Original Article

Sustained NMDA receptor hypofunction impairs brain-derived neurotropic factor (BDNF) signalling in the prefrontal cortex (PFC), but not in the hippocampus, and disturbs PFC-dependent cognition in mice

Running head: BDNF function is lost in PFC of PCP-treated mice

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Abstract

Background: Cognitive deficits profoundly impact on the quality of life of patients with schizophrenia. Alterations in Brain Derived Neurotrophic Factor (BDNF) signalling, which regulates synaptic function through the activation of full-length tropomyosin-related kinase B receptors (TrkB-FL), are implicated in the aetiology of schizophrenia, as is NMDA receptor (NMDA-R) hypofunction. However, whether NMDA-R hypofunction contributes to the disrupted BDNF signalling seen in patients remains unknown.

Aims: The purpose of this study was to characterise BDNF signalling and function in a preclinical rodent model relevant to schizophrenia induced by prolonged NMDA-R hypofunction.

Methods: Using the subchronic phencyclidine (PCP) model, we performed electrophysiology approaches, molecular characterisation and behavioural analysis.

Results: The data showed that prolonged NMDA-R antagonism, induced by subchronic PCP treatment, impairs long-term potentiation (LTP) and the facilitatory effect of BDNF upon LTP in the medial prefrontal cortex (mPFC) of adult mice. In addition, TrkB-FL receptor expression is decreased in the PFC of these animals. By contrast, these changes were not present in the hippocampus of PCP-treated mice. Moreover, BDNF levels were not altered in the hippocampus or PFC of PCP-treated mice. Interestingly, these observations are paralleled by impaired performance in PFC-dependent cognitive tests in mice treated with PCP.

Conclusions: Overall, these data suggest that NMDA-R hypofunction induces dysfunctional BDNF signalling in the PFC, but not in the hippocampus, which may contribute to the PFC-dependent cognitive deficits seen in the subchronic PCP model. In addition, the data suggest that targeting BDNF signalling may be a mechanism to improve PFC-dependent cognitive dysfunction in schizophrenia.

Keywords

BDNF, cognitive deficits, PFC, Phencyclidine, TrkB-FL

Introduction

Schizophrenia is characterized by positive and negative symptoms and pronounced cognitive deficits (Marder and Cannon, 2019). The drugs currently used relieve the positive symptoms (Huhn et al., 2019) but have limited efficacy for the negative symptoms and cognitive deficits (Fusar-Poli et al., 2015; Krause et al., 2018). Thus, there is an urgent need to understand the mechanisms contributing to these symptoms and for new effective treatments.

A key role for glutamatergic dysfunction in schizophrenia is supported. In particular, there is evidence for N-methyl-D-aspartate receptor (NMDA-R) hypofunction in the prefrontal cortex (PFC) and hippocampus, brain regions dysfunctional in the disorder (Dauvermann et al., 2017; Lee and Zhou, 2019). Furthermore, prolonged NMDA-R hypofunction induced by subchronic PCP administration in rodents induces cognitive, behavioural and neurochemical alterations, along with deficits in PFC metabolism ("hypofrontality") and brain network connectivity, that have translational relevance to those seen in patients (Cochran et al., 2003; Egerton et al., 2008; Dawson et al., 2012, 2014; Neill et al., 2014).

BDNF is a neurotrophic factor that, through the activation of the full length kinase receptor B (TrkB-FL), promotes neuronal survival, differentiation, and synaptic plasticity (Lee et al., 2001; Chao, 2003). Truncated isoforms of the receptor (TrkB-TC) act to limit BDNF signaling (Dorsey et al., 2006; Eide et al., 1996). Alterations in BDNF signaling are implicated in schizophrenia pathogenesis (Nieto et al., 2013; Palomino et al., 2006). Reduced BDNF and TrkB-

FL expression, and increased TrkB-TC expression, in the PFC (Weickert et al., 2005; Wong et al., 2013) and hippocampus (Durany et al., 2001; Iritani et al., 2003; Thompson Ray, 2011) of patients support reduced BDNF signalling in the disorder (Sigurdsson and Duvarci, 2015). Interestingly, serum BDNF levels are also positively associated with cognitive function in patients (Ahmed et al., 2015) and a functional single-nucleotide polymorphism (rs6265) in the *BDNF* gene is linked to neurocognitive deficits in patients(Egan et al., 2003). Overall, the data suggests that BDNF signalling may play a key role in the cognitive deficits seen in the disorder. Evidence from relevant animal models also supports a role for disrupted BDNF signalling in the disorder (Faatehi et al., 2019; Fiore et al., 2004; Pillai and Mahadik, 2008). Despite these observations the potential contribution of sustained NMDA-R hypofunction to the disturbed BDNF signalling seen in schizophrenia remains to be adequately defined. Thus, here we characterize the molecular and functional deficits in BDNF signalling seen in the PFC and hippocampus of the subchronic PCP mouse model.

Methods

Animals

Male (2-3 months old) C57BL/6 mice (Charles River, Spain) were used. All procedures were carried out under European Community Guidelines (Directive, 2010/63/EU) and the Portuguese law (DL 113/2013) for Animal Care for Research Purposes. Animals were tagged and distributed in groups of five animals to each housing cage, kept in a controlled environment (14-10 h light/dark cycle, 45–65% humidity and 22-24 °C room temperature, RT) with food and water provided *ad libitum*. To determine allocation to either the control or experimental treatment group a pseudo-randomization procedure was employed. Animals were habituated to the presence of the investigator and handled for 5-days before testing. Animal behaviour experiments were performed during the light phase and around the same time each day.

Drug Treatments and Experimental Timeline

Animals treated subchronically with PCP received 10 mg/kg PCP.HCl (2 ml/kg injection volume) (Tocris, UK, in 0.9% w/v sterile saline, *intraperitoneally*, *i.p.*) over 12 days (one daily administration on days 1-5 and 8-12, with no treatment on days 6 and 7). Controls received saline treatment with the same protocol. The PCP dosing regimen was based on published work showing that this induces cognitive, behavioural and neurochemical alterations in mice relevant to schizophrenia, that last for at least 7 days after the final PCP administration (Hashimoto et al., 2007, 2008; Fujita et al., 2008; Tanibuchi et al., 2009; Santini et al., 2013). Thus, to make sure that all primary endpoint measures of interest were taken between 3-5 days after the final PCP injection, we performed the Morris Water Maze Test (MWMT) and other behavioural tests in two separate cohorts of animals. Therefore, the Open Field Test (OFT), Novel Object Recognition

Test (NORT) and Y-Maze Spontaneous Alternation Test (YMT) were conducted in one cohort of animals and the MWMT was performed in a separate cohort of animals. This also ensures that the results obtained in the OFT, NORT and YMT are not influenced by the stress induced by the MWMT.

The aim of the present study was to determine the primary endpoint measures of interest in the behavioural tests, and electrophysiology measurements, at around 72 hours after the final PCP administration, where the conformational changes induced by the drug are more prominent than its acute effects. This timepoint aligns with that measured in a broad base of published literature utilising subchronic PCP administration in both mice (Hashimoto et al., 2007, 2008; Fujita et al., 2008; Tanibuchi et al., 2009; Santini et al., 2013) and also in rats, using lower PCP doses (Cochran et al., 2003; Egerton et al., 2008; Dawson et al., 2012). The available pharmacokinetic information on repeated PCP administration in mice shows that animals are indeed "drug-free" at this timepoint. Nabeshima *et al*., 1987 reported the half-life of PCP in brain tissue in mice, at the end of a repeated dosing schedule (10 mg/kg, i.p., 14 days of treatment), to be approximately 30 minutes. Thus, this is the expected half-life of PCP in the brain associated with an *i.p*. injection of the same PCP dose used in our study in mice. Therefore, at 72 hours after the final PCP injection (approximately 142 half-lives), when we take the primary measures of interest in our study, the brain concentration of PCP in our treated mice would be negligible. Similar information supports subchronic PCP-treated rats being drug-free at this timepoint, albeit with the lower PCP doses applied (Kalinichev et al., 2008). In accordance with this, we aligned the test phase of NORT and the probe trial of the MWMT to be completed 72 hours after the final PCP administration. This meant that some aspects of the training for these tests must overlap with part of the PCP administration protocol. The PCP treatment and testing protocol

employed ensures that animals were not under the acute effects of PCP administration during behavioural testing for the primary end-point measures (the training and test phase of the NORT and MWMT probe trial). However, during the initial training phase of the MWMT, the habituation phase of the NORT and the OFT (no alterations detected), PCP treatment was ongoing. To ensure that animals were not under the effects of acute PCP treatment at these time points behavioural measurements were all conducted at least 20h after the previous PCP injection.

The OFT was conducted on day 11. The probe trial of the MWMT was conducted on day 15, 3 days after the final PCP administration. The test phase of the NORT and YMT were conducted on day 16, 4 days after the final PCP administration. For behavioural analysis 10-12 PCP and 9-10 saline-treated mice were used.

Electrophysiological and molecular characterisation was performed during the same time interval. However, a separate cohort of animals, 16 PCP-treated (10, hippocampus; 6, mPFC) and 20 controls (10, hippocampus; 10, mPFC) was used to prevent the potential impact of animal manipulation and behavioural testing on electrophysiology. The experimental design is detailed in Figure 1.

[insert Figure 1.]

Ex Vivo Electrophysiology Recordings in medial prefrontal cortex

Animals treated with saline or PCP were sacrificed by decapitation under deep isoflurane (Esteve, Barcelona, Spain) anaesthesia, and the brain was rapidly removed from the skull and submerged in ice-cold dissecting solution containing: 110 mM sucrose; 2.5 mM KCl; 0.5 mM $CaCl_2$; 7 mM $MgCl_2$; 25 mM $NaHCO_3$; 1.25 mM NaH_2PO_4; 7 mM glucose, pH 7.4, gassed with 95% O_2 and 5% CO_2. For the electrophysiology in the medial prefrontal cortex (mPFC), coronal slices

(300 µm thick) were cut with a Vibratome (VT 1000S; Leica, Nