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L-Theanine Prevents Long-Term Affective and Cognitive Side-Effects of Adolescent Δ-9-Tetrahydrocannabinol Exposure and Blocks Associated Molecular and Neuronal Abnormalities in the Mesocorticolimbic Circuitry

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ABSTRACT

Chronic adolescent exposure to Δ-9-Tetrahydrocannabinol (THC) is linked to elevated neuropsychiatric risk and induces neuronal, molecular and behavioural abnormalities resembling neuropsychiatric endophenotypes. Previous evidence has revealed that the mesocorticolimbic circuitry, including the prefrontal cortex (PFC) and mesolimbic dopamine (DA) pathway are particularly susceptible to THC-induced pathological alterations, including dysregulation of DAergic activity states, loss of PFC GABAergic inhibitory control and affective and cognitive abnormalities. There are currently limited pharmacological intervention strategies capable of preventing THC-induced neuropathological adaptations. L-theanine is an amino acid analogue of L-glutamate and L-glutamine derived from various plant sources, including green tea leaves. L-theanine has previously been shown to modulate levels of GABA, DA and glutamate in various neural regions and to possess neuroprotective properties. Using a pre-clinical model of adolescent THC exposure in male rats, we report that L-theanine pre-treatment prior to adolescent THC exposure is capable of preventing long-term, THC-induced dysregulation of both PFC and VTA DAergic activity states, a neuroprotective effect which persists into adulthood. In addition, pre-treatment with L-theanine blocked THC-induced downregulation of local GSK-3 and Akt signaling pathways directly in the PFC, two biomarkers previously associated with cannabis-related psychiatric risk and sub-cortical DAergic dysregulation. Finally, L-theanine powerfully blocked the development of both affective and cognitive abnormalities commonly associated with adolescent THC exposure, further demonstrating functional and long-term neuroprotective effects of L-theanine in the mesocorticolimbic system.

SIGNIFICANCE STATEMENT

With the increasing trend of cannabis legalization and consumption during adolescence, it is essential to expand knowledge on the potential effects of adolescent cannabis exposure on brain development and identify potential pharmacological strategies to minimize THC-induced neuropathology. Previous evidence demonstrates that adolescent THC exposure induces long-lasting affective and cognitive abnormalities, mesocorticolimbic dysregulation and schizophrenia-like molecular biomarkers that persist into adulthood. We demonstrate for the first time that L-theanine, an amino acid analogue of L-glutamate and L-glutamine, is capable of preventing long-term THC side-effects. L-theanine prevented development of THC-induced behavioral aberrations, blocked cortical downregulation of local GSK-3 and Akt signaling pathways and normalized dysregulation of both PFC and VTA DAergic activity, demonstrating powerful and functional neuroprotective effects against THC-induced developmental neuropathology.

63 **INTRODUCTION**

64 Previous evidence has shown that adolescent THC exposure increases long-term
65 vulnerability to various neuropsychiatric disorders, including schizophrenia, anxiety and
66 cognitive impairments (Andréasson et al., 1987; Arseneault et al., 2002; Weiser et al., 2002;
67 Zammit et al., 2002; Fergusson et al., 2003; Stefanis et al., 2004; Ferdinand et al., 2005; Malone
68 et al., 2010; Murray et al., 2017; Krebs et al., 2019). With increasing trends towards cannabis
69 legalization and adolescent consumption, it is critical to characterize the effects of adolescent
70 THC exposure on the developing brain. Moreover, there is an urgent need to identify potential
71 compounds capable of preventing the long-term effects of THC exposure on adolescent brain
72 development.

73 Given that THC is a partial CB1 receptor (CB1R) agonist and CB1R is enriched in the
74 mesocorticolimbic system (Herkenham et al., 1990), these neural circuits are particularly
75 vulnerable to THC during adolescent neurodevelopment. Translational research has revealed that
76 THC induces long-lasting dysregulation of PFC neuronal activity and oscillatory states
77 resembling schizophrenia-like endophenotypes. These effects include loss of GABAergic
78 inhibitory control of pyramidal neuron activity and hyperactive DAergic activity persisting into
79 adulthood (Renard et al., 2017b, 2017a, 2018). These disturbances are accompanied by profound
80 cortical molecular adaptations, resembling phenotypes observed in schizophrenia and mood
81 disorders, such as the loss of GSK-3α/β, Akt, p70S6K, mTOR and GAD67, all critical
82 biomarkers for increased neuropsychiatric risk (Alimohamad et al., 2005; Gururajan and Van
83 Den Buuse, 2014; Renard et al., 2017a, 2017b).

84 Translational animal models demonstrate a range of behavioural abnormalities following
85 adolescent THC exposure, resulting in sensory filtering deficits (Schneider and Koch, 2003;

Wegener and Koch, 2009; Rubino and Parolaro, 2016; Renard et al., 2017a), working memory impairments (Schneider and Koch, 2003; O'Shea et al., 2006; Rubino et al., 2009; Renard et al., 2013; Zamberletti et al., 2014), and social interaction and cognitive disturbances (O'Shea et al., 2006; Renard et al., 2017a). Furthermore, THC exposure is linked to affective dysregulation including heightened anxiety (Llorente-berzal et al., 2013; Renard et al., 2017a) and the development of anhedonia-like behaviours (Rubino et al., 2008; Bambico et al., 2010). Despite clinical and pre-clinical characterization of these phenotypes following adolescent THC exposure, there are limited adjunct pharmacological interventions that can mitigate or prevent these potential neuropathological and/or psychiatric side-effects (Murphy et al., 2017; Segal-Gavish et al., 2017; Cuccurazzu et al., 2018).

L-theanine, an amino acid analogue of L-glutamate and L-glutamine, possesses significant neuroprotective properties (Kakuda, 2011; Zukhurova et al., 2013; Chen et al., 2018). For example, L-theanine has been found to have ameliorative effects on positive and anxiety-related symptoms in schizophrenia (Ritsner et al., 2011; Wakabayashi et al., 2012; Ota et al., 2015). A significant improvement in positive and negative syndrome scale (PANSS) scores were observed in schizophrenia patients receiving adjunct L-theanine treatment with antipsychotic medications (Lardner, 2014). While the neurobiological mechanisms underlying the neuroprotective effects of L-theanine have not been clearly elucidated, it has been shown to normalize DA, serotonin and GABA signalling disturbances (Nathan et al., 2006; Lardner, 2014). In addition, due to its structural similarity to glutamate, it has been proposed to produce its therapeutic effects via modulation of glutamatergic dysfunction, critically involved in the pathophysiology of schizophrenia (Ota et al., 2015). Indeed, L-theanine has affinities for AMPA,

NMDA and kainate receptors (Kakuda et al., 2002) and has been shown to have long-term inhibitory effects on glutamate release (Kakuda et al., 2008).

