Research paper

Anxiety-like behavior induced by salicylate depends on age and can be prevented by a single dose of 5-MeO-DMT

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A B S T R A C T

Salicylate intoxication is a cause of tinnitus and comorbidly associated with anxiety in humans. In a previous work, we showed that salicylate induces anxiety-like behavior and hippocampal type 2 theta oscillations (theta2) in mice. Here we investigate if the anxiogenic effect of salicylate is dependent on age and previous tinnitus experience. We also tested whether a single dose of DMT can prevent this effect. Using microwire electrode arrays, we recorded local field potential in young (4–5-month-old) and old (11–13-month-old) mice to study the electrophysiological effect of tinnitus in the ventral hippocampus (vHipp) and medial prefrontal cortex (mPFC) in an open field arena and elevated plus maze 1h after salicylate (300mg/kg) injection. We found that anxiety-like behavior and increase in theta2 oscillations (4–6 Hz), following salicylate pre-treatment, only occurs in young (normal hearing) mice. We also show that theta2 and slow gamma oscillations increase in the vHipp and mPFC in a complementary manner during anxiety tests in the presence of salicylate. Finally, we show that pre-treating mice with a single dose of the hallucinogenic 5-MeO-DMT prevents anxiety-like behavior and the increase in theta2 and slow gamma oscillations after salicylate injection in normal hearing young mice. This work further support the hypothesis that anxiety-like behavior after salicylate injection is triggered by tinnitus and require normal hearing. Moreover, our results show that hallucinogenic compounds can be effective in treating tinnitus-related anxiety.

1. Introduction

Tinnitus is a high prevalence sign that commit 11.9–30.3% of the general population (McCormack et al., 2016). Within this group, 3–3.9% of the subjects report, long-lasting, bothersome tinnitus (McCormack et al., 2016). The condition is often associated with adverse emotional responses, including increased stress, irritability, sleep disorders, anxiety and depression (Belli et al., 2008; Bhatt et al., 2017; Izuhara et al., 2013; Lauer et al., 2018; Pattyn et al., 2016; Winne et al., 2019). In a previous study, we have shown that salicylate, a known cause of tinnitus (Pearlman and Gambhir, 2009), induces anxiety-like behavior in mice (Winne et al., 2019). Salicylate can generate tinnitus in both humans and animals (Eggermont and Roberts, 2015) yet, few studies explore anxiety-like behavior in the salicylate tinnitus models (Lauer et al., 2018; Winne et al., 2019). It is known, however, that noise-induced tinnitus augment aggressive behavior but does not change spatial performance (Zheng et al., 2011a, 2011b; Zhang et al., 2016).

Type 2 theta oscillation (theta2) can appear when rodents are exposed anxiogenic environments or in the presence of predator odor (Mikulovic et al., 2018; Sainsbury and Montoya, 1984). Anxiety in the salicylate tinnitus model also generates theta2 in the ventral hippocampus (vHipp) during open-field test (Winne et al., 2019). Given the strong projections from the vHipp to the mPFC, theta2 generated in the vHipp could drive the mPFC, and, consequently the amygdala (Adhikari et al., 2010). In fact, theta2 coherence increases between the hippocampus and mPFC during anxiety-like behavior (Adhikari et al., 2010). However, the effect of tinnitus-induced anxiety in the vHipp/mPFC pathway is yet to be explored.

Subjective tinnitus is primarily associated with sensorineural hearing loss (Cima et al., 2019; Moon et al., 2018; Norena and Eggermont, 2003; Weisz et al., 2006) which may be the consequence of...
noise trauma, ototoxic drugs, or aging. Here, we used acute salicylate injection as a tinnitus model (Rüttiger et al., 2003). Mechanisms by which salicylate induces tinnitus are unclear. Salicylate has well-established effects on cochlear function. It has been suggested that salicylate induces tinnitus through the activation of cochlear NMDA receptors (Guitton et al., 2003). In addition, salicylate modifies the expression of the protein prestin on the outer wall of hair cells (Yang et al., 2009; Yu et al., 2008). However, recent experiments have highlighted direct modulation of neural activity in other auditory areas (Guitton et al., 2003; Sheppard et al., 2014; Sun et al., 2009). Salicylate can also have direct effects on limbic system neurons (Sun et al., 2009; Winne et al., 2019) and therefore could directly alter limbic function. Interestingly, inflammatory reaction can trigger anxiety (Haj-Mirzaiaei et al., 2016; Haj-Mirzaiaei et al., 2017). Hence, attenuated salicylate-triggered anxiety-like behavior in animals suffering tinnitus/hearing loss as a precondition could help to distinguish anxiety triggered by tinnitus from anxiety generated by changes in inflammatory pathways.

We have previously hypothesized that salicylate induces anxiety-like behavior due to the acute generation of tinnitus (Winne et al., 2019). Hence, previous tinnitus perception and/or hearing loss could potentially minimize anxiety after acute salicylate injection, precluding a direct anxiogenic effect of the drug itself. Salicylate could also fail to trigger anxiety in older mice as several strains suffer from age-related hearing loss due to degeneration of peripheral auditory neurons (Jison et al., 2007). However, to our knowledge, no study that use acute salicylate injection to induce tinnitus has explored age related effects on anxiety.

Recent studies evinced the potential of hallucinogens in treating mood disorders (Davis et al., 2019; Dos Santos et al., 2016; Palhano-Fontes et al., 2019; Reiche et al., 2018). Results in clinical trials were also replicated in animal models of depression (Buchborn et al., 2014). Rats submitted to olfactory bulbectomy (depression model) treated with lysergic acid diethylamide (LSD) showed near normal active avoidance (a major sign of anxiety-like behavior) and learning similar to animals treated with classical antidepressants (Buchborn et al., 2014). Davis and others described that one to four sessions of 5-MeO-DMT administration therapy lead to 79% decrease in depression and 69% in anxiety symptoms (Davis et al., 2018). Moreover, hippocampal neurogenesis, a hallmark of long-term antidepressant treatment, is also increased after a single dose of the hallucigen 5-methoxy-N,N-di-methyltryptamine (5-MeO-DMT) (Lima da Cruz et al., 2018). Clinical assays that examined antidepressant effects of DMT analogs described no significant side effects following their administration (Davis et al., 2018; Palhano-Fontes et al., 2019). It is important to note that hallucinations caused by psychedelics can be aversive to certain groups of patients. (Baumeister et al., 2014). In addition, there is a lack of long-term studies assessing chronic adverse effects of hallucinogen therapy. Electrophysiological assays exploring the effect of hallucinogens in animal models of mood disorders are essential to assess whether anxiolytic/antidepressant effects are accompanied by the absence of oscillatory patterns inherent to mood disorders (e.g. theta2) and if other rhythms that encode behaviors like running, foraging etc. (e.g. type 1 theta) are not affected.

In this work we used implanted microwire arrays in young (4–5-month-old) and old (11–13-month-old) mice to assess changes in local field potentials (LFPs) triggered by tinnitus and anxiety in the ventral hippocampus (vHipp) and the medial prefrontal cortex (mPFC). In addition, we tested if pre-treating mice with 5-MeO-DMT could provide resilience to anxiety comorbidly linked to tinnitus.

2. Material and methods

2.1. Mice

All procedures were approved by the Animal Ethics Committee (CEUA) of the Federal University of Rio Grande do Norte (Protocol number 052/2015). All effort was made to minimize suffering and discomfort of animals and to reduce the number of the animals used. Young (4–5 month-old) and old (11–13 month old) C57BL/6 male mice were implanted with microwire arrays targeting the vHipp and mPFC (Winne et al., 2019). Mice were individually housed after surgeries (Adhikari et al., 2010). Electrode arrays were fabricated from insulated tungsten wires (50 μm, impedance between 100 and 400kΩ, California Fine Wire). Array configuration: 6 electrodes (2 × 3, electrode spacing 200 μm) targeting the ventral hippocampus and 4 electrodes (2 × 2, electrode spacing 200 μm) for targeting the pre-limbic cortex. Animals were anesthetised with isoflurane 2% oxygen and placed on a heat pad maintained at 37 °C by a temperature controller (Supertec). The head was then fixed to a stereotaxic frame and an incision was made to expose the skull and three holes were drilled to implant the electrodes. Coordinates for array implantation were −3.4 mm AP, 3.5 mm ML, 3.5 mm DV (vHipp), and 2.1 AP, 0.3 ML, 1.6 DV (mPFC). A screw placed over the cerebellum served as reference electrode and three additional screws anchored the implant (held with dental cement). Animals were then housed individually and allowed to recover for at least 10 days before recordings. Animals were housed on a 12 h/12 h day/night cycle to maintain their normal biorhythms and had free access to food and water.

2.2. Auditory brainstem responses

Auditory brainstem response (ABR) was recorded in mice before and after acoustic noise trauma (see below). Mice were anesthetized with a mixture of ketamine/xylazine 90/6 mg/kg and placed in a stereotaxic apparatus with thermal pad to avoid heat loss. A speaker was positioned in front and 11 cm away from the head of the animal. To record the ABR, two pre-chlorinated Ag/AgCl electrodes with ~1 kΩ impedance were used, one recording electrode and one reference. Electrodes were placed subdermally; small incisions were made in the skin covering the bregma region (reference) and another in the region of lambda (recording). The ground was connected to the system ground. Sound stimulus consisted of narrow-band Gaussian white noise pulses with 3 ms duration each, presented at 10 Hz for 529 repetitions for each frequency and intensity tested. The frequency bands tested were 8 ~ 10 kHz, 9 ~ 11 kHz, 10 ~ 12 kHz, 12 ~ 14 kHz and 14 ~ 16 kHz (same as for GPIAS, see below). Pulses were presented initially at 80 dB sound pressure level (dBSPL) and decreasing in 5 dBSPL steps down to 45 dBSPL. All sound equipment was calibrated with an ultrasonic microphone (4939-A-011, Bruel and Kjaer).

2.3. Tinnitus induced by salicylate

For salicylate-induced tinnitus, mice received an injection of 300 mg/kg sodium salicylate (Sigma) diluted in saline or saline (control) intraperitoneal injection 1 h prior to the experiments (Brozoski and Bauer, 2016; Lauer et al., 2018; Winne et al., 2019).

2.4. Tinnitus induced by acoustic trauma

Acoustic over-exposure to cause noise-induced tinnitus were carried out in a sound shielded room, inside a sound-isolated cabinet (44x33x24cm). Mice were habituated for 5 days and were then placed inside an acrylic cylinder with restraining doors perforated at regular intervals, to maintain in front of the speaker (4 cm) during the noise-overexposure (broadband noise of 4–20 ± 1 kHz, 90 dBSPL, 90 min). This intensity is enough to generate tinnitus perception without causing permanent hearing loss (T. Malfatti, unpublished data). After the acoustic trauma, mice were kept in the sound shielded room for another hour to ensure development of tinnitus, since recent studies have shown that increased ambient noise and acoustic enrichment immediately after noise trauma prevents circuit reorganization of the inferior colliculus and thereby can prevent noise-induced tinnitus (Sturm et al., 2018).
2.5. Gap prepulse inhibition of acoustic startle

Gap prepulse inhibition of acoustic startle (GPIAS) test was performed to assess salicylate- or noise-induced tinnitus perception. Animals were placed in custom-made acrylic restraining boxes perforated at regular intervals. The restraining boxes were placed in a sound-shielded custom-made cabinet (44x33x24cm) with low-intensity LED lights in a sound-shielded room. A loudspeaker (Super tweeter ST400 trio, Selenium Pro) was placed horizontally 4.5 cm in front of the restraining cylinder, and startle responses were recorded using a digital accelerometer (MMAS452Q, NXP Semiconductors, Netherlands) mounted to the base plate of the restraining cylinder and connected to an Arduino Uno, which provides the accelerometer signal outputs to a data acquisition board (Open-ephys). Sound stimulus consisted of 60 dB SPL narrow-band Gaussian white noise (background noise); 40 ms of silence (Gap trials) or background noise (No-Gap trials); 100 ms of background noise; and 50 ms of background noise at 105 dBSPL (startle pulse). The intensity of the background noise presented was adjusted to the animal can hear the stimulus. Trials of initial background noise were pseudo-randomized (interval of 12 to 22 s), and trials were played continuously. Frequencies between 8 and 10, 9–11, 10–12, 12–14 and 14–16, as well as 8–16 kHz were tested. The full session consisted of 18 trials per band of frequency tested, 9 Gap and 9 No-Gap trials, presented pseudo-randomly. All sound equipment was calibrated with an ultrasonic microphone (4939-A-011, Bruel and Kjaer).

2.6. Open field test (OFT)

In the open-field test, each mouse received an injection of sodium salicylate dilute in saline or a control saline injection. Tests order was randomized with the second session 1 week following the first. In general, 1 h following ip injection (salicylate or saline) each mouse was placed in a rectangular open field arena (40 cm × 32 cm × 15 cm) made of opaque white plastic for electrophysiological and video recordings. Two sessions of ten minutes each were recorded. At the beginning of each session animals were connected to the headstage and lightweight recording cable and placed individually in the center of the open field. The following behaviors were quantified: total distance travelled, and time spent in the center of the arena during the ten minutes of the test (Lopatina et al., 2014; Seibenhener and Wooten, 2015; Wang et al., 2011). Between each tested animal, the apparatus were cleaned with water. For DMT experiments, 5-MeO-DMT (sigma) was first dissolved in DMSO and then in 0.9% saline (1:500) to a final dose of 20 mg/kg and injected i.P. 7 days before the re-exposed to open-filed test (4 weeks after the first open-field experiment). It is important to note that salicylate induces anxiety in the open-field even if animals are re-tested in < 15 days (Winne et al., 2019).

2.7. Elevated plus maze (EPM)

In the EPM, the mice received salicylate injection 1 h before the test and each animal were exposure only once to the EPM. The apparatus has four arms of equal dimensions (6.5 × 62 cm), two of them open and two closed (walls with 20 cm height) elevated 52 cm above the floor. The animals connected to the headstage and cables were placed individually in the center of the EPM and allowed to explore the maze for 10 min. Total time in the open or closed arms and exploration patterns were quantified (Buccafusco and Buccafusco, 2009; Komada et al., 2008; Walf and Frye, 2007). EPM exploration sessions were synchronized with electrophysiological recordings and the apparatus were cleaned with water between each tested animal.

2.8. Histology

To verify electrode position after the experiments, animals were deeply anesthetized with ketamine/xylazine (80 mg/kg) and transcardiac perfusion was carried out with cold saline solution followed by 4% paraformaldehyde (PFA). Brains were dissected and placed in PFA with 30% sucrose for hydration for (two days). Next brains were frozen in isopentane and sectioned on a cryostat (50 μm thick coronal slices) and sections of areas of interest were processed using Nissl colorimetric staining (Paul et al., 2008). In summary, sections were placed in xylol for 10 min, gradually rehydrated through increasing concentrations of water in the alcohol, next stained with cresyl violet, and gradually dehydrated through decreasing concentrations of water in the alcohol followed by xylene and finally mounted on glass slides using the Permount mounting medium. Images were taken with a microscope (Zeiss, Berlin, Germany) at 10 × magnification.

