng Biological Technology Co., Ltd, ShangHai, China). The TMB chromogen solution was used as detectionsubstrate. The optical density was measured at 450 nm with microtiter plate reader. The content of TH was calculated by standard curve (Mouse TH ELISA Kit: 25–800pg/ml).

**2.9 The confirmation of ICV and EEG by Histology**

At the end of the microinjection and EEG experiment, ICV injection and EEG electrode site was verified using histology, respectively. DAPI staining for mark the ventricular space and determine the guide cannula and placement of EEG electrode. Aiming at the brain harvest, each mouse was deeply anesthetized with an overdose of chloral hydrate and transcardially perfused with 10 ml PBS (pH 7.4), followed by 10 ml 4% paraformaldehyde. After removing, the brain was stored in 4% paraformaldehyde at 4°C. Each DBA/1 mouse brain was sectioned into 60-μm thickness of coronal slices with a freezing microtome, and the location of the guide cannula track was observed using a Nikon Eclipse TS100 light microscope (A516, Olympus BX61, Japan). The mice with outplace of implantation of ICV and EEG was

abandoned.

**3.0 Statistical analysis**

All data are presented as the mean ± SEM. The incidence of S-IRA was compared among different groups using Wilcoxon Signed Rank test. The treatment of data on the seizure score and the latency to AGSZs, the duration of wild running, clonic seizures, tonic-clonic using one-way ANOVA and post-hoc Tukey's test. Statistical significance was inferred if $p < 0.05$.

**4.Results**

**4.1 The lower incidence of S-IRA evoked by acoustic stimulation through IP administration with atomoxetine was significantly reversed by IP prazosin**

Based on our previous study, we test and address whether the incidence of S-IRA evoked by acoustic stimulation was reduced by IP atomoxetine through acting on NEa-1R in brain in DBA/1 mice. Compared with vehicle control in primed DBA/1 mice, the incidence of S-IRA evoked by acoustic stimulation was significantly reduced by atomoxetine with the doseage of 15 mg/kg, i.p. ( $p < 0.01$). The lower incidence of S-IRA by atomoxetine was markedly prazosin i.p ($p < 0.01$). The latency to AGSZs , the duration of wild running , clonic seizures, tonic-clonic and seizure score from different groups was no significantly difference ($p > 0.05$).The above data suggest that no obvious intervention in seizure behavior of the reversed effects by prazosin was to be fund. Our results demonstrated that the lower incidence of S-IRA evoked by acoustic stimulation through IP administration with atomoxetine can be specifically reversed by prazosin. (Fig 1)

**4.2 The effects of prazosin on S-IRA in the acoustic stimulation DBA/1 model**

To exclude the effects of prazosin on the incidence of S-IRA evoked by acoustic stimulation in DBA/1 mice, the DBA/1 mice was IP with prazosin at the different dosage in different groups. Compared with vehicle control in primed DBA/1 mice, the incidence of S-IRA evoked by acoustic stimulation was not significantly reduced by prazosin at the dosage of 0.01-2mg/kg ($p > 0.05$). The latency to AGSZs , the duration of wild running , clonic seizures, tonic-clonic and seizure score from different groups was no significantly difference ($p > 0.05$). These data suggest that prazosin exerts no effects on the S-IRA **evoked by** acoustic stimulation. (Fig 2)

**4.3 The effects of prazosin on blood pressure and heart rate in DBA/1 mice**

Given that prazosin can markedly reduce the peripheral blood pressures and affect heart rate at a high dose as a common clinical drug and reduce the cerebral perfusion pressure to produce the effects on S-IRA in our model. We choose measured the changes of SBP, DBP, MAP and HR.There is no significant changes of SBP, DBP, MAP and HR to be found between the vehicle control where atomoxetine (15 mg/kg) was given 120 min and 25% DMSO 30 min prior to acoustic stimulation by IP injection and 5 min post acoustic stimulation the same mouse and the experimental group (n = 6) where atomoxetine (15 mg/kg) was given 120 min and prazosin 30 min prior to acoustic stimulation by IP injection and 5 min post acoustic stimulation in the same mouse, respectively. Our data showed that prazosin significantly reverses the suppressant effects of S-IRA by atomoxetine independence of affecting the change of cerebral perfusion pressure.(Fig 3)

**4.4 The effects of ICV prazosin on the lower incidence of S-IRA by atomoxetine in the model of PTZ injection**

Considering the difference of SUDEP model to affects the effects of prazosin to reverse the suppressant effects of S-IRA by atomoxetine (15mg/kg, i.p.). We accepted another SUDEP model established by PTZ with IP to test. Compared with vehicle control group, the high incidence of S-IRA by PTZ was markedly reduce by atomoxetine in another treatment group.($p< 0.01$). Compared with the group (atomoxetine, 15mg/kg, i.p, MDSO,icv, n=6) , prazosin (atomoxetine, 15mg/kg, i.p, prazosin, 4.764 nmol,icv) can't markedly reverse the suppressant effects of S-IRA by atomoxetine ($p>0.05$). However, the suppressant effects of S-IRA by atomoxetine was significantly reversed by the dosage of prazosin (9.528 nmol,icv) ($p < 0.01$).(Fig 4)

**4.5 Repetitive acoustic stimulation significantly produce no effects on the of TH protein in DBA/1 mice**

We examined the expression of TH2 in the brainstem of DBA/1mice from 3 groups including naive group without acoustic stimulation, S-IRA group by acoustic stimulation without resuscitation, respectively. As compared with the relative optical density of DBA/1mice without acoustic stimulation, to which that of two groups was normalized, the relative optical density of TH was not markedly reduced in the brainstem of primed DBA/1 mice ($p > 0.05$, n=6). The TH protein relative expression levels were not significantly different between

S-IRA without resuscitation and S-IRA with resuscitation groups ($p > 0.05$, n=6).