In the present study, we hypothesized that L-theanine administration would mitigate the pathological effects of THC exposure during adolescent neurodevelopment, by preventing a battery of neuronal, molecular and behavioural pathophysiological sequelae linked to THC-related neuropsychiatric side-effects. Using an established rodent model of adolescent THC exposure and a combination of behavioural pharmacology, *in vivo* electrophysiology and localized molecular pathway analyses in the mesocorticolimbic circuitry, we report for the first time that L-theanine administration powerfully mitigates the long-term negative effects of THC exposure during vulnerable periods of adolescent brain development at the molecular, neuronal and behavioural levels of pathology.

MATERIAL AND METHODS

Animals and housing: Male Sprague-Dawley rats were obtained at PND 28 from Charles River Laboratories (Quebec, Canada). Rats were pair housed in controlled conditions (constant temperature and humidity, 12 h light/dark cycle) with free access to food and water. All procedures and protocols were approved by appropriate Governmental and Institutional guidelines.

Drugs: L-theanine (Cayman Chemical) was diluted in physiological saline. THC (Cayman Chemical) was dissolved in an ethanol, cremophor, and saline (1:1:18) dilution. Ethanol was evaporated using nitrogen gas to remove it from the final THC solution.

Adolescent THC exposure protocol: Our adolescent THC exposure protocol has been previously described (Renard et al., 2017a). Rats were treated twice daily from postnatal day (PND) 35 to

45 with L-theanine (10 mg/kg i.p.) or saline, 10 minutes prior i.p. injections of escalating doses of THC (2.5 mg/kg; Days 1–3; 5 mg/kg; Days 4–7; 10 mg/kg, Days 8–11) or vehicle. The protocol was adapted from the previous rodent studies in order to overcome CB1 receptor de-sensitization at chronic exposure phase and that was shown to produce profound and enduring neuropsychiatric phenotypes selectively during adolescent neurodevelopment, but not during adulthood (Renard et al., 2017a). The THC dosing range is designed to mimic the effects of a moderate to heavy use regiment of marijuana in human adolescent (Renard et al., 2017b, 2017a). All experimental procedures started after a 30-day-drug free washout period (PND 75).

Behavioral Assays:

Social Motivation and Cognition. Social interaction testing was performed as described previously (Renard et al., 2017a). 24 h before testing rats were habituated to a test arena for 13 min (5 min in the center + 8 min in the entire apparatus). The following day, rats were acclimated for 5 min and then tested for 2 consecutive 8 min phases. In phase 1, two wire cages, one empty and one containing an unfamiliar male rat, were placed in the apparatus. We measured the propensity of the rat to explore the stranger vs. the empty cage and calculated a social motivation index for each test rat (i.e. time spent with the stranger/total time exploring both rats x 100). In phase 2, a new unfamiliar rat was placed in the previously empty cage and the tested rat had a choice between the previously encountered rat versus the novel one. Time spent exploring rats in both chambers was analyzed and a social memory index was calculated for each test rat (i.e. time spent with the novel rat/total time exploring both rats, x 100). Locations of stranger and novel rats were randomly counterbalanced between trials. After each test, chambers were cleaned with ethanol to avoid olfactory cue bias. The test was videorecorded and analyzed offline with a video-tracking system (ANY-maze; Stoelting).

154 ***Light–Dark Box Test:*** The light-dark box test measures anxiety level, based upon the innate
155 aversion of rats to bright environments, as previously described (Renard et al., 2017a). The test
156 apparatus consisted of two separated 50×25×37 cm compartments, connected by 10×10 cm
157 opening. One compartment was black and covered with a black lid (the dark box), while the
158 other compartment was white with an open top and brightly illuminated by a lamp located 120
159 cm above the apparatus floor, providing 1500 lux at floor level. The test rat was allowed to freely
160 explore both compartments for a period of 8 min. We analyzed times spent in the dark vs. the
161 light box measuring total times in either environment, latency to entry for each environment and
162 number of transitions between environments. The test was videorecorded and analyzed offline
163 with a video-tracking system (ANY-maze; Stoelting).
164 ***Prepulse Inhibition of Startle Reflex:*** Startle and PPI testing was performed as previously
165 described (Renard et al., 2017a). Rats were placed in a startle chamber (Med Associates, USA)
166 for a 5 min acclimatization period over 3 days. On the last day of acclimatization, rats were
167 tested in an input/output (I/O) function consisting of 12 increasing startle pulses (from 65 to 120
168 dB, 5 dB increments) to determine the appropriate gain setting for each individual rat. The
169 testing paradigm consisted of three phases: acclimatization, habituation (Block 1), and PPI
170 measurement (Block 2). During acclimatization, rats were exposed to the chambers and white
171 background noise (68 dB) for 5 min. During Block 1, 10 pulse alone trials (110 dB white noise,
172 20 ms duration) were delivered at 15–20 s intervals. Block 2 consisted of 9 trials presented 10
173 times in a pseudorandomized order, and at 30 s intervals: 10 pulse-alone trials and 10 of each of
174 3 different prepulse–pulse trial types (72, 76, 80) with interstimulus intervals (ISI) of 100ms. PPI
175 was calculated for each animal and each trial condition as PPI (%) = (1 − average startle
176 amplitude to pulse with prepulse/average startle amplitude to pulse only) x 100.

Object recognition test: Rats were tested using the object recognition task as described previously (Renard et al., 2017b). The test sessions consisted of two 3-min trials with an intersession interval of 60 min. During the acquisition phase (trial 1), each rat was allowed to explore two identical objects placed 15 cm from the side wall. For the second trial, one of the objects was replaced with a novel one. The two objects and their location were randomized and counterbalanced. Object exploration was considered when rat was sniffing the object. The test was videorecorded and analyzed with a video-tracking system (ANY-maze; Stoelting). Exploration times were recorded and used to calculate object recognition index (time spent with novel object/total time exploring both objects)*100.

Sucrose preference test: The sucrose preference test is used to investigate anhedonia (i.e. inability to experience pleasure). Rats were given access in their home cage to two bottles, containing 2% sucrose solution or plain water, respectively, for a period of 4 days. The animals were not isolated, and the test was carried out in their home cages. No food and/or water restriction has been applied prior to the test and the position of two bottles was randomized daily to avoid any side bias. Water and sucrose intake were measured daily, and the sucrose preference index was calculated for each rat as the percentage of the volume of sucrose solution intake over the total volume of fluid intake averaged across the 4 testing days and across groups.