2.9. Immunohistochemistry for c-Fos and analysis

For c-Fos immunohistochemistry, 60 min after the salicylate injection, young mice (4–5-month-old) and old mice (11–13-month-old) were deeply anesthetized with ketamine and transcardially perfused. The brains were sectioned in 50 μm slices. Sections containing the vHipp were sequentially incubated in blocking solution (Phosphate buffer saline (PBS), 3% bovine serum albumin and 0.2% Triton-X-100). Sections were next drained and incubated in primary antibody rabbit anti-cFos (1:1000, Santa Cruz, California, USA) overnight at 4 °C. Next sections were washed 3 × in PBS and incubated in secondary antibody (Goat anti-rabbit IgG 555, 1:1000, Invitrogen). Finally, sections were washed 3 × in PBS and coveredslipped with 10 μl antifade mounting medium. Images were acquired using a conventional upright microscope (equipped with epifluorescence) (Zeiss) and analyzed in ImageJ (Rueden et al., 2017).

2.10. Data acquisition and statistical analysis

For each frequency tested in the Gap detection test, Gap and No-Gap trials responses were separated and the signal was filtered with a Butterworth low pass filter at 50 Hz. The absolute values of the accelerometer axis were averaged and sliced 400 ms around the startle pulse (200 ms before and 200 ms after). The root-mean-square (RMS) of the sliced signal before the sound pulse (baseline) was subtracted from the RMS after the startle pulse (response). The GPIAS index for each frequency was calculated as (1-(GapRMS/NoGapRMS))×100, meaning the percentage of suppression induced by the gap.

For each intensity of each frequency tested in ABR tests, all trials were averaged, filtered using a Butterworth bandpass filter of 3rd order from 500 ~ 1500 Hz, and sliced 12 ms after the sound pulse onset. Thresholds were defined by visually inspecting the lowest intensity that can elicit a wave peak in response to sound. Python scripts for ABRs, GPIAS and sound calibration signal generation and analysis can be found at https://gitlab.com/malfatti/SciScripts.

Local field potentials (LFPs) were acquired by a 16-channel amplifier OpenEphys system (Siegle et al., 2017) with an Intan RHD headstage set to high pass filter from 0.1 Hz during open field tests. Video was simultaneously recorded using a Basler camera (model acA1300-30um) triggered (frame by frame) by an Arduino board (precisely at 20 frames per second) also recorded using the OpenEphys. A maximum of three mice were recorded per day and the recordings were done during the night. Power spectral densities (PSD) for all channels were computed using the Welch method (pwelch Matlab command). A custom Matlab program (using the Matlab Imaging Processing Toolbox) was used for tracking the animal in the field by thresholding the animal compared with the background. We calculated the coherence between mPFC and vHipp channels using the Matlab command mscohere. Data is presented by mean ± standard error of the mean (SEM). We tested for
normality of the data using the Matlab command vartest2 to calculate statistical significance with paired t-test. For non-normal distributed data, we used the non-parametrical Friedman’s test to calculate statistical significance (Friedman, 1940).

The expression of c-Fos in the ventral hippocampus of animals of both groups was quantified by optical densitometry using ImageJ,
following a protocol adapted from (Biran et al., 2005; Budoff et al., 2019). Mann-Whitney U tests were performed after a Kolmogorov-Smirnov test that demonstrated the necessity of non-parametric analysis in this case (statistically significant difference is indicated for $p < .05$).

3. Results

3.1. Auditory brainstem responses, gap detection and c-Fos immunohistochemistry

We first analyzed auditory brainstem responses (ABRs) in young (age 4–5 month) and old (age 11–13 month) as the C57BL/6 J mouse line is known to develop age-related hearing loss (Ison et al., 2007). As expected, old animals showed an increased hearing threshold to higher frequencies (Fig. 1A), specifically in response to frequencies of 12–14 kHz and 14–16 kHz (64.5 ± 4.0 dBSPL and 67.0 ± 3.6 dBSPL, respectively) when compared to young animals (53.3 ± 2.1 dBSPL and 49.4 ± 2.0 dBSPL; $p = .02556$ and 0.00602, Student’s t-test, Bonferroni-corrected for 5 comparisons, Fig. 1A). Next, we wanted to test if old animals have an increased tinnitus perception compared to young mice, and here we evaluated tinnitus-like behavior using a gap detection test. The GPIAS index shows the level of suppression (in percent) generated by the silent gap. Low GPIAS index indicates that the animal does not perceive the gap, which may be due to a persistent tinnitus in the same frequency as the background noise band. We did not find any significant difference in suppression of the startle response by the silent gap related to any specific frequency between old (n = 5) and young (n = 5) mice (Fig. 1B, middle), however when grouping the most affected frequency for each animal a trend of decrease in GPIAS suppression was seen in the old mice (Fig. 1B, right, $p = .05209$, Student’s t-test, Bonferroni-corrected for 6 comparisons, Fig. 1B). This suggests that old mice tend to have a decreased gap suppression, which may indicate age-related tinnitus perception, compared to young animals.