**4.6 The effects of ICV prazosin on EEG data in the DBA/1 mice SUDEP mode by PTZ**

To evaluate the effects of prazosin EEG, we choose the PTZ model to test. As the Fig 5 showed, the EEG voltage amplitude peak (power) post PTZ injection was significantly higher than before PTZ injection in 3 groups . Meanwhile, low frequency oscillations peak appeared before PTZ injection and high-frequency oscillations peak occurred post PTZ injection in 3 groups. Our data showed that prazosin reverse the lower incidence of S-IRA by atomoxetine without inhibiting and/or exciting seizure firing by EEG data analysis, meaning the suppressant effects of S-IRA by atomoxetine can be specifically reversed by prazosin. (Fig 5)

**5. Discussion**

Although some emerging advancements had been obtained in the cause leads to SUDEP, the underling mechanism concerning it seem to still need to be explored ( (Bateman,et al., 2010; Tomson, et al., 2008; Lhatoo , et al., 2015; Devinsky,et al., 2016; Zhang,et al., 2016). Furthermore, no obvious mechanisms were to be established to understand the SUDEP with the co-morbidity of ADHD and epilepsy for child and pediatric epilepsy patients (Verrotti A, et al., 2018; Socanski D, et al., 2013 ; Donner EJ, et al., 2017; Keller AE, et al., 2018). Here, our current results first provided the new insight into the role by NE neurotransmission in brain to understand and decode the cause resulting in SUDEP in the DBA/1 mice SUDEP model for child and pediatric epilepsy patients with ADHD. Based on our previous findings that the incidence of S-IRA in DBA/1 mice was significantly reduced by atomoxetine and the role by norepinephrine α-1 receptor in control respiration rhythm, we choose the acoustic stimulation and PTZ SUDEP model to test whether the effects of atomoxetine reducing S-IRA depend on the norepinephrine α-1 receptor in brain. Our data showed that the suppressant effects of S-IRA by atomoxetine was significantly reversed by administering prazosin with IP and ICV injection in AGS DBA/1 and PTZ mice model, respectively.

Much as other laboratory took advantage of our previous study concerning atomoxetine to reduced the incidence of S-IRA of DBA/1 mice in DAB/1 mouse SUDEP model to test the role of NEα-1R to modulate S-IRA and SUDEP in lmx1bf/f/p mice with deletition of 5‐HT neurons in maximal electroshock (MES) model( Zhang,et al, 2017; Zhao ,et al, 2017; Kruseet al., 2019), some possible and potential problems existing in their study need to work out.

Namely, based on the results from our previous finding in DBA/1 mice SUDEP model to test the role of NEα-1R in mediating S-IRA in MES model(Kruseet al., 2019). In MES model, the dose of prazosin reversing the suppressant effects of S-IRA by atomoxetine was 1000 times higher than in our DBA/1 mice SUDEP model. The higher dosage of prazosin may produce other side-effects on reversing the lower incidence of S-IRA by atomoxetine in MES model and the reversed effects by prazosin will be short of the specificity for understanding S-IRA and SUDEP. What's more, as the paper stressed that the MES model is not a model of epilepsy itself and can't perform experiments as own control (Kruseet al., 2019).

To a certain degree, our work will be beneficial to address this problem using two models. For one thing, we used DBA/1 mice SUDEP model by acoustic stimulation to test the role of NEα-1R in modulating S-IRA as own control. For another, we choose the SUDEP model by PTZ which can simulate the generalized seizures of epilepsy patients to test the action of NEα-1R in modulating S-IRA (White, 1997). Furthermore, we explored the specificity of reversed effects by prazosin from behavioral and electrophysiological level as discussed below. Importantly, we provided the first evidence concerning the effects changes of TH content, a rate-limiting enzyme for synthesizing norepinephrine, in the brainstem brain in DBA/1 mice on the S-IRA. Our results suggested that inhibition of the incidence of S-IRA by atomoxetine depended on enhancing the norepinephrine level of synaptic space rather on TH content in the lower brainstem of DBA/1 mice. Although the we didn't measure the activity of TH, there is basically TH protein expression consistent with the change of TH activity just as we measuring the TPH2 protein and another laboratory measured the TPH2 activity in DBA/1 mice SUDEP model (Zhang,et al., 2016; Chen,et al., 2019). Thus, combing our findings with their study data about the role of NEα-1R in modulating S-IRA in DBA/1 mouse and MES model will be helpful to understand the pathogenesis of S-IRA and SUDEP.

In our model, considering the property of prazosin to markedly reduce the peripheral blood pressures and affect heart rate at a high dose, which will reduce the cerebral perfusion pressure to produce the effects on S-IRA and to exclude the circulation effects on the reversed the lower incidence of S-IRA by prazosin , we choose to measure the changes of SBP, DBP, MAP and HR among different treatment groups of DBA/1 mice, which shows prazosin within the range of our dose without affecting the obvious changes of SBP, DBP, MAP and HR

among different groups as a common medication widely used to reduce high blood pressure in clinical practise will be potentially used in clinical epilepsy patients who holds the risk for SUDEP to prevent the occurrence of SUDEP. However, the factor of arrhythmia can't ruled out in our model. Some studies showed that the arrhythmia can result in the occurrence of SUDEP(Devinsky,et al., 2016; Massey, et al., 2014; Chahal, et al., 2020). Meanwhile, our previous finding demonstrated that the repeated generalized seizures can produce calcified cardiac lesions in DBA/1 mice (Zhao,et al., 2017 ). Thus, it is essential for us to figure out the role of circulation system in SUDEP in the near future.

Additionally, aiming at the specificity of atomoxetine reducing the incidence of S-IRA and the reversed effects by prazosin, we accepted the seizure score and measured the AGSZs latency and duration of w/c and or tonic-clonics to analyze the seizure behaviour and no obvious difference among groups to be found. By analyzing the EEG data from the PTZ model, the power and frequency were no obvious difference to be found in the treatment with atomoxetine and prazosin,respectively. Therefore, in our model , the lower incidence of S-IRA by atomoxetine was specifically reversed by prazosin in the manner of dose-dependence with ceiling effects, suggesting that NEa-1R in brain in our model is a specific target to prevent the occurrence of SUDEP. Interestingly, a recent study showed that the suppressant effects of S-IRA by atomoxetine was not significantly reversed by prazosin (1mg/kg,i.p.) in the same model (Zhang, et al., 2019). However, in our study the suppressant effects of S-IRA by atomoxetine was markedly reversed by prazosin at the dose 0.01mg/kg (i.p.) (Shen, et al., 2019). Analyzing of the cause for this, the time of administration with prazosin my be a critical cause (Shen, et al., 2019). In their study, the lower incidence of S-IRA by atomoxetine was significantly reversed by yohimbine, a NEa-2 receptor antagonist (Zhang, et al., 2019) . Of course, it is possible for the NEa-1 and NEa-2 receptor to mediate SUDEP by mutual interaction, which will be explored in the near future.

In summary, our current data demonstrated that NE transmission in brain is of great significance to mediate the pathogenesis of S-IRA and SUDEP in two models. The lower incidence of S-IRA by atomoxetine was significantly as well as specifically reversed by the lower dosage of prazosin with peripheral and central pathway. Importantly, we addressed the issue of suitable SUDEP model to study SUDEP. Different from other study in the other

model, we established the import role of NEa-1R in modulating SUDEP in DBA/1mice, which will lay down the foundation to last studying the role of NE neurotransmission in DBA/1mice SUDEP model. Taken together, our findings suggest that intervening the synaptic transmission of NE in brain will be a potential target to prevent SUDEP.

**Limitations of the Study**

In this work , we failed to explore the further role of NEa-1R in brain including its neural circuit by specific manner jus as we previously attempted to suppress the S-IRA evoked by acoustic stimulation or PTZ injection in DBA/1 mice by activating 5-HT neuron in dorsal raphe nucleus using optogenetics technology (Zhang, et al., 2018). Currently, optogenetics technology had been accepted in epilepsy research (Cela, et al., 2019). Inaddtion, we didn't establish the model of co-morbidity of ADHD and epilepsy to test our results. Future experiments will be designed to verify the action of NEa-1R in brain to modulate S-IRA and SUDEP by optogenetics technology in the co-morbidity of ADHD and epilepsy model. Nevertheless, our results will shed a light on the decoding the development of SUDEP and atomoxetine holds its further translational investigation as potential medications to prevent SUDEP with the co-morbidity of ADHD and epilepsy.

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## 7. Figure legend

## Figure 1

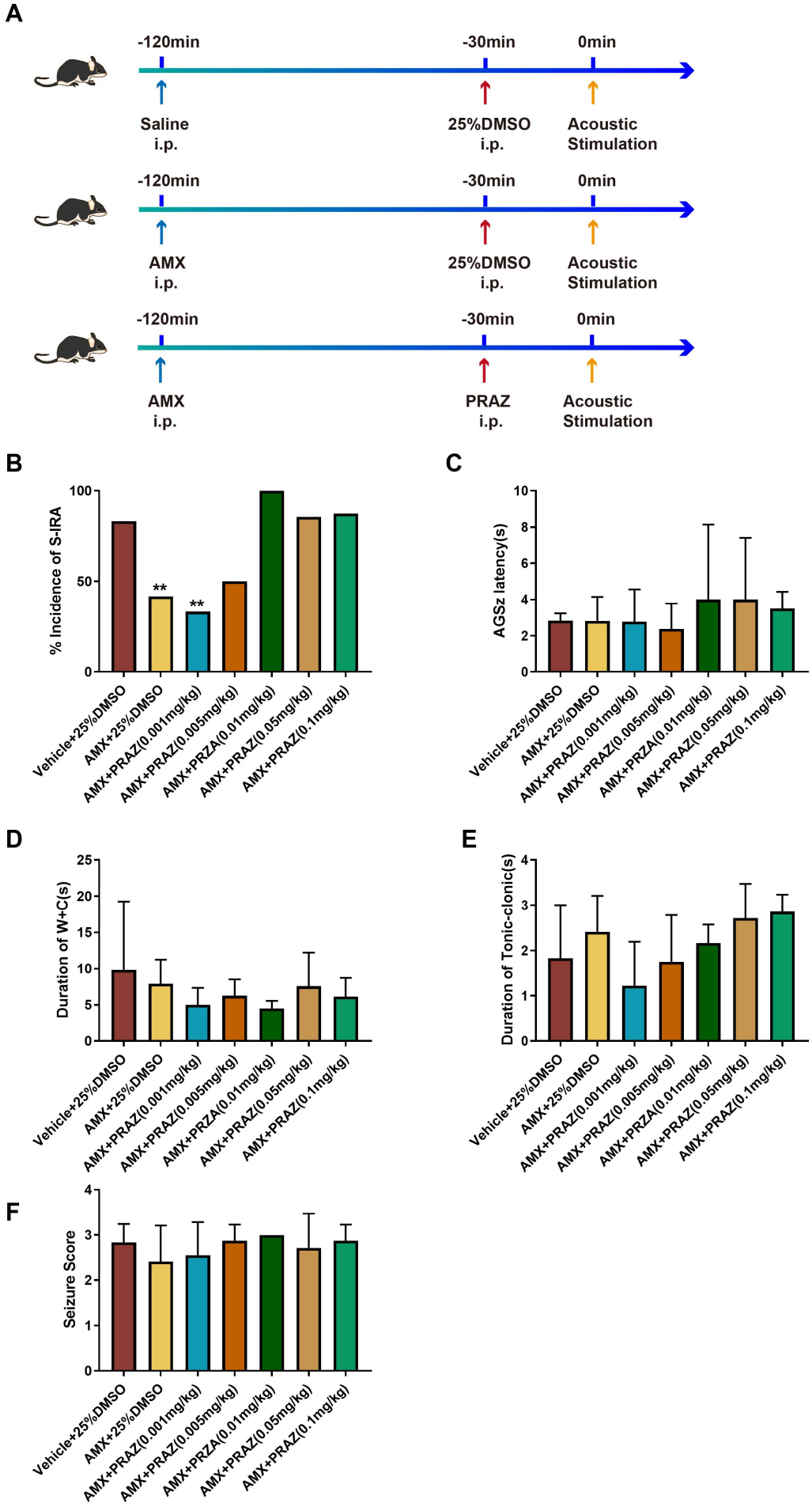
A
-120min
-30min
0min
Saline
i.p.
25%DMSO
i.p.
Acoustic
Stimulation
-120min
-30min
0min
AMX
i.p.
25%DMSO
i.p.
Acoustic
Stimulation
-120min
-30min
0min
AMX
i.p.
PRAZ
i.p.
Acoustic
Stimulation
B
% Incidence of S-IRA
100
50
0
**
**
C
AGSz latency(s)
10
8
6
4
2
0
D
Duration of W+C(s)
25
20
15
10
5
0
E
Duration of Tonic-clonic(s)
4
3
2
1
0
F
Seizure Score
4
3
2
1
0
Vehicle+25%DMSO
AMX+25%DMSO
AMX+PRAZ(0.001mg/kg)
AMX+PRAZ(0.005mg/kg)
AMX+PRZA(0.01mg/kg)
AMX+PRAZ(0.05mg/kg)
AMX+PRAZ(0.1mg/kg)

Figure 1

The effects of atomoxetine reducing S-IRA evoked by acoustic stimulation was significantly reversed by prazosin in DBA/1 mice

B.Compared with the control group (n=6) treated with vehicle (saline), the incidence of S-IRA evoked by acoustic stimulation was markedly lower in groups by IP with atomoxetine (AMX) at 15 mg/kg (n=12) or AMX and prazosin (PRAZ) at 0.01-0.05mg/kg (n=9,8)in primed DBA/1 mice (** $p < 0.01$). However, the lower incidence of S-IRA by atomoxetine (AMX) at 15 mg/kg or AMX at 15 mg/kg in primed DBA/1 mice was significantly reversed by prazosin at a dosage of 0.01-0.1mg/kg (n=9,8,6,7,8)(** $p < 0.01$). C-F. There is no significant different changes of the latency to AGSZs , the duration of wild running plus clonic seizures (W+C), tonic-clonic and seizure score from different groups ( $p > 0.05$). Atomoxetine was administered IP 2 hr and prazosin 30min prior to induction of audiogenic seizures.