In vivo electrophysiology: Extracellular single unit recordings in the PFC and VTA and Local field potential (LFP) signals were carried out as described previously (Renard et al., 2017b, 2017a). At adulthood, THC or vehicle treated rats were anesthetized with urethane (1.3 g/kg, i.p.) and placed in a stereotaxic apparatus (KOPF instruments), with body temperature maintained at 37±1ºC by a heating pad. The scalp was retracted, and one burr hole was drilled above the targeted areas (PFC AP: +2.7 to +3.5 mm, L: ±0.8 to ±1 mm from bregma; VTA: AP: −5.0 to 5.2

mm, L: ±0.8 to ±1 mm from bregma), according to the Atlas of Paxinos and Watson (Paxinos and Watson, 2007). Single-unit activity of putative pyramidal neurons in the PFC (DV: −2.5 to −4 mm from the dural surface) and putative DA cells in the VTA (DV: −7 to −9 mm from the dural surface) were recorded extracellularly with glass microelectrodes (average impedance of 6–10 MΩ) filled with 0.5M sodium acetate solution containing 2% pontamine sky blue (Sigma-Aldrich).

Population spontaneous activity was determined in 4-6 predetermined recording tracks separated by 200 µm and the activity of each neuron was recorded for 5 min. Putative PFC pyramidal cells were identified according to previously established criteria: firing frequency < 10 Hz, waveform shape, and action potential duration > 2.5 ms. Cells exhibiting 3 consecutive spikes with inter-spike intervals < 45 ms were classified as burst-firing cells. Putative VTA DA neurons were identified according to previously established electrophysiological features for in vivo extracellular recordings: action potential width > 2.5 ms, spontaneous firing rate between 2–5 Hz, a triphasic waveform consisting of a notch on the rising phase followed by a delayed after potential, and a single irregular or burst firing pattern. Single-unit neuronal activity was filtered (bandpass 0.3–5 kHz) and individual action potentials were isolated and amplified (MultiClamp 700B amplifier, Molecular Devices), digitized at 25kHz and recorded using a Digidata 1440 A and pClamp software (Molecular Devices).

Local field potential (LFP) signals were analyzed using NeuroExplorer (Nex Technologies). LFP were decimated to 1 kHz, and lowpass filtered (IIR Butterworth filter at 170 Hz; filter order set to 3). Subsequently, a spectrogram function was used to calculate the power of oscillations at frequencies between 0–100 Hz (window length 2 s; shift 0.5 s). One-minute long recording epochs were used for estimating the average power spectrum distributions. Epochs were selected

such as either the desynchronized (relatively small signal amplitude and fast oscillations) or synchronized (relatively large signal amplitude with slow oscillations) cortical state could be easily distinguished. Power values for a given frequency were averaged over time of the recording epoch and normalized so that the sum of all power spectrum values equals 1. The total power was calculated by adding all the power values at frequencies between 0–59 and 61–100 Hz. Power values at 60 ± 1 Hz were excluded from all the calculations. Gamma band was defined as frequency between 30–80 Hz, as a result of two subcategories, low gamma: 30-59 Hz and high gamma: 61-80 Hz.

To perform histological analyses, at the end of recording sessions, DC current (20 mA for 15 min) was passed through the recording micropipette in order to mark the recording site with an iontophoretic deposit of pontamine sky blue dye.

Western blots. The Western blotting procedure was performed as previously described (Renard et al., 2017b, 2017a). At the conclusion of experiments, rats received an overdose of sodium pentobarbital (240 mg/kg, i.p., EuthanylTM) and brains were removed and flash frozen. Bilateral micro-punches of the PFC were obtained for protein isolation. Primary antibody dilutions were as follows: α-tubulin (1:120000; Sigma-Aldrich), phosphorylated GSK-3α/β ser21/9 (p-GSK-3α/β; 1:1000; Cell Signaling Technology), total GSK-3α/β ser21/9 (t-GSK-3α/β; 1:1000; Cell Signaling Technology), phosphorylated AKT-Thr308 (p-AKT-Thr308; 1:1000, Cell Signaling Technology), phosphorylated AKT-Ser473 (p-AKT-Ser473; 1:1000, Cell Signaling Technology), total AKT (t-AKT; 1:1000, Cell Signaling Technology). Species appropriate fluorophore-conjugated secondary antibodies (LI-COR IRDye 680RD and IRDye 800CW; Thermo Scientific) were used at a dilution of 1:10000. Membranes were scanned using a LI-COR Odyssey Infrared Imaging System and densitometry measurements were obtained using

Image Studio analysis software. Target protein bands were normalized to the intensity of the respective α-tubulin.

Statistical analysis. Averaged data from different experiments are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA) and SPSS (IBM). The data were analyzed using two-way ANOVA and two-way ANCOVA, where appropriate. *Post-hoc* analyses were calculated using Fisher's LSD. The significance level was established at $P<0.05$.

RESULTS

L-theanine prevents the development of adolescent THC-induced social interaction and memory deficits in adulthood

First, we examined the neuroprotective potential of L-theanine on aberrant social behavior at adulthood (PND 75) following adolescent THC exposure. Two-way ANOVA revealed that social motivation (**Fig. 1A**) was not affected of either adolescent treatment or interaction between factors, as the animals in all of the experimental groups showed preference toward compartment containing stranger rat vs. empty cage (VEH: n=8, THC: n=11, thea + THC: n=12, theanine: n=11; interaction: $F_{(1,38)} = 0.003$, THC: $F_{(1,38)} = 2.95$, theanine: $F_{(1,38)} = 0.05$, $P>0.05$ for all; **Fig. 1B**). On the contrary, there was a significant effect of adolescent drugs exposure and interaction between factors on the recall of social memory (phase 2 of the test) at adulthood (interaction: $F_{(1,38)} = 11.52$, $P=0.0016$, THC: $F_{(1,38)} = 8.86$, $P=0.0051$, theanine: $F_{(1,38)} = 6.77$, $P=0.0131$; **Fig. 1C**). *Post-hoc* analysis revealed that whereas THC-exposed rats spent less time exploring the novel rat vs. the previously encountered rat comparing to controls ($P<0.001$; **Fig. 1C**), this effect was fully prevented by co-exposure with L-theanine ($P<0.001$; **Fig. 1C**).

Moreover, THC rats showed a significant reduction of the recognition score when compared to theanine group ($P<0.001$; **Fig. 1C**).