We then investigated whether salicylate (300 mg/kg, i.p.) and noise overexposure (broadband noise of 4–20 ± 1 kHz, 90 dBSPL, 90 min) produced changes in gap startle suppression. The GPIAS index for the most affected frequency showed a significant decrease in suppression for the salicylate-injected group compared to saline-injected group ($p = .00521$, Student’s t-test, Bonferroni-corrected for 6 comparisons; Fig. 1C, right). Similarly, young mice showed a significant decrease in startle suppression following noise overexposure when compared to non-exposed young animals ($p = .02104$, Student’s t-test, Bonferroni-corrected for 6 comparisons; Fig. 1D, right) for the most affected frequency, but not for any particular frequency band (Fig. 1D, center). Taken together, this data supports the notion that both salicylate and noise overexposure can cause tinnitus, but that the frequency of tinnitus perception is different among individual animals (Longenecker and Galazzy, 2016).

We next used c-Fos immunohistochemistry to investigate whether salicylate differentially affect neuronal activity of cells in the ventral hippocampus of young (age 4–5-month, n = 3) and old mice (age 11–13-month, n = 3, Fig. 2A). Neurons with increased activity were detected by c-Fos-immunoreactivity within the ventral CA1 region. Animals sacrificed 1 h following salicylate injection showed no statistical difference in staining density median between the vHipp of young mice (21.06 ± 7.43 A.U) and old mice (16.64 ± 7.55 A.U, $p = .059$, Fig. 2B). These results suggest that salicylate injection have similar immediate effects on overall neuronal activity in the vHipp of young and old mice.

3.2. Open field test

Anxiety-like behavior in animals of different ages was assessed using the open field test (young mice: 4–5-month-old, n = 10 and old mice: 11–13-month-old, n = 7). Animals were placed in an open field arena 1 h after i.p. injection of saline or salicylate (Fig. 3A). Following the salicylate injection, young animals spent significantly less time in the center of the arena compared to the saline group (mean center time, saline group: 192.61 ± 44 s; salicylate group: 72.41 ± 34.74 s, for a 10 min session, $p = .003$, n = 10 mice, Fig. 3C). In addition, these mice showed decreased locomotion (mean travelled distance saline group: 41.11 ± 10.79 m; salicylate group 21.24 ± 5.78 m, $p = .0001$, Fig. 3C). When testing old animals, salicylate injected mice spent more time in the center (saline group: 192.61 ± 58.26 s; salicylate group: 246.71 ± 76.72 s, $p = .04$, n = 7 mice, Fig. 3D). Locomotion of old animals showed no difference between saline and salicylate groups (total travelled distance saline group: 54.84 ± 9.53 m; salicylate group: 41.87 ± 6.2 m, p = .14, Fig. 3D). As reduced time in the center and reduction in exploration indicates anxiety-like behavior and our results suggest that acute salicylate administration produce anxiety-like behavior preferentially in younger mice.

The resilience of old mice in displaying anxiety-like behavior following salicylate treatment could be explained by hearing loss (and possible pre-existing tinnitus). In a reversed scenario, young animals with tinnitus as a (precondition induced by noise trauma) would not show anxiety-like behavior after salicylate injection (in case anxiety is directly related to tinnitus perception rather than salicylate itself). We, therefore, injected salicylate in animals previously exposed to acoustic trauma with signs of tinnitus screened by GPIAS followed by the open field test (n = 9 mice, Fig. 3B). Young mice with preconditioned tinnitus (acoustic trauma) injected with salicylate spent more time in the center than saline treated mice (time in the center acoustic trauma/salicylate: 198.63 ± 80.57 s; acoustic trauma/salicylate: 304.68 ± 73.48 s, p = .04, Fig. 3E). However, acoustic trauma animals showed less locomotion following salicylate injection (mean travelled distance acoustic trauma/saline: 55.10 ± 17.40 m; acoustic trauma/salicylate: 39.29 ± 10.51 m, p = .01, n = 9 mice, Fig. 3E). These findings showed that salicylate conversely increased the time spent in the center, indicating reduced anxiety. However, due to less distance travelled, this could also indicate a hypolocomotion rather than reduced anxiety in the acoustic trauma + salicylate animals.

Next we compared the age-effect of salicylates by comparing old mice versus young mice post-acoustic trauma (Fig. 3F). Interestingly, old animals treated with salicylate showed similar values to young acoustic trauma animals administered salicylates when comparing mean time in the center of the arena (acoustic trauma salicylate group: 304.68 ± 73.48 s; old salicylate group: 246.71 ± 76.72 s in a 10 min session, $p = .34$, n = 9 and 7 mice respectively, Fig. 3F) and locomotion (total travelled distance post-acoustic trauma animals after salicylate 39.29 ± 10.51 m, old mice after salicylate: 41.87 ± 6.2 m, p = .48, Fig. 3F). These results show that, similar to old mice, young mice that had a previous tinnitus perception are less prone to display anxiety-like behavior after salicylate injection cause (when time in center and distance travelled is taken into account). As salicylate alone in young mice cause less time spent in the center and less distance travelled (Fig. 3C) these results could indicate that previously altered auditory perception caused by noise trauma masks salicylate-induced tinnitus effects on anxiety.