## Figure 2

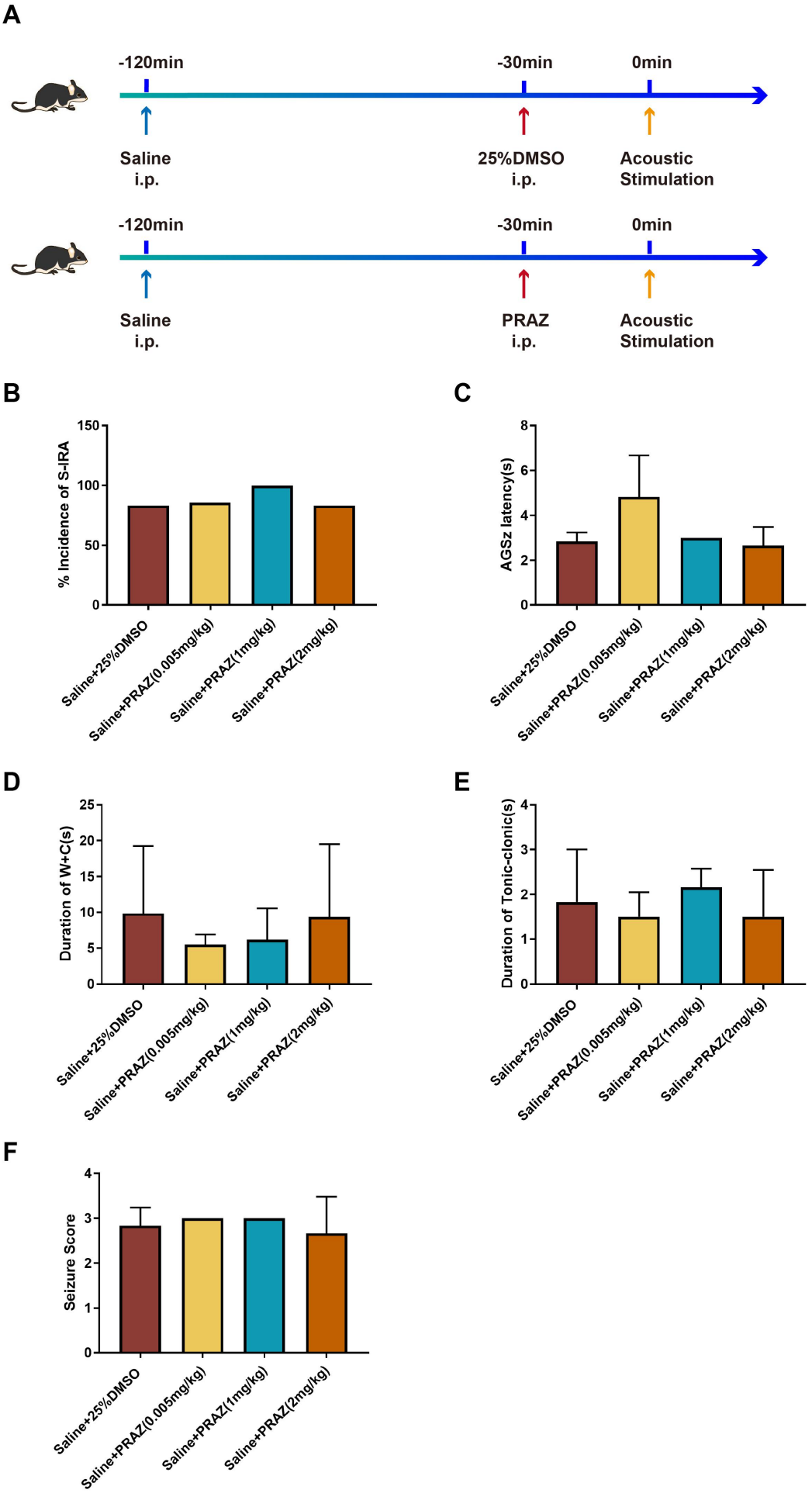
A
-120min
-30min
0min
Saline
i.p.
25%DMSO
i.p.
Acoustic
Stimulation
-120min
-30min
0min
Saline
i.p.
PRAZ
i.p.
Acoustic
Stimulation
B
% Incidence of S-IRA
150
100
50
0
Saline+25%DMSO
Saline+PRAZ(0.005mg/kg)
Saline+PRAZ(1mg/kg)
Saline+PRAZ(2mg/kg)
C
AGSz latency(s)
8
6
4
2
0
Saline+25%DMSO
Saline+PRAZ(0.005mg/kg)
Saline+PRAZ(1mg/kg)
Saline+PRAZ(2mg/kg)
D
Duration of W+C(s)
25
20
15
10
5
0
Saline+25%DMSO
Saline+PRAZ(0.005mg/kg)
Saline+PRAZ(1mg/kg)
Saline+PRAZ(2mg/kg)
E
Duration of Tonic-clonic(s)
4
3
2
1
0
Saline+25%DMSO
Saline+PRAZ(0.005mg/kg)
Saline+PRAZ(1mg/kg)
Saline+PRAZ(2mg/kg)
F
Seizure Score
4
3
2
1
0
Saline+25%DMSO
Saline+PRAZ(0.005mg/kg)
Saline+PRAZ(1mg/kg)
Saline+PRAZ(2mg/kg)