L-theanine prevents the development of long-term adolescent THC-induced memory deficits

Acute or chronic THC exposure has been linked with significant deficits in short-term memory formation (Quinn et al., 2008). Thus, we next examined the effects of adolescent THC exposure on novel object memory formation (**Fig. 1D**). Consistent with previous reports (Zamberletti et al., 2014; Renard et al., 2017b), significant effects of adolescent treatment and interaction between factors were observed in short-term memory performance (VEH: n=10, THC: n=11, thea+THC: n=11, theanine: n=10; interaction: $F_{(1,38)} = 51.79$, THC: $F_{(1,38)} = 39.34$, theanine: $F_{(1,38)} = 18.55$, $P \leq 0.0001$ for all; two-way ANOVA; **Fig. 1E**). *Post-hoc* comparisons revealed short-term memory deficits in adult rats following THC exposure during adolescence when compared to vehicle ($P<0.001$) and theanine ($P<0.001$) groups (**Fig. 1E**). Co-administration of THC with L-theanine was able to restore the short-term memory deficits observed in THC-exposed rats ($P<0.001$; **Fig. 1E**).

L-theanine prevents the development of adolescent THC-induced anhedonic and anxiety-like behaviours

Another shared endophenotype of schizophrenia and chronic THC exposure is anhedonia, defined as a diminished ability to experience pleasure (Rubino et al., 2008). To examine anhedonic-like behaviours we used the sucrose preference test (**Fig. 1F**). Two-way ANOVA revealed a significant main effect of adolescent THC exposure in sucrose preference index

(VEH: n=8, THC: n=11, thea+THC: n=12, theanine: n=11; $F_{(1,38)} = 5.68$, P=0.0222; **Fig. 1G**). *Post-hoc* analyses revealed that while adolescent THC exposed rats showed decreased sucrose preference relative to VEH controls (P<0.05; **Fig. 1G**), this effect was blocked in rats receiving L-theanine pre-treatment (P<0.05; **Fig. 1G**). In addition, THC group exhibited a significant reduction when compared to theanine rats (P<0.01; **Fig. 1G**). While chronic THC exposure has previously been reported to influence feeding behaviours in rats (Rubino et al., 2008), we found that prior to commencing behavioural testing in adulthood, there were no significant body weight differences between experimental cohorts, suggesting that THC exposure likely had no impact on sucrose preference consumption behaviours (data not shown).

We next evaluated the potential effects of L-theanine on THC-induced anxiogenic behaviours using the light-dark box test (**Fig. 2A**). A main effect of THC treatment and interaction between factors were observed in time spent in the dark environment (VEH: n=10, THC: n=12, thea+THC: n=11, theanine: n=8; interaction: $F_{(1,37)} = 4.30$, P=0.0452, THC: $F_{(1,37)} = 6.12$, P=0.0181; two-way ANOVA; **Fig. 2B**), while a significant effect of adolescent THC exposure was found in latency to re-enter into the light environment ($F_{(1,37)} = 5.50$, P=0.0245; two-way ANOVA; **Fig. 2C**). *Post-hoc* analyses revealed that THC-treated rats spent more time in the dark side (P<0.01; **Fig. 2B**) and re-emerged later (P<0.05; **Fig. 2C**) when compared to VEH controls. Similarly, THC rats exhibited a significant anxiogenic behaviour in comparison with theanine group in time spent in the dark (P<0.05; **Fig. 2B**), as well as in second transition from dark to light (P<0.05; **Fig. 2C**). L-theanine prevented THC-induced anxiogenic effects in terms of times spent in the dark zone and in latency of dark/light environmental transitions (P<0.05 for both; **Fig. 2B, C**).

L-theanine prevents the development of adolescent THC-induced sensorimotor gating deficits in adulthood

Core deficits in sensorimotor filtering are found in both schizophrenia and following chronic THC exposure (Braff and Geyer, 1990; Renard et al., 2017a). Thus, we next used a paired-pulse inhibition procedure to measure this endophenotype (**Fig. 2D**). As shown in **figure 2E**, baseline startle amplitudes were not affected by adolescent treatment (VEH: n=8, THC: n=11, thea+THC: n=11, theanine: n=8; interaction: $F_{(1,34)} = 0.81$, THC: $F_{(1,34)} = 2.03$, theanine: $F_{(1,34)} = 0.03$, $P>0.05$ for all; two-way ANOVA). However, two-way ANOVA revealed a significant interaction between treatment and prepulse intensities ($F_{(9,102)} = 2.41$; $P=0.0160$; **Fig. 2F**) as well as a main effect of treatment ($F_{(3,34)} = 3.59$; $P=0.0235$) and prepulse intensities ($F_{(3,102)} = 32.92$; $P<0.0001$). *Post-hoc* comparisons revealed that whereas THC rats showed significant PPI deficits at prepulse intensity levels of 72 ($P<0.05$), 76 ($P<0.001$) and 80 dB ($P<0.05$), L-theanine treatment with THC was able to reverse THC-induced impairments at same intensity levels (THC vs thea+THC: 72 dB, $P<0.05$; 76 dB, $P<0.01$ and 80 dB, $P<0.05$; **Fig. 2F**). Moreover, THC rats showed a significant impairment at 76 dB, in comparison with theanine group ($P<0.05$; **Fig. 2F**).

L-theanine treatment normalizes THC-induced mesocorticolimbic neuronal aberrations

Dysregulation of cortical pyramidal neuron and sub-cortical DAergic neuronal activities are core endophenotypes associated with both schizophrenia and chronic THC exposure and are mechanistically linked to their effects on affective and cognitive abnormalities (Kambeitz et al., 2014; Renard et al., 2017b, 2017a). Given our above described findings showing that L-theanine + THC treatment effectively blocked THC-induced behavioural abnormalities, we next examined

337 if L-theanine may similarly block PFC or sub-cortical VTA DA neuronal dysregulations
338 following adolescent THC exposure.

339 Since multiple cells were recorded from each individual rat and considered as
340 independent observations, we assess the variability among subjects including their identity as
341 covariate in two-way ANCOVA. While statistical analysis did not reveal a significant effect of
342 subjects ($F_{(1,129)}$ = 1.95, P=0.165), sampling population of VTA revealed a main effect of
343 adolescent THC treatment and interaction between fixed factors (cells/rats: VEH: n=36/6, THC:
344 n=26/5, thea+THC: n=41/4, theanine: n=31/5; THC: $F_{(1,129)}$ = 5.23, P=0.024, interaction: $F_{(1,129)}$
345 = 4.39, P=0.038; two-way ANCOVA; **Fig. 3A**). *Post-hoc* comparisons showed an increase in
346 DA neuronal frequency in THC-treated rats compared with the vehicles (P<0.01; **Fig. 3A**) and
347 theanine by itself (P<0.05; **Fig. 3A**), while co-administration with L-theanine was able to
348 normalize this effect (P<0.05; **Fig. 3A**). Similarly, two-way ANCOVA revealed no effects of
349 subjects on bursting activity of putative VTA DA neurons ($F_{(1,129)}$ = 0.001, P=0.975), and a
350 significant interaction between theanine and THC treatments ($F_{(1,129)}$ = 14.89, P=0.000; **Fig. 3B**).
351 *Post-hoc* comparisons demonstrated that THC rats showed an increase in bursting activity when
352 compared with vehicle group (P<0.01 for both; **Fig. 3B**), which was not prevented by L-theanine
353 co-exposure (P>0.05; **Fig. 3B**). As shown in **fig. 3B**, adolescent treatment with L-theanine
354 increased DA burst firing when compared with vehicles (P<0.001; **Fig. 3B**) and theanine+THC
355 combined group (P<0.01; **Fig. 3B**). **Figure 3C** shows representative traces and rate histograms
356 of VTA DA neurons recorded from each treatment group.