3.3. Type 2 theta and slow gamma oscillations in open field

In addition to behavioral data, LFP was recorded in young mice (n = 10 mice) and old mice (n = 7 mice). We histologically and electrophysiology confirmed the electrode placement at the radial stratum CA1 (SR) (Schomburg et al., 2014) (Fig. 4A and B). Theta oscillations were separated in 2 bands, 4–6 Hz and 7–10 Hz, for type 2 and type 1 theta bands (Kramis et al., 1975; Sainsbury and Montoya, 1984; Montoya et al., 1989). Increased gamma oscillations were also observed in the power spectrum density (PSD) plots. These oscillations are classically separated in 2 bands, ~30–60 Hz (slow gamma) and ~65–100 Hz (fast gamma) (Colgin, 2015; Hsiao et al., 2016). In young
animals, during free exploration in the open field following salicylate injection, power spectrum analysis revealed an increase in amplitude of frequency between 4 and 6 Hz and 30–60 Hz (slow gamma) in comparison to injection of saline. Mean 4–6 Hz power was equal to 2335.98 ± 1161.61 μV²/Hz for the salicylate group and 2311.33 ± 390.56 μV²/Hz for the saline group (p = .496, Fig. 4E) or mean power of slow gamma (30–60 Hz) (slow gamma) in comparison to injection of saline. Mean 30–60 Hz power was equal to 75.16 ± 41.44 μV²/Hz for the saline group and 107.16 ± 58.83 μV²/Hz for the saline group (p = .004, Fig. 4D). However, in old mice, salicylate injections did not cause a significant difference in the mean power of theta2 (4–6 Hz; 2258.48 ± 1161.61 μV²/Hz for the saline solution and 2344.78 ± 1061.01 μV²/Hz for salicylate, p = .496, Fig. 4E) or mean power of slow gamma (30–60 Hz; 108.44 ± 34.37 μV²/Hz for saline solution and 118.55 ± 43.86 μV²/Hz for salicylate, p = .38, Fig. 4F). These data indicate that salicylate modulates circuits associated with type 2 theta and slow gamma oscillation in the ventral hippocampus specifically in young mice naive to tinnitus.

3.4. Complementary appearance of theta2 and slow gamma in the elevated plus maze

To test if theta2 and slow gamma encode different states of the animal in anxiogenic environments, we use the elevated plus maze test (EPM) for a clear separation of perception of safety (closed arms) or eminent danger (open arms) during salicylate-induced tinnitus. Salicylate injected young mice (n = 6) spend significantly less time in the open arms (117 ± 55, 8s) compared in the close arms (459 ± 73.8s), p = .001, (Fig. 5A) of the EPM. However, old mice (n = 6) treated with salicylate did not show significant difference in time spent in the open arms compared with closed arms (258.54 ± 55.67 s for opened arms and 323.09 ± 90.81 s for closed arms, p = .42, Fig. 5B). LFP recordings during the exploration in the EPM showed that in salicylate-treated young mice an increase in theta2 amplitude (4–6 Hz) in the closed arms in comparison to the open arms as shown by power spectrum analysis (mean 4–6 Hz power was equal to 3582.02 ± 1654.33 μV²/Hz in the closed arms and 1869.56 ± 993.51 μV²/Hz in the open arms, p = .008, Fig. 5C). On the other hand, there was a significant increase in the mean 30–60 Hz oscillation (slow gamma) power in the open arms of the EPM (mean 30–60 Hz power was equal to 154.96 ± 73.09 μV²/Hz for the closed arms and 243.87 ± 106.20 μV²/Hz for the open arms, p = .047, Fig. 5C). However, in old mice injected with salicylate showed no significant difference in the mean power of 4–6 Hz (1901.93 ± 1206.79 μV²/Hz for closed arms and 1847.99 ± 1255.21 μV²/Hz for open arms, p = .165, Fig. 5D) and 30–60 Hz between opened and closed arms (123.14 ± 27.47 μV²/Hz for closed arms and 149.99 ± 52.80 μV²/Hz for open arms, p = .165, Fig. 5D). Taken together, this data shows that old mice do not show “anxiety-signaling” theta2 after salicylate injection and theta2 and slow gamma encode different states in anxiogenic environments.

3.5. Complementary theta2 and slow gamma in the open field

Anxious-like young mice displayed higher theta2 power “safer” regions of the EPM (closed arms) while slow gamma power increased in the most anxiogenic regions (open arms). To further demonstrate the complementary appearance of these two rhythms, we investigated if young animals (n = 10) presented similar results when exploring the center (“anxiogenic” area) and border (“safer” area) of the open field. Similar to the EPM, after salicylate injections, mice in the open field arena showed a significant increase in the mean 4–6 Hz oscillation power in the border (2639.38 ± 950.71 μV²/Hz) in comparison to the center (2223.51 ± 854.21 μV²/Hz) of the open field (p = .006, Fig. 5E). We have also found a significant increase in the mean 30–60 Hz oscillation power in the center compared of the border (mean 30–60 Hz power was equal to 134.87 ± 27.00 μV²/Hz and in the center and 114.52 ± 28.69 μV²/Hz in the border and, p = .01, Fig. 5E). Taken together, these results show that theta2 and slow gamma oscillations increase in the vHipp and after salicylate injections in anxiety tests in a complementary manner.

3.6. mPFC oscillations and coherence with vHipp in salicylate-induced anxiety

As the old animal group did not show anxiety-like behavior, further experiments only explore effect of salicylate on theta and gamma oscillations in the mPFC of young animals (n = 10). We used LFP and post-hoc histology to confirm the placement of electrodes mPFC (Winne et al., Experimental Neurology 326 (2020) 113175).
et al., 2019), (Fig. 6A and B). In the open field, power spectrum analysis revealed an increase of frequency peak between 4 and 6 Hz and 30–60 Hz when animals receive salicylate injection in comparison to injection of saline. Mean 4–6 Hz power was equal to 758.63 ± 231.60 μV^2 /Hz for the saline group and 898.23 ± 268.64 μV^2 /Hz for the salicylate group (p = .008, Fig. 6C) and mean 30–60 Hz power was equal to 32.62 ± 6.52 μV^2 /Hz for the saline group and 42.67 ± 9.19 μV^2 /Hz for the salicylate group (p = .0002, Fig. 6D). In the elevated plus maze test, when previously injected with salicylate, the power spectrum analysis revealed an increase in amplitude of frequency between 4 and 6 Hz in the closed arms (587.70 ± 183.50 μV^2 /Hz) in comparison to the open arms (376.20 ± 151.21 μV^2 /Hz), p = .0004, Fig. 6E. There was a significant increase in the mean 30–60 Hz oscillation power in the open arms of the EPM (42.38 ± 20.87 μV^2 /Hz) in comparison to the open arms (32.29 ± 15.53 μV^2 /Hz, p = .03, Fig. 6F). We then asked if the mPFC and ventral hippocampus show coherent salicylate induced theta oscillation in young animals (n = 10). We measure coherence in theta oscillations from pairs of electrodes implanted in the mPFC and vHipp (Fig. 7A). Ventral hippocampus/mPFC theta2 coherence was significantly higher in the salicylate condition (mean coherence for the saline group was equal to 0.28 ± 0.12 for the saline group and 0.51 ± 0.06 for the salicylate group, p = .0001, n = 10, Fig. 7B). Thus, coherence analysis indicates that salicylate-induced theta oscillations are significantly enhanced in the salicylate condition compared to the saline condition.
oscillation is efficiently transmitted from the ventral hippocampus to the mPFC.

3.7. Behavior and LFP recordings in mice pretreated with 5-MeO-DMT

Lastly, we investigated whether a single dose of 5-MeO-DMT would have a short-term anxiolytic effect (4 days after DMT injection) on the behavior of young mice ($n=8$) after salicylate injection (Fig. 8A, B). We found that 20 mg/kg 5-MeO-DMT reversed the salicylate anxiogenic effect (Fig. 8C). The animals treated with DMT four days prior to the test showed increased locomotion after salicylate administration (1 h prior to test) when compared to animals exposed to salicylate without previous DMT treatment (total distance walked 22.52 ± 6.07 m and 40.00 ± 14.82 m for salicylate and salicylate after DMT respectively, $p = .04$, Fig. 8C). Pre-treatment with DMT also caused the animals to spend more time in the center of the arena (69.06 ± 34.03 s for salicylate and 120.29 ± 44.29 s salicylate after DMT, respectively, $p = .001$, Fig. 8C). In addition to reversing the anxiogenic effect of salicylate, mean theta2 power was also significantly reduced in animals pre-treated with DMT (19.59 ± 67.32 μV^2/Hz for the salicylate post DMT group and 33.75 ± 108.43 μV^2/Hz for the salicylate group, $p = .01$, $n=8$, Fig. 8D). The mean of 30–60 Hz of slow gamma power was also significantly reduced (11.52 ± 43.25 μV^2/Hz for salicylate post DMT group and 13.93 ± 53.94 μV^2/Hz for the salicylate group, $p = .04$, $n=8$, Fig. 8D). These results demonstrate that pre-treatment with DMT can prevent anxiety related changes in the type 2 theta and slow gamma oscillation in the ventral hippocampus in young mice produced by salicylate.

Fig. 4. Salicylate increases type 2 theta and gamma rhythms in the ventral hippocampus depends on age. A. (left) Schematic drawing of the localization of electrodes in the ventral hippocampus (*). (right) Bright field image showing positioning of electrode in stratum radiatum (arrow) on the vHipp. B. Representable example of a raw trace signal from one channel from the same animal for saline (top) and salicylate (bottom). C. Young animals: Mean power spectrum density for all animals showing an increase in type 2 theta (4–6 Hz) in salicylate (red) compared to saline (gray). Boxplots showing the mean power was significantly different between saline and the salicylate group for 4–7 Hz oscillations (type 2 theta), $^p < .05$, $^*^p < .01$, $n=10$ mice. D. Young animals: Mean power spectrum density for all animals showing an increase in gamma (30–60 Hz) in salicylate (red) compared to saline (gray). Boxplots showing the mean power was significantly different between saline and the salicylate group for gamma oscillations, $^p < .05$, $^*^p < .01$, $n=10$ mice. E. Old animals: Mean power spectrum density for saline (gray) vs. salicylate (blue) for 4–7 Hz oscillations (type 2 theta). Boxplots showing the mean power for saline and salicylate groups for 4–7 Hz oscillation (type 2 theta), $^p < .05$, $^*^p < .01$. F. Old animals: Mean power spectrum density for saline (gray) vs. salicylate (blue) for 30–60 Hz oscillations. Boxplots showing the mean power for saline and salicylate groups for gamma oscillations, $^p < .05$, $^*^p < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4. Discussion

In this work, we show that anxiety-like behavior observed in animals acutely exposed to salicylate only occurs in animals naive to tinnitus. In addition, we describe that during anxiety both theta2 and slow gamma can serve as biomarkers encoding safety and danger, Fig. 5.
respectively. These two rhythms were augmented in both the vHipp and mPFC. However, only theta2 was coherent between these two regions.

Lastly, we demonstrate that a single, pre-salicylate, injection of 5-MeO-DMT provides resilience to the anxiety generate by tinnitus in the salicylate animal model.

Our GPAS experiments show that salicylate induced tinnitus do not show specific frequency responses across different subjects. Salicylate can cause a variety of types tinnitus, especially in relation to the center frequency of tinnitus (~7 kHz, Jastreboff et al., 1988; ~10 kHz, Guitton et al., 2003 and Zheng et al., 2006; ~15 kHz, Bauer et al., 1999; ~16 kHz, Yang et al., 2007, Kizawa et al., 2010 and Ralli et al., 2010; ~12, ~16, ~20 and ~24 kHz, Su et al., 2012; broadband noise-like, Rüttiger et al., 2003 and Turner and Parrish, 2008; or BBN-like 2 h after and 8–10 kHz 5 h after salicylate injection, Berger et al., 2013). Still there is variability in tinnitus-like behavior observed when different animals are subjected to the same acoustic trauma (noise-induced tinnitus), showing altered Gap-detection in response to different frequencies (Coomber et al., 2014). Therefore, it is not possible to predict in which frequency each animal will perceive tinnitus.

Salicylate poisoning is a known cause of tinnitus in humans (Pearlman and Gambhir, 2009; Puel and Guitton, 2007) but the drug has diverse effects in non-auditory neurons (Pearlman and Gambhir, 2009; Wu et al., 2015). Hence, anxiety-like behavior found in animals treated with salicylate could be a direct effect of the compound on limbic neurons. However, young animals subjected to acoustic trauma or older animals suffering of age-related hearing loss showed no signs of anxiety after salicylate injection. Besides, there was no difference in the c-Fos expression in the vHipp of young versus old animals following

![Diagram](image)

**Fig. 6.** Salicylate increases type 2 theta and gamma rhythms in the prelimbic cortex: A. left: Schematic drawing of the localization of electrodes in the prelimbic cortex (*). Right: Bright field image showing positioning of electrode in the PL cortex. B. Example of a LFP recording in the PL cortex from one animal after saline (top) and salicylate (bottom) injection. C. Mean power spectrum density for all animals showing an increase in type 2 theta (4–6 Hz) following salicylate (purple) compared to saline (gray) (n = 10) administration. Boxplots showing that mean power was significantly different between saline and the salicylate group for 4–7 Hz oscillations (type 2 theta), *p < .05, **p < .01. D. Mean power spectrum density for saline (gray) vs. salicylate (purple) for 30–60 Hz oscillations (gamma). Mean normalized power was significantly different between saline and the salicylate group for 4–6 Hz oscillations (type 2 theta), *p < .05, **p < .01. E. Mean power spectrum density for all animals showing an increase in type 2 theta (4–6 Hz) in closed arms (gray) compared to opened arms (purple) in salicylate group (n = 10). Boxplots showing that mean power was significantly different between closed arms and opened arms for 4–7 Hz oscillations (type 2 theta), *p < .05, **p < .01. Mean power spectrum density in the closed arms (gray) vs. opened arms (purple) for 30–60 Hz oscillations (gamma). Mean normalized power was significantly different between closed arms and the opened arms for 30–60 Hz oscillations (gamma) *p < .05, **p < .01.
salicylate injections. Old mice injected with salicylate, however, stayed longer in the center of the field and showed greater locomotion than salicylate-treated young mice. A recent study has shown that old and young normal male animals show no difference in performance in EPM and open-field tests (Nolte et al., 2019). Thus, it seems that previous tinnitus perception and/or hearing loss protect animals from anxiogenic effects of salicylate. Our data also strengthen the hypothesis that after salicylate injection, tinnitus perception rather than the drug itself cause anxiety.

Our previous work show that anxiety-like behavior elicits theta2 oscillation in the vHipp (Winne et al., 2019). Here, we show that the mPFC show coherent theta2 with vHipp. Other studies described synchrony between vHipp to mPFC is observed in anxiety-related and spatial representations of aversion (Adhikari et al., 2010; Padilla-Coreano et al., 2016). As a significant anxiety-like behavior was only observed in normal-hearing (young) animals, theta2 was not observed in old mice. Another important finding of this paper is the increase in slow gamma in both vHipp and mPFC when animals display anxiety-like behavior. Interestingly, we found that these two rhythms encode different states (theta2: safety/slow gamma: danger). We have previously suggested that theta2 could encode safety in anxiogenic situations (Mikulovic et al., 2018). Here, we not only confirm this idea but also describe another rhythm that is triggered by eminent danger.
gap detection and ABR data with input from KEL. EB participated in DMT data collection. JD and EB collected c-Fos data.

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**Declaration of Competing Interest**

The authors declare no conflict of interest.

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