Figure 2

The effect of prazosin on the S-IRA evoked by acoustic stimulation in DBA/1 mice

B.Compared with the control group (n=6) treated with vehicle (saline), the incidence of S-IRA evoked by acoustic stimulation was not markedly lower or higher in groups by IP with prazosin (PRAZ) at 0.005-2mg/kg (n=6, 6) in primed DBA/1 mice ($p > 0.05$). C-F. There is no significant different changes of the latency to AGSZs , the duration of wild running plus clonic seizures (W+C), tonic-clonic and seizure score from different groups ( $p > 0.05$).

**Figure 3**

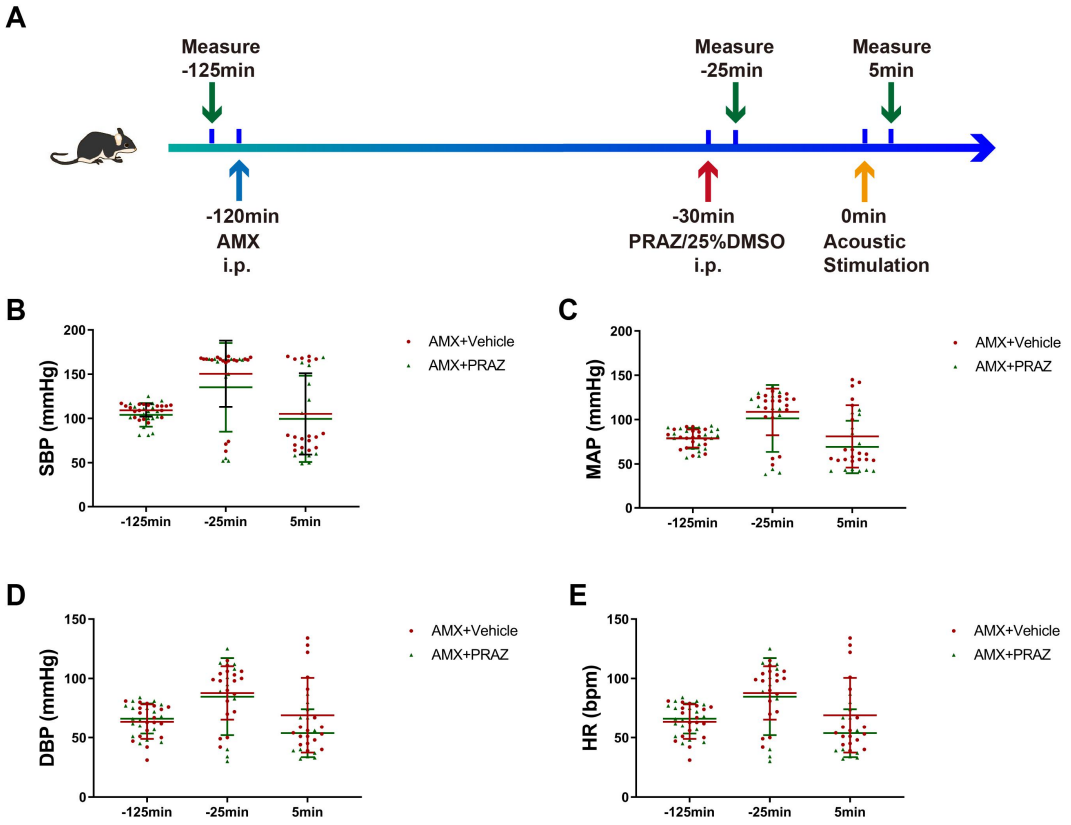


Figure 3

The effect of prazosin on the blood pressure and heart rate in DBA/1 mice

B-E. Compared with the control group (n=6) where atomoxetine (AMX, 15 mg/kg, i.p.) was given 120 min and 25% DMSO 30 min prior to acoustic stimulation and 5 min post acoustic stimulation, there is no significant changes of SBP, DBP, MAP and HR to be found in the control where atomoxetine (AMX, 15 mg/kg, i.p) was given 120 min, prazosin (PRAZ,

2mg/kg, i.p.) (n=6) 30 min prior to acoustic stimulation and 5 min post acoustic stimulation in DBA/1 mice ( p >0.05).

**Figure 4**

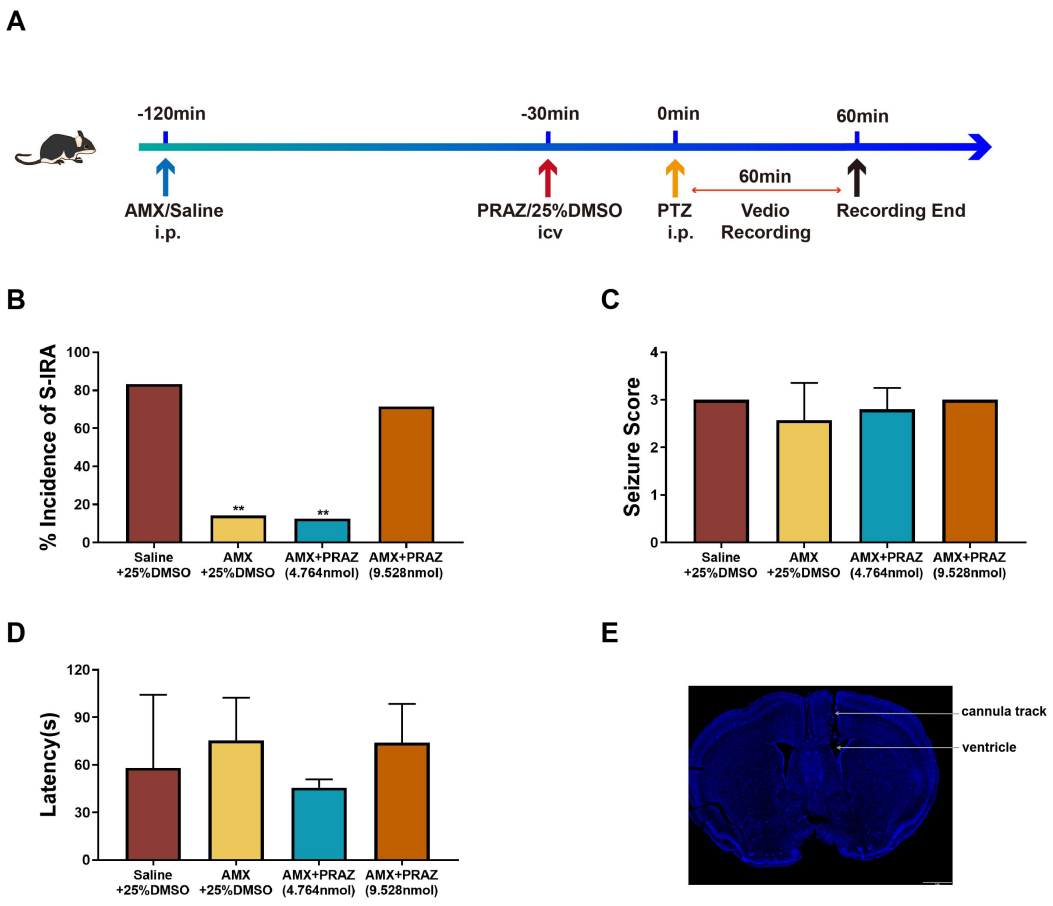


Figure 4

The effect of ICV injection of prazosin on S-IRA evoked by PTZ

B. Compared with the control group (n=6) treated with vehicle (IP saline and ICV 25%DMSO), the incidence of S-IRA evoked by PTZ was markedly lower in groups by IP with atomoxetine (AMX, 15 mg/kg,i.p,n=7) and ICV with 25%DMSO and the group treated with (AMX, 15 mg/kg,i.p) and prazosin (PRAZ) ICV of 4.764 nmol(n=5) (**p < 0.01). The lower incidence of S-IRA by atomoxetine was significantly reversed by and prazosin (PRAZ) ICV of 9.528 nmol (n=7) (**p < 0.01). C-D. There is no significant difference of seizure score and latency among different treated groups ( p> 0.01). E. a representative example for histology indicating that the vehicle or prazosin (PRAZ) was successfully delivered into the ventricle.

Figure 5

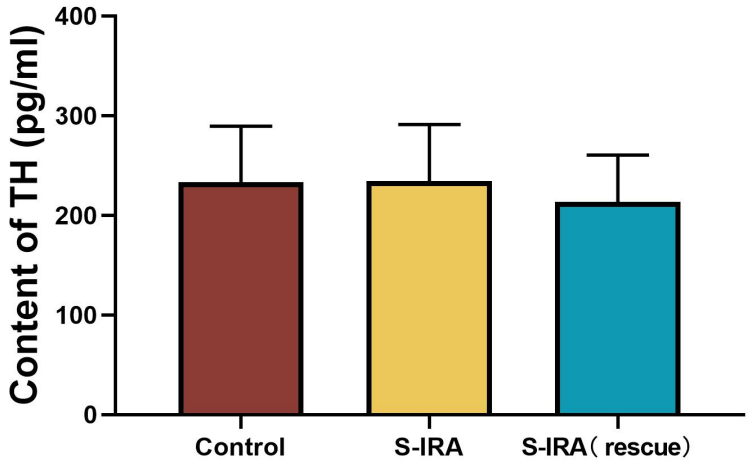


Figure 5

The effects of repetitive acoustic stimulation on the change of the TH protein level in DBA/1 mice

Compared with the control group without acoustic stimulation, there was no significant changes in S-IRA without resuscitation and S-IRA with resuscitation group ($p > 0.05$, n=6).

There were not obvious changed of TH protein level between S-IRA without resuscitation and S-IRA with resuscitation group ($p > 0.05$, n=6).