357 Subsequently, we analyzed the spontaneous activity of putative pyramidal neurons and
358 LFP in PFC. The study of the whole population, including tonic and bursting cells, revealed
359 significant effects of subjects and treatments (cells/rats; VEH: n=45/7, THC: n=37/5, thea+THC:

n=74/6, theanine: n=40/5; rats: $F_{(1,191)}$ = 4.83, P=0.029, theanine: $F_{(1,191)}$ = 6.09, P=0.014, THC: $F_{(1,191)}$ = 5.94, P=0.016; two-way ANCOVA; **Fig. 3D**). However, *post-hoc* comparisons did not reveal any differences between groups.

When a more detailed analysis of bursting in PFC putative pyramidal neurons was performed, a notable effect of THC treatment, but not of the individual subjects, emerged (cells/rats: VEH: n=37/7, THC: n=31/5, thea+THC: n=57/6, theanine: n=20/5; rats: $F_{(1,140)}$ = 0.57, P=0.453, THC: $F_{(1,140)}$ = 8.08, P=0.005, two-way ANCOVA; **Fig. 3E**). *Post-hoc* comparisons indicated that bursting rate was enhanced in adolescent THC-exposed rat compared to the vehicle and theanine groups (P<0.001 for both; **Fig. 3E**), and co-administration with L-theanine fully prevented this hyperactive PFC bursting state (P<0.001; **Fig. 3E**). Traces and rate histograms of representative PFC pyramidal neurons recorded from each group are shown in **figure 3F**.

Aberrant gamma-band oscillations in the PFC are well-established functional correlates of both schizophrenia and chronic neurodevelopmental THC exposure (Williams and Boksa, 2010; Raver et al., 2013). Accordingly, we next analyzed PFC LFP recordings to examine potential effects on gamma-band oscillation patterns. Epochs of desynchronized (i.e. small-voltage <0.5 mV and fast oscillations) state were examined (**Fig. 4A**). Two-way ANCOVA did not reveal any effect of subjects ($F_{(1,72)}$ = 1.46, P=0.231). However, there was a significant interaction between THC and theanine treatment in gamma-band desynchronized activity, calculated as the sum of the frequency power values between 30 and 80 Hz (recording sites/rats: VEH: n=20/6, THC: n=17/3, thea+THC: n=23/4, theanine: n=17/4; $F_{(1,72)}$ = 8.73, P=0.004; **Fig. 4C**). *Post-hoc* analysis revealed that, during the desynchronized states, THC-rats showed a significant reduction in gamma oscillations when compared to the vehicle counterparts (P<0.05;

383 **Fig. 4C**), which was fully normalized by L-theanine pre-exposure (P<0.001; **Fig. 4C**). Moreover,
384 co-application of theanine and THC was yielding significantly higher gamma power scores than
385 theanine alone (P<0.05; **Fig. 4C**).

386 **L-theanine prevents the development of maladaptations in schizophrenia-related PFC**
387 **molecular biomarkers following adolescent THC exposure**

388 We next examined protein expression levels of select molecular signaling pathways
389 associated with both schizophrenia and neurodevelopmental THC exposure. Previous reports
390 have demonstrated a dysregulation of both the GSK-3α/β and AKT signaling pathways in post-
391 mortem cortical tissue samples of schizophrenic subjects (Emamian, 2012) as well as in adult
392 rats following THC adolescent exposure (Renard et al., 2017a), demonstrating their importance
393 as common neuropathological biomarkers. PFC Western blots measuring phosphorylated GSK-
394 3α (p-GSK-3α) revealed a significant interaction between adolescent THC and theanine
395 treatment (VEH: n=8, THC: n=7, thea+THC: n=8, theanine: n=8; $F_{(1,27)} = 8.03$, P=0.0086; two-
396 way ANOVA; **Fig. 5A**). *Post-hoc* comparisons revealed that THC exposure during adolescence
397 induced long-term reduction in p-GSK-3α expression level (P<0.05; **Fig. 5A**) and L-theanine
398 pre-treatment reversed this downregulation effect (P<0.05; **Fig. 5A**). Expression of total GSK-3α
399 was unaffected (n=8 for each group; interaction: $F_{(1,28)} = 0.85$, THC: $F_{(1,28)} = 0.10$, theanine: $F_{(1,28)}$
400 $= 0.59$, P>0.05 for all; two-way ANOVA; **Fig. 5A**). A significant interaction between factors
401 were observed in the ratio of p-GSK-3α/total GSK-3α expression (VEH: n=8, THC: n=7,
402 thea+THC: n=8, theanine: n=8; $F_{(1,27)} = 8.54$, P=0.0069; two-way ANOVA; **Fig. 5A**). *Post-hoc*
403 analysis revealed that the ratio of p-GSK-3α/total GSK-3α expression was reduced following
404 adolescent THC exposure (P<0.05; **Fig. 5A**) and prevented by the co-administration of L-
405 theanine (P<0.05; **Fig. 5A**).

