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NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment

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NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a $\mathbf{2}$ 6 novel environment $\mathbf{5}$ Tetsushi Hirano^a, Shogo Yanai^a, Tadashi Takada^a, Naoki Yoneda^a, Takuya Omotehara^a, Naoto Kubota^a, Kiichi Minami^a, Anzu Yamamoto^a, Youhei Mantani^b, Toshifumi Yokoyama^a, $\mathbf{7}$ Hiroshi Kitagawa^b, Nobuhiko Hoshi^{a, *} ^aLaboratory of Animal Molecular Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, Kobe, Hyogo 657-8501, Japan ^bLaboratory of Histophysiology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, Kobe, Hyogo 657-8501, Japan * Corresponding author at: Laboratory of Molecular Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan. Tel.: +81 78 803 5811; Fax: +81 78 803 5811. E-mail address: nobhoshi@kobe-u.ac.jp (N. Hoshi). Keywords: Neonicotinoid Clothianidin Neurobehavioral effect Elevated plus-maze test Anxiety-related behavior Human-audible vocalization

33 Abstract

Neonicotinoids are novel systemic pesticides acting as agonists on the nicotinic acetylcholine receptors (nAChRs) of insects. Experimental studies have revealed that neonicotinoids pose potential risks for the nervous systems of non-target species, but the brain regions responsible for their behavioral effects remain incompletely understood. This study aimed to assess the neurobehavioral effects of clothianidin (CTD), a later neonicotinoid developed in 2001 and widely used worldwide, and to explore the target regions of neonicotinoids in the mammalian brain. A single-administration of 5 or 50 mg/kg CTD to male C57BL/6N mice at or below the no-observed-adverse-effect level (NOAEL) induced an acute increase in anxiety during the elevated plus-maze test. In addition, mice in the CTD-administered group spontaneously emitted human-audible vocalizations (4-16 kHz), which are behavioral signs of aversive emotions, and showed increased numbers of c-fos immunoreactive cells in the paraventricular thalamic nucleus and dentate gyrus of the hippocampus. In conclusion, mice exposed to NOAEL-dose CTD would be rendered vulnerable to a novel environment via the activation of thalamic and hippocampal regions related to stress responses. These findings should provide critical insight into the neurobehavioral effects of neonicotinoids on mammals.

1. Introduction

Neonicotinoids are widely used systemic pesticides characterized by high water solubility, residual efficacy and selective toxicity for insects. They have agonistic effects on-and high affinity for-the nicotinic acetylcholine receptors (nAChRs) of insects (Tomizawa and $\mathbf{54}$ Casida, 2005), but thought to be safe for humans and other vertebrates. Nevertheless, a pioneering study demonstrated that acetamiprid (ACE) and imidacloprid (IMI), earlier types of neonicotinoids, induced an excitatory calcium influx mediated by mammal nAChRs into cerebellar neurons from neonatal rats (Kimura-Kuroda et al., 2012), raising the question of whether neonicotinoids would affect the nervous systems of vertebrates. At the same time, it has been reported that neonicotinoids have adverse effects on the reproductive, immune and nerve functions of non-target species such as fish, amphibians, reptiles, birds and mammals (Tokumoto et al., 2013; Hoshi et al., 2014; Gibbons et al., 2015).

Clothianidin (CTD) was developed in 2001 and is thus one of the latest neonicotinoids; it is used for pest control and seed treatment around the world. Unlike IMI, which suppresses the responses to acetylcholine (ACh) in HEK cells expressing human $\alpha 4\beta 2$ nAChRs, CTD augments and sustains the responses to ACh (Li et al., 2011). In addition, in vivo studies showed that CTD triggered transient dopamine releases through extracellular calcium influx in the rat striatum (Falo et al., 2012), and changed several developmental behaviors of the offspring following maternal exposure (Tanaka, 2012). We recently reported that subchronic exposure of CTD resulted in anxiety-like behavior in mature male mice in an open field test, which became more serious under mildly stressed conditions (Hirano et al., 2015). Taken together, these facts indicate that CTD exerts excitatory effects on the neurobehavioral function of mammals at both the cellular and individual levels.

The endogenous target of neonicotinoids, nAChRs are ion channel-linked receptors that are

partially conserved during the evolution from invertebrates to vertebrates (Bossy *et al.*, 1988). Their properties vary according to their subunit compositions, and they play diverse roles such as controlling the inflammation and immune responses in the peripheral tissues, in addition to their primary role of transmitting nerve impulses by acetylcholine (Wang et al., 2003). There are two major types of neural nAChRs in the mammalian brain, the α 7 homopentamers and $\alpha 4\beta 2$ heteropentamers, and both types are expressed in the olfactory bulbs, cortex, striatum, hippocampus, thalamus, hypothalamus, amygdala, midbrain, cerebellum and pons on the central nervous system (Gotti et al., 2006). They function not only in cholinergic neurotransmission at the postsynaptic membranes, but also modulate the release of other neurotransmitters at the presynaptic terminals (Marchi and Grilli, 2010). Disturbances of the signals mediated by nAChRs have been associated with psychiatric disorders including Alzheimer's disease, schizophrenia, depression, attention deficit hyperactivity disorder and nicotine addiction (Taly et al., 2009), and thus agents that disturb these signals could pose a significant risk to public health.

As previously mentioned, there are different types of nAChRs broadly expressed in various brain regions, and it remains unclear which brain regions neonicotinoids may affect. Therefore, the purposes of this study were (i) to elucidate whether acute CTD exposure has anxiogenic effects in mammals, and (ii) to reveal the possible brain regions of the mammals related to the neurobehavioral changes in a novel environment under the influence of CTD. To this end, we conducted behavioral and neuroactivity analyses focusing on anxiety-related phenotypes such as the abnormal vocalizations observed in mice administered CTD during the elevated plus-maze test.

97 2. Materials and methods

Male C57BL/6N mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were ear-punched for identification and group-housed (5–6 mice per a cage) in $40.5 \times 20.5 \times 18.5$ cm individually ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature $(23 \pm 2^{\circ}C)$ and humidity $(50 \pm 10\%)$ on a 12-h light/dark cycle at the Kobe University Life-Science Laboratory with ad libitum access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and filtered water. At 9-10 weeks of age (body weight: 22-25 g), all mice in a cage were randomly oral-administered CTD (purity: 95%, extracted from Dantotsu® Sumitomo Chemical Co., Tokyo, Japan; Hirano et al., 2015) or vehicle (0.5% carboxymethylcellulose, 10 mL/kg) at a dose of 0, 5 or 50 mg/kg body weight with reference to the no-observed-adverse-effect level (NOAEL) of 47.2 mg/kg from 78-week dietary carcinogenicity study in male mice (Food and Agriculture Organization of the United Nations; Uneme et al., 2006). These groups were defined as the control, CTD-5 and CTD-50 groups, respectively. All chemicals were administered to mice using flexible administration tubes under light and short isoflurane anesthesia to minimize excessive stress by handling, restraint, and injection. The experimental design of this study was summarized in Figure 1. This study was approved by the Institutional Animal Care and Use Committee (Permission #26-05-07) and carried out according to the Regulation on Animal Experimentation at Kobe University.

2-2. Elevated plus-maze test

After the CTD administration, all mice in their home cages were allowed to acclimate to the conditions in a dimly-illuminated experimental room (10–20 lux, $23 \pm 2^{\circ}$ C) for 1 h. The elevated plus-maze test was then conducted as described previously (Walf and Frye, 2007)

with some modifications. Briefly, the apparatus was constructed of white acrylic plates and consisted of two opposite open arms $(30 \times 5 \text{ cm})$ and two opposite enclosed arms (the same size with walls 15 cm high) extending from a common central platform (5 \times 5 cm) (Tom Product, Tokyo, Japan). Mice were individually placed on the central platform facing an open arm, and all their activities were recorded by a video camera for the subsequent 10 min. The total distance traveled, the total number of arm entries, the percentage of open arm entries, and the percentage of time spent in open arms were scored by using Image EP software (Komada et al., 2008).

2-3. Vocalization analysis

All sounds during each elevated plus-maze test were recorded by a PCM recorder (96 kHz sampling rate, 24 bit depth) to visualize the chirp-like vocalizations emitted by CTD-administrated mice in human-audible range (< 20 kHz). Frequency spectrograms (hamming window, FFT length = 256, frame size = 100%, overlap = 75%, cut-off frequency <3 kHz) were made by using SAS Lab software (Avisoft Bioacoustics, Berlin, Germany). Human-audible vocalizations were manually checked and counted for each minute of the behavioral tests.

2-4. Neuroactivity analysis

Two hours after the behavioral test, all mice were deeply anesthetized with isoflurane and transcardially perfused with 0.9% normal saline, then with ice-cold 4% paraformaldehyde in phosphate buffer. The brains were cryoprotected in ascending solutions of sucrose (10%, 20%, 30%) in 0.1 M phosphate buffer (PB) overnight and then frozen in liquid nitrogen in an embedding solution consisting of Tissue-Tek® O.C.T. compound (Sakura Finetek, Tokyo,

3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan).

The sections were immersed in absolute methanol and 0.5% H₂O₂ for 30 min, respectively, at room temperature (RT) to quench the endogenous peroxidase activity. They were then incubated with Blocking OneHisto (Nacalai Tesque Inc., Kyoto, Japan) for 1 h at RT for protein blocking, followed by incubation with rabbit polyclonal anti-c-fos antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1:2,000 in phosphate buffered saline with 0.05% Tween-20 (PBST; pH 7.4) for 18 h at 4°C. After being washed with PBST, the sections were reacted with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer in tris (hydroxymethyl) aminomethane-HCl buffer (EnVision+; Dako, Glostrup, Denmark) for 1 h at RT. Immunoreactivity was then detected by incubation with 3,3'-diaminobenzidine solution (EnVision+ kit/HRP[DAB]; Dako). Next, the sections were placed in a graded series of ethanol, dehydrated with absolute ethanol, cleared in xylene and coverslipped with Eukitt mounting medium (O. Kindler GmbH, Freiburg, Germany). Sections were photographed with an Olympus BX61 microscope equipped with a DP-70 digital camera (Olympus Japan Co., Tokyo, Japan) under bright-field. We determined the brain regions anatomically according to a brain atlas (Paxinos and Franklin, 2001) and calculated the number of c-fos immunopositive nuclei per area by using ImageJ software. At least 3 sections per mouse were analyzed in this way.

2-5. Statistical analysis

Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00; SSRI,

Tokyo, Japan). According to the reference (Walf and Frye, 2007), in the elevated plus-maze test, the experimental abnormal values such as mice fell off arms or freezing in arms for a considerable time were excluded from the analysis (Control: n = 3, CTD-5: n = 1, CTD-50: n= 3). The behavioral data were analyzed by one-way ANOVA followed by the Dunnett's post hoc test. The histological data were analyzed by Welch's *t*-test. The results were considered significant when the *p*-value was less than 0.05.

3. Results

3-1. Behavioral effects of acute CTD in the elevated plus-maze test

To measure the anxiogenic effect of CTD in a novel and stressful environment, all mice were subjected to the elevated plus-maze test. There were no marked physical signs of acute toxicity in the CTD-administered mice during the 1-h period spent in their home cages before the test. The representative trajectory maps showed that the CTD-administered mice seldom walked in the open arms (Fig. 2A). One-way ANOVA showed that there are significant effects of CTD on the locomotor activities measured by the total distance traveled [F(2, 31 =33.16) p < 0.01 and the total number of arm entries [F(2, 31 = 4.49) p < 0.05]. The locomotor activities of mice were not changed in the CTD-5 groups, but were significantly lower in the CTD-50 group [p < 0.01] than the control group (Fig. 2B, C). At the same time, one-way ANOVA showed that there are significant effects of CTD on the open arm activities measured by the percentage of time spent in the open arms [F(2, 31 = 9.87) p < 0.01] and the percentage of open arm entries [F(2, 31 = 8.02) p < 0.01]. The open arm activities of mice were significantly decreased in both the CTD-5 and CTD-50 groups [p < 0.01], indicating an increased level of anxiety (Fig. 2D, E). In addition, mice in the CTD-50 group showed several signs of excessive anxiety, such as teeth chattering, freezing and human-audible

3-2. Vocalization analysis

All mice in the CTD-50 group spontaneously emitted chirp-like vocalizations on the elevated plus-maze when they were selecting arms on the center platform or looking down from open arms (Supplementary movie). The spectrogram revealed that the human-audible vocalizations produced by mice consisted of harmonics at a constant frequency between 4 and 16 kHz with durations between 75 and 120 msec (Fig. 3A). There were 70.6 ±19.5 instances of vocalizations in the elevated plus-maze test in the CTD-50 group, but no vocalizations in the control and CTD-5 groups (Fig. 3B). It was also noted that most of the vocalizations were observed immediately after the elevated plus-maze test started and decreased drastically within 1 min (Fig. 3C).

3-3. The effects of CTD on c-fos expression induced by the elevated plus-maze test

There was no marked difference in the neural morphology and arrangement of neural nucleus of brain between the control and the CTD-administrated groups. To identify the brain regions involved in excessive behavioral responses to the elevated plus-maze test in the CTD-50 group, neuroactivity analyses visualizing c-fos immunoreactivity were performed (Fig. 4A-C). There were c-fos immunoreactive cells detected in parts of the cortex, hippocampus, thalamus, amygdala and hypothalamus-namely, the piriform cortex, hippocampal CA1 and CA3 regions, dentate gyrus of the hippocampus (DG), paraventricular thalamic nucleus (PVT), habenular nucleus, laterodorsal thalamic nucleus, medial nucleus of amygdala, paraventricular hypothalamic nucleus, dorsomedial hypothalamic nucleus, and ventromedial hypothalamic nucleus-in both the control and CTD-50 groups, which was in

agreement with the previous report (Silveira *et al.*, 1993). Notably, in the medial blade of DG (Fig. 4D–F) [t(9) = -2.169, p = 0.058] and PVT (Fig. 4G–I) [t(9) = -3.128, p < 0.05], there were significant increases in the c-fos immunoreactive nucleus per area in the CTD-50 group compared to the control group.

4. Discussion

In this study, we demonstrated the acute effect of CTD at the NOAEL-dose on the behavioral responses to a novel environment and explored the brain regions related to the anxiety-like behaviors in the elevated plus-maze test. Intraperitoneally-administered CTD (20 mg/kg) was immediately absorbed and was detected in the mouse brain at a level on the order of ppm for at least 4 h (Ford and Casida, 2006); therefore, CTD was considered to be present in the brains of CTD-administered mice at a concentration that was previously shown to be sufficient to cause neuronal excitation in vitro (Kimura-Kuroda et al., 2012). Although the expression of the apoptotic marker (immunoreactivity of cleaved caspase-3) were seldom observed in the brain of CTD-administrated mice (data not shown), the behavioral effects observed in this study strongly suggest that NOAEL-dose CTD has anxiogenic effects in mammals. These findings agree closely with a previous study on the behavioral effects of thiamethoxam (TMX; a metabolic precursor of CTD) showed that 50 mg/kg of TMX decreased the open arm activity in an elevated plus-maze test and 100 mg/kg of TMX also decrease locomotor activity in an open field test (Rodrigues et al., 2010).

As mentioned above, neural nAChRs are involved with emotional reactions such as mood and anxiety (Picciotto *et al.*, 2015). Nicotine, a typical agonist of nAChRs, exerts both anxiolytic and anxiogenic effects depending on the dose of exposure, which are known to be largely mediated through the $\alpha 4\beta 2$ type of nAChRs (Anderson and Brunzell, 2015). Both the

 α 4 subunit knock-out mice (Ross *et al.*, 2000) and hypersensitive α 4 receptor mice (Labarca et al., 2001) have been reported to show strong anxiety in the elevated plus-maze test. Importantly, exposures to nicotinic agonists in milliseconds to minutes strongly desensitize nAChRs (Giniatullin et al., 2005), implying that the anxiogenic effects of CTD would be responsible for the dysfunction of nAChRs involved in the modulation of other neurotransmitters in presynaptic terminals (Marchi and Grilli, 2010).

One of the most distinctive findings in this study was the observation of human-audible vocalization behaviors in the CTD-50-group mice during the elevated plus-maze test. To the best of our knowledge, there have been no reports of mice expressing such a pronounced phenotype on this behavioral test. It is well known that adult experimental mice rarely make chirping sounds in the human-audible range except under stressful and crisis situations (Sánchez, 2003). According to the latest reports, the vocalizations of mice are indicators of emotional states and can be classified by frequency as follows: (i) ultrasonic vocalizations (>20 kHz) are used to communicate with others such as in mother-pup and male-female interactions; (ii) mid-frequency vocalizations (12 kHz) are observed under restraint-stress conditions; and (iii) low-frequency harmonics (5-40 kHz, broad harmonic structure) are emitted during strong pain or aggressive encounters (Scattoni et al., 2009; Grimsley et al., 2016). The latter two types of vocalizations are also described as "distress calls" of mice experiencing negative emotions and are similar to the 22 kHz ultrasonic vocalizations of rats in stressful situations. Although the human-audible chirps made by CTD-administered mice in this study were characterized by a low and harmonic frequency (4-16 kHz) that was similar to that of mice making distress calls, they might be categorized as another type of mouse vocalization in an aversive behavioral context. Taken together, these findings indicate that CTD rendered mice irritable in the presence of what would otherwise have been a

tolerable level of stress, leading them to emit human-audible calls as signs of excessive stressor anxiety early in the course of the elevated plus-maze test.

It remains a critical question which neural circuits were involved in the unique behavioral changes that the anxiety-like behavior with the human-audible vocalization induced by CTD. Cholinergic neurons in the mammalian brain are divided into two major populations: (i) neurons of the basal forebrain region, which mainly project to the olfactory bulbs, frontal cortex, amygdala, and hippocampus, and (ii) those of the pontomesencephalic tegmental region, which project to the thalamus, hypothalamus, cerebellum, midbrain and pons (Woolf, 1991). In these possible target regions receiving cholinergic innervations and expressing nAChRs, we demonstrated that the level of excitability of parts of the thalamus and hippocampal areas was elevated in response to the novel environment after CTD administration, suggesting that the cholinergic signals from both the major populations could be disrupted by CTD.

PVT, a small dorsal nucleus of thalamus, has recently been shown to modulate negative emotional behaviors such as fear, anxiety and stress response (Hsu et al., 2014). Consistent with our results, rodent studies showed that various stressors and nicotine both activated the PVT (Bubser and Deutch, 1999, Pasumarthi and Fadel, 2008), and the c-fos expressions induced by restraint stress were much greater under nicotine treatment (Pagliusi et al., 1996). The PTV anatomically receives the afferent fibers from the brainstem, diencephalon and telencephalon, and project to forebrain regions (Kirouac, 2015). The main role of the PVT could be described as "coordinating" the emotional responses that integrate information from neurotransmitters and neuropeptides including serotonin, dopamine, norepinephrine, corticotropin releasing hormone and orexin from the caudal brain regions. In addition, according to lesion studies, the PVT is required for down-regulating the stress response

(Bhatnagar *et al.*, 2002; Spencer *et al.*, 2004). Moreover, a persuasive study described that the PVT-amygdala pathway controls the fear circuit (Penzo *et al.*, 2015). Although there were no changes of the neural activities in amygdaloidal regions by CTD, the activation of PVT and subsequent enhancement of the input to PVT-innervating regions such as the central nucleus of the amygdala, bed nucleus of the stria terminalis and nucleus accumbens may be among the factors involved in the emotional changes observed in CTD-administered mice.

The hippocampus is important not only for spatial and episodic memory, but also stress responses. Cholinergic blockade in the hippocampus activates the physical-stress response of the hypothalamic-pituitary-adrenal axis and cholinergic stimulation decreases anxiety (Bhatnagar et al., 1997; Degroot and Treit, 2002). Either region-specific administration of nicotine or knockout of acetylcholinesterase in the hippocampus results in an elevated level of anxiety in rodents (Ouagazzal et al., 1999; Mineur et al., 2013), suggesting that abnormal states of cholinergic signaling in the hippocampus result in aversive behaviors similar to those observed in the present study. In particular, the DG is involved in an event called adult neurogenesis in the granular cell layer, in which only the immature granular neurons have been shown to express functional a7 type of nAChRs (John et al., 2015), and therefore vulnerable to environmental chemicals and stressors (Heine et al., 2004; Kobayashi et al., 2015). Anatomical studies have shown that the DG is described as an input region of the hippocampal formation (Jonas and Lisman, 2014) which is innervated by multiple neurotransmitters and projects to the hippocampal CA3 regions. The cholinergic afferents from the medial septal nucleus and nucleus of the diagonal band of Broca are important as excitatory inputs to the DG (Amaral et al., 2007). In this study, mice in the CTD-50 group showed an increase in the number of c-fos-positive nuclei in granule cells in the DG, and this change may have had an impact on the entire hippocampus and contributed to the behavioral

314 effects of neonicotinoids.

According to the latest studies, other brain regions may also be targeted by neonicotinoids. Kimura-Kuroda et al. (2016) recently reported an alteration in the transcriptome of cerebellar cultures exposed to ACE and IMI, including changes in genes essential for brain development. Terayama *et al.* (2016) revealed that ACE decreased the β 2 type of nAChRs in many brain regions and tended to be residual in the midbrain. In other studies, stress-induced 22 kHz vocalizations of rats were attributed to the activation of cholinergic neurons in the midbrain and pons (Kroes et al., 2007; Borges et al., 2010). Considering all of these evidences, it cannot be denied that the cerebellum and midbrain innervated by cholinergic projection could also be upstream targets of neonicotinoids.

As noted before, the cholinergic signals in the brain are important for stress responses (Picciotto *et al.*, 2012). For instance, nicotine is known to enhance the responses to various stressors (Yu and Sharp, 2012). However, in the risk assessment for new chemical substances, there have been no studies measuring their disruption of the stress reactivity in mammals. This is crucial information, since excessive stress responses are known to suppress the physical condition of mammals, including impairments in reproductive, immune and metabolic function (Chrousos and Gold, 1992; Hirano *et al.*, 2014), and thus an increase in the threshold for novel environments and the activation of stress reactivity may be helpful endpoints when evaluating the systemic effects of environmental factors.

In the current study, we present new evidence that neural circuits involved in controlling the stress reactivity, including the PVT and DG, would become susceptible to a novel environment under CTD at NOAEL-dose resulting in excessive anxiety-like behavior in the elevated plus-maze test. Epidemiological studies revealed that the detection rates and amounts of neonicotinoids in urine in Japanese adults became increased in proportion to the

domestic usage (Ueyama et al., 2015), suggesting that further information is needed for the proper global risk assessment of neonicotinoids. These results contribute toward evaluating the neurobehavioral effects of neonicotinoids.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

> Fig. 1. Scheme showing the overall experimental design. One hour after oral administration of CTD (0, 5, 50 mg/kg) to mature male mice, behavioral effects were assessed by an elevated plus-maze test for 10 min. After 2 h of the behavioral test, mice were sacrificed for the neuroactivity analysis.

> Fig. 2. Behavioral effects of acute CTD observed in the elevated plus-maze test. (A) Representative trajectory maps showed that the exploratory behaviors in the open arms were suppressed in the CTD-administered groups compared to the control group. (B) The total distance traveled and (C) the total number of arm entries were not changed in the CTD-5 groups, but were significantly lower in the CTD-50 group relative to those of the control group. (D) The percentage of time spent in the open arms and (E) the percentage of open arm entries were significantly lower in both the CTD-5 and CTD-50 groups than the control group. Columns showed the group mean \pm SE and circles showed individual values of mice (Control: n = 14, CTD-5: n = 10, CTD-50: n = 10), *p < 0.05, **p < 0.01 vs. control group (one-way ANOVA followed by Dunnett's post-hoc test).

Fig. 3. Representative spectrograms of human-audible vocalizations emitted by mice in the CTD-50 group during the elevated plus-maze (A); no vocalizations were emitted in the control group (B). The frequency distribution of the vocalizations was in the human-audible frequency range (<20 kHz). (C) Histogram showing the number of the vocalizations observed during the elevated plus-maze test. Columns showed the group mean \pm SE (n =10 mice in the CTD-50 group).

Fig. 4. Representative histology of the c-fos immunoreactivities of the control (A) and CTD-50 (B) groups in -1.06 mm of bregma of mice brain according to the brain atlas (Paxinos and Watson, 2001; C). Higher magnification pictures showed the hippocampal dentate gyrus (DG, surrounded by a dotted line) (D, E) and paraventricular thalamic nucleus (PVT, surrounded by a solid line) (G, H) of the control (D, G) and CTD-50 (E, H) groups. The number of c-fos-immunoreactive cells per area in PVT (F) and DG (I) were significantly increased in the CTD-50 group compared to the control group. Bar = $100 \mu m$. Columns showed the group mean \pm SE and circles showed individual values of mice (Control: n = 6, CTD-50: n = 5), *p < 0.05 vs. control group (Welch's *t*-test).

CTD administration

(0, 5, 50 mg/kg, i.o.)







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