**Figure 6**

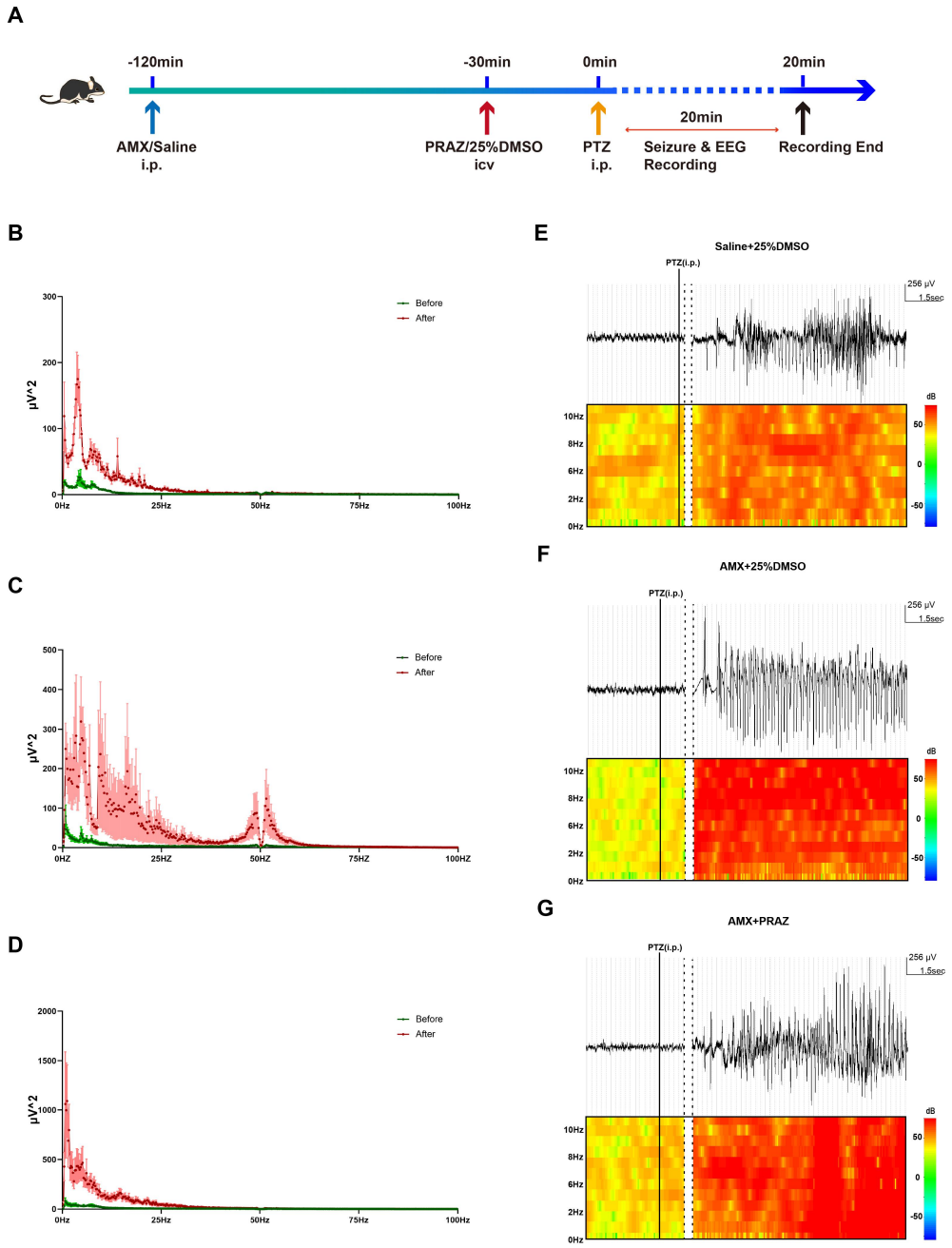


Figure 6

The effect of ICV injection of prazosin on EEG in the SUDEP model by PTZ

A-F, Sample EEG trace illustrating an seizure induced by PTZ (75mg/kg,i.p) in 3 different treatment groups in DBA/1 mice. B and E, the changes of voltage amplitude peak (power) frequency oscillations peak was significantly lower before PTZ than after PTZ injection in the group saline +25% DMSO (n=3) in which saline (i.p)120 min prior to PTZ (75mg/kg ,i.p) was administered and 25% DMSO at 2 μl volume was administered ICV at a rate of 0.5

μl/min 15 min prior to PTZ injection in DBA/1 mice as control. C and F, the changes of power and frequency oscillations peak in the group treated with atomoxetine (AMX)+ 25% DMSO group (n=5)in which Atomoxetine (15mg/kg ,i.p)120 min prior to PTZ (75mg/kg,i.p.) was administered and 25% DMSO was administered ICV in DBA/1 mice 15 min prior to PTZ injection was similar to control group. D and G, the changes of voltage amplitude and frequency oscillations peak in the group treated with AMX+PRAZ(prazosin) group (n=3) in which Atomoxetine (15mg/kg ,i.p)120 min prior to PTZ (75mg/kg ,i.p) was administered and prasion ( 9.528 nmol) was administered ICV15 min prior to PTZ injection were roughly consistent with the group of control and AMX+ DMSO group.

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