Similarly, we found a significant interaction between THC and theanine treatment in p-GSK-3β expression level (VEH: n=8, THC: n=7, thea+THC: n=8, theanine: n=8; $F_{(1,27)}$ = 12.57, P=0.0015; two-way ANOVA; **Fig. 5A**). *Post-hoc* analysis revealed that THC administration during adolescence reduced PFC p-GSK-3β expression (P<0.05; **Fig. 5A**), whereas L-theanine treatment prevented this effect (P<0.001; **Fig. 5A**). Moreover, PFC p-GSK-3β expression was significantly higher in rats exposed to the combination of theanine and THC, in comparisons with theanine alone group (P<0.05; **Fig. 5A**). Total GSK-3β PFC expression was not significantly affected by adolescent treatment (n=8 for each group; interaction: $F_{(1,28)}$ = 0.83, THC: $F_{(1,28)}$ = 0.16, theanine: $F_{(1,28)}$ = 0.49, P>0.05 for all; two-way ANOVA; **Fig. 5A**). A significant interaction between treatments was observed in the ratio of p-GSK-3β/total GSK-3β expression (VEH: n=8, THC: n=7, thea+THC: n=8, theanine: n=8; $F_{(1,27)}$ = 8.04, P=0.0086; two-way ANOVA; **Fig. 5A**). *Post-hoc* analysis revealed a significant increase following the co-exposure of theanine and THC in comparison with THC-treated (P<0.01; **Fig. 5A**) and theanine alone (P<0.05; **Fig. 5A**) groups. Analysis of PFC AKT expression patterns revealed a significant interaction between factors in phosphorylated AKT-Thr308 (p-AKT-Thr308) (VEH: n=8, THC: n=7, thea+THC: n=8, theanine: n=8; $F_{(1,27)}$ = 9.14, P=0.0054; two-way ANOVA; **Fig. 5B**). *Post-hoc* comparisons revealed that THC exposure strongly reduced PFC p-AKT-Thr308 expression relative to VEH controls (P<0.01; **Fig. 5B**). Moreover, theanine group showed a decrease in p-AKT-Thr308 expression level when compared to controls (P<0.05; **Fig. 5B**). Interaction between theanine and THC was observed in the ratio of p-AKT-Thr308/total AKT-Thr308 expression ($F_{(1,27)}$ = 6.87, P=0.0143; two-way ANOVA; **Fig. 5B**). *Post-hoc* comparison revealed that the ratio of p-AKT-Thr308/total AKT-Thr308 expression was significantly reduced following THC exposure during adolescence (P<0.05; **Fig. 5B**). Total Akt protein expression level was not

affected by the treatment (n=8 for each group; interaction: $F_{(1,28)} = 0.01$, THC: $F_{(1,28)} = 0.21$, theanine: $F_{(1,28)} = 0.12$, $P>0.05$ for all; two-way ANOVA; **Fig. 5B**). Moreover, no difference was observed in phosphorylated AKT-Thr473 (p-AKT-Thr473) expression level (n=8 for each group; interaction: $F_{(1,28)} = 0.28$, THC: $F_{(1,28)} = 0.33$, theanine: $F_{(1,28)} = 1.30$, $P>0.05$ for all; two-way ANOVA; **Fig. 5B**) as well as in the ratio of p-AKT-Thr473/total AKT-Thr473 expression (interaction: $F_{(1,28)} = 0.60$, THC: $F_{(1,28)} = 0.35$, theanine: $F_{(1,28)} = 0.47$, $P>0.05$ for all; two-way ANOVA; **Fig. 5B**).

DISCUSSION

Clinical and pre-clinical evidence has demonstrated that adolescent THC exposure induces a wide range of neuropsychiatric side-effects including psychotomimetic, mood/anxiety-related disturbances and cognitive deficits (Rubino et al., 2008; Llorente-berzal et al., 2013; Renard et al., 2013, 2017a; Rubino and Parolaro, 2016). However, there are currently limited adjunct pharmacological treatments capable of preventing these long-term side-effects (Murphy et al., 2017; Segal-Gavish et al., 2017; Cuccurazzu et al., 2018). Our findings demonstrate for the first time, that administration of L-theanine, prior to THC exposure, can powerfully prevent a host of well-established neuronal, behavioural and molecular biomarkers in the mesocorticolimbic circuitry, associated with THC-induced neuropathophysiology.

In line with previous reports (Renard et al., 2017a, 2017b), adolescent THC exposure induced a range of long-term behavioural symptoms resembling neuropsychiatric phenotypes, including deficits in social recognition and short-term memory, anhedonia, sensorimotor gating impairments and increased anxiety. Remarkably, L-theanine significantly normalized all these THC-related neurodevelopmental behavioural phenotypes into adulthood. Previously, acute L-

theanine exposure has been found to ameliorate select cognitive deficits and to have pro-attentional effects independently of cannabis-related phenomena. For example, Tamano *et al*. (Tamano et al., 2013) found that acute stress-induced object recognition memory impairments and associated hippocampal synaptic plasticity deficits could be reversed by L-theanine administration in rats. Social interaction memory deficits are cardinal features of schizophrenia-related disorders and episodic deficits in memories for social interactions are perturbed during all phases of the illness (Lee et al., 2018). Consistent with previous reports (Renard et al., 2017a), we observed significant social memory deficits following adolescent THC exposure. Similar to object recognition deficits, these social cognition impairments were completely reversed by L-theanine administration. While no previous reports have examined the effects of L-theanine specifically on social memory processing, a previous report (Park et al., 2011) demonstrated that L-theanine administration significantly improved semantic and episodic memory indices in patients with mild cognitive impairments, an effect that correlated with increased theta wave activity in several neural regions, including the frontal cortices.

Consistent with clinical and pre-clinical findings (Rubino et al., 2008; Bambico et al., 2010; Renard et al., 2017a), we observed long-term anhedonia and increased anxiety-like behaviours following adolescent THC exposure. Remarkably, L-theanine blocked both anhedonic and anxiety-related phenotypes induced by adolescent THC. Previous evidence has suggested that acute L-theanine may possess both mood-enhancing and anxiolytic effects (Ritsner et al., 2011; Yin et al., 2011; Lardner, 2014; Ota et al., 2015). Moreover, anti-depressant-like effects of L-theanine have been demonstrated in the forced swim and tail suspension tests in pre-clinical studies (Yin et al., 2011; Wakabayashi et al., 2012). Pre-clinical studies have also found that the acute anti-depressant and anxiolytic effects of L-theanine may be

related to its ability to increase levels of DA and serotonin, directly within the mesocorticolimbic circuitry (Shen et al., 2019). Furthermore, long-term administration of L-theanine has been found to reduce anxiety-related symptoms in schizophrenia, as measured by the Hamilton anxiety rating scale (HARS) (Ritsner et al., 2011; Lardner, 2014; Ota et al., 2015).

While the precise mechanisms underlying adolescent THC-induced anxiogenic and anhedonic-like effects are not entirely understood, previous evidence has demonstrated that acute activation of CB1Rs in the rat PFC and modulation of PFC neuronal firing and bursting rates can strongly modulate fear-related memory formation and sensitivity to fear-related cues (Laviolette and Grace, 2006; Tan et al., 2011; Draycott et al., 2014). CB1R-mediated potentiation of fear-related memory encoding depends upon a pathological amplification of PFC neuron associative bursting rates (Laviolette and Grace, 2006; Tan et al., 2011). In addition, intra-PFC CB1R activation has been shown to potentiate the anxiogenic properties of associative footshock via hyper-activating sub-cortical VTA DA neuron firing and bursting rates (Draycott et al., 2014). While the present study used long-term, neurodevelopmental protocols for THC and L-theanine exposure, our finding that L-theanine prevented the effects of chronic CB1R stimulation (via adolescent THC exposure) on inducing PFC and VTA-related neuronal pathologies, may suggest a mechanism by which L-theanine may block longer term dysregulation of anxiety and mood-related phenotypes via normalization of PFC/VTA-related neuronal dysregulation.

The inability to filtered-out irrelevant sensory stimuli, demonstrated by deficits in sensorimotor gating, represents a crucial endophenotype of schizophrenia (Braff and Geyer, 1990). The PFC, via complex interactions with brainstem circuitry, is a critical component of normal PPI behavioural function (Swerdlow et al., 2001). As previously reported (Renard et al., 2017a), adolescent THC exposure significantly impairs sensorimotor gating in adulthood. We

found that L-theanine fully prevented this THC-induced sensory gating deficit. While the precise mechanisms underlying THC-induced PPI deficits are not currently understood, normal gamma-oscillation activity in the sensory cortex is necessary for effective sensorimotor gating (Cheng et al., 2016). Given that L-theanine was able to prevent the development of PFC-related gamma-oscillation disturbances, this effect may relate to theanine's preventative effects on THC-induced cognitive filtering impairments. Moreover, both the Akt and GSK-3 PFC signaling pathways are crucial for regulating normal sensorimotor gating behaviours. For example, Kapfhamer *et al*. (Kapfhamer et al., 2010) reported that GSK-3 signaling was critical for modulating M-type potassium channel function on PFC pyramidal neuron activity during PPI behavioural processing, such that inhibition of GSK-3 signaling led to substantial PPI deficits. In addition, Chen and Lai (Chen and Lai, 2011) reported that genetic knockdown of Akt1 caused profound impairments in PPI behaviours which were reversed with GSK-3 inhibition, further underscoring the importance of these pathways in sensorimotor gating. We observed profound reductions in phosphorylated levels of PFC GSK-3α/β and Akt-Thr308 levels following adolescent THC exposure, concomitant with dysregulated PFC neuronal activity states. While future studies are required to examine the causal mechanisms associated with these molecular adaptations, the ability of L-theanine to prevent these THC-induced molecular and neuronal phenotypes may underlie the normalization of long-term sensorimotor gating impairments. Interestingly, a previous report demonstrated that a single administration of L-theanine was able to ameliorate PPI impairments induced by MK-801 (Wakabayashi et al., 2012), suggesting that the potential benefits of L-theanine on THC-induced cognitive impairments may extend beyond cannabinoid-related signaling mechanisms.

Cognitive impairments associated with schizophrenia have been correlated with abnormal cortical oscillation patterns, which are crucial for the normal coordination of excitatory vs. inhibitory neural elements within many neural circuits, including the mesocorticolimbic system (Uhlhaas and Singer, 2010). Clinical and preclinical studies have described dysregulation of cortical gamma-band frequencies linked to schizophrenia-related cognitive abnormalities (Uhlhaas and Singer, 2010; Williams and Boksa, 2010; Raver et al., 2013). In the present study, we observed a persistent dysregulation in gamma-band (frequency powers between 30-80 Hz) in desynchronized states, in the PFC of adolescent THC-exposed rats. Interestingly, L-theanine administration was able to prevent these THC-induced oscillatory disturbances. While we are not aware of any previous studies examining the role of L-theanine in cortical gamma-oscillatory regulation, the effects of L-theanine may relate to the normalization of THC-induced pyramidal neuron bursting disturbances and concomitant normalization of GABAergic/glutamatergic signaling disturbances following adolescent THC exposure. For example, acute activation of PFC CB1R transmission has been reported to cause profound disturbances in associative emotional memory formation by causing abnormal associative bursting activity states in PFC neuronal populations (Laviolette and Grace, 2006; Tan et al., 2010), likely due to a loss of normal GABAergic inhibition of PFC pyramidal output neurons. Moreover, adolescent THC exposure causes long-term loss of GABAergic molecular markers directly in the PFC and a concomitant increase in PFC pyramidal neuron bursting levels (Renard et al., 2017b). Other neurodevelopmental animal models of schizophrenia have shown that reduced cortical density of parvalbumin-positive interneurons was associated with a reduction in gamma-band activity patterns (Lodge et al., 2009). Given our findings that L-theanine prevented THC-induced gamma-band disturbances as well as blocked the development of long-term hyper-bursting levels

in the PFC, one possibility is that L-theanine prevents THC-induced disruptions in inhibitory/excitatory balance within the PFC by its ability to prevent sub-cortical overdrive of DA signals to the PFC.

Such a mechanism is supported by the present molecular findings in the PFC Akt-GSK signaling pathway. For example, both phosphorylated Akt and GSK-3 are strongly reduced in post-mortem cortical schizophrenia tissue (Emamian, 2012) as well as in the rat PFC following chronic adolescent THC exposure (Renard et al., 2017a). Changes in cortical GSK-3α/β and AKT, particularly in Thr308, are associated with hyperdopaminergic states, as observed following adolescent THC exposure (Renard et al., 2017b, 2017a). Moreover, PFC GSK-3α/β and Akt is reduced following chronic DA D2 receptor activation (Sutton and Rushlow, 2012), consistent with sub-cortical overdrive of VTA DAergic signals observed in the present study and the ability of L-theanine to prevent this hyperactive DA signal. Importantly, adolescent THC exposure induced a profound reduction in p-GSK-3α/β expression and in the ratio of both (p-GSK-3α/total p-GSK-3α and p-GSK-3β/total GSK-3β) as well as in p-Akt and its ratio, exclusively at Thr308. The selective effects of L-theanine at Akt Thr308 is of particular translational significance given that genetic biomarkers associated with Akt-Thr308 are directly linked to increased risk of cannabis-related psychosis vulnerability (Di Forti et al., 2012). Finally, L-theanine protects against DA-dependent neurotoxic effects *in vitro*. For example, L-theanine blocked excess DA-related toxicity in cultured mesencephalic neurons by increasing glutathione levels in surrounding astrocytes (Takeshima et al., 2016). While the present studies were performed *in vivo*, such evidence may suggest that L-theanine might similarly protect against DAergic toxicity in frontal cortical regions through similar modulation of astrocytic populations. Future studies are required to further explore these possibilities.

In conclusion, we report a novel, neuroprotective role for L-theanine in mitigating the neuropsychiatric side-effects of chronic adolescent THC exposure. The range of neuroprotective effects induced by L-theanine were remarkable not only for their persistence beyond the adolescent THC exposure period, but for the comprehensive nature of its protective effects. These benefits extended beyond the prevention of THC-induced affective and cognitive disturbances and included the prevention of long-term molecular and neuronal adaptations within the PFC and a concomitant normalization of sub-cortical DAergic abnormalities.

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774 **Figure legends**

775 **Figure 1.** Effects of L-theanine pre-exposure on social, cognitive and depressive aberrations
776 induced by adolescent THC exposure. **(A)** Schematic representation of the social interaction
777 apparatus. Chronic THC exposure did not influence social motivation **(B)** but induced significant
778 deficits in social memory **(C)**. L-theanine prevents the THC-induced impairments in social
779 recognition (n=8 vehicle, n=11 THC, n=12 thea + THC, n=11 theanine; Two-way ANOVA,
780 Post-hoc Fisher's LSD; ***p<0.001). **(D)** Schematic protocol representation for object memory
781 test. **(E)** L-theanine prevented THC-induced short-term memory impairments (n=10 vehicle,
782 n=11 THC, n=11 thea + THC, n=10 theanine; Two-way ANOVA, Post-hoc Fisher's LSD;
783 ***p<0.001). **(F)** Schematic representation of the two-bottle sucrose preference test. **(G)** L-
784 theanine prevent THC-induced anhedonia effects (n=8 vehicle, n=11 THC, n=12 thea + THC,
785 n=11 theanine; Two-way ANOVA, Post-hoc Fisher's LSD; *p<0.05, **p<0.01).

786 **Figure 2.** Effects of L-theanine pre-exposure on affective and sensorimotor gating impairments
787 induced by adolescent THC exposure. **(A)** Schematic representation of light-dark box anxiety
788 test. Adolescent THC-treated rats spent more time in the dark **(B)**, and re-emerged later **(C)**,
789 indicating increased anxiety levels. L-theanine administration partially prevents long-term THC-
790 induced anxiety (n=10 vehicle, n=12 THC, n=11 thea + THC, n=8 theanine; Two-way ANOVA,
791 Post-hoc Fisher's LSD; *p<0.05, **p<0.01). **(D)** Schematic representation of PPI apparatus set-
792 up. **(E)** Average startle amplitude response was not affected by any treatment. **(F)** Comparing to
793 vehicle controls, THC-treated rats exhibited impairments in sensory motor gating, which were
794 fully prevented by L-theanine (n=8 vehicle, n=11 THC, n=11 thea + THC, n=8 theanine; Two-
795 way repeated measures ANOVA, Post-hoc Fisher's LSD; *p<0.05, **p<0.01, ***p<0.001).

796 **Figure 3.** Effects of L-theanine exposure on adolescent THC-induced alterations of VTA DA
797 and PFC pyramidal activity. **(A)** L-theanine prevents the sub-cortical hyperdopaminergic state
798 induced by THC in terms of firing frequency. **(B)** THC-treated rats exhibited an increase in
799 bursting activity, which was not prevented by L-theanine+THC or L-theanine alone. Rats
800 exposed to L-theanine alone showed a higher bursting activity when compared with the other
801 groups (n=36 cells from 6 vehicle rats, n=26 cells from 5 THC rats, n=41 cells from 4 thea +
802 THC rats, n=31 cells from 5 theanine rats; Two-way ANCOVA, Post-hoc Fisher's LSD;
803 *p<0.05, **p<0.01, ***p<0.001). **(C)** Traces and rate histograms of representative VTA DA
804 neurons recorded from each group. **(D)** The firing rate of spontaneous PFC putative pyramidal
805 neurons was not altered by adolescent THC treatment (n=45 cells from 7 vehicle rats, n=37 cells
806 from 5 THC rats, n=74 cells from 6 thea + THC rats, n=40 cells from 5 theanine rats; Two-way
807 ANCOVA, Post-hoc Fisher's LSD; p>0.05). **(E)** However, analysis of bursting activity revealed
808 that L-theanine significantly prevented hyper-bursting phenotypes induced by adolescent THC
809 (n=37 cells from 7 vehicle rats, n=31 cells from 5 THC rats, n=57 cells from 6 thea + THC rats,
810 n=20 cells from 5 theanine rats; Two-way ANCOVA, Post-hoc Fisher's LSD; ***p<0.001). **(F)**
811 Traces and rate histograms of representative PFC pyramidal neurons recorded from each group.

812 **Figure 4.** Effect of L-theanine on THC-induced abnormalities in spontaneous cortical gamma
813 oscillations (30-80 Hz). **(A)** Representative spectrogram of a five-minutes recording during
814 desynchronized state, characterized by small-amplitude fast oscillations (trace on the top). LFP
815 power values between 59-61 Hz were excluded since they were reflecting the power line
816 frequency. **(B)** Average normalized power spectra representing LFP during the desynchronized
817 state in prefrontal cortex of the four experimental groups. The THC-treated group displayed a
818 decrease in total gamma oscillations (30-80 Hz) during the desynchronized state. L-theanine

prevented THC-induced abnormalities in prefrontal LFP gamma oscillations. **(C)** Group summary comparing LFP power following adolescent treatments. The THC-induced reduction in the total power of gamma oscillations in desynchronized state was fully prevented by pre-exposure to L-theanine (n=20 recording sites from 6 vehicle rats, n=17 recording sites from 3 THC rats, n=23 recording sites from 4 thea + THC rats, n=17 recording sites from 4 theanine rats; Two-way ANCOVA, Post-hoc Fisher's LSD; *p<0.05, ***p<0.001).

Figure 5. Effects of L-theanine on the expression of THC-induced schizophrenia-like abnormalities in molecular biomarkers. **(A)** Representative Western blots for phosphorylated and total GSK-3α and β expression (left) in the PFC. Densitometry analysis revealed that L-theanine reverts THC-induced decreases in both GSK-3 isoforms, α and β, and in the ratio of phosphorylated to total GSK-3 α and β (n=8 vehicle, n=7 THC, n=8 thea + THC, n=8 theanine; Two-way ANOVA, Post-hoc Fisher's LSD; *p<0.05, **p<0.01, ***p<0.001). No differences were detected in total GKS-3α and β (n=8 for each group). **(B)** Representative Western blots for phosphorylated and total AKT expression in PFC (top panel). L-theanine co-administration normalized reductions in phosphorylated AKT-Thr308 and in the ratio of phosphorylated to total AKT-Thr308 induced by THC exposure during adolescence (n=8 vehicle, n=7 THC, n=8 thea + THC, n=8 theanine; Two-way ANOVA, Post-hoc Fisher's LSD; *p<0.05, **p<0.01). No significant changes in phosphorylated AKT-Ser473 and in the ratio of phosphorylated to total AKT-Ser473 as well as in total AKT were detected (n=8 for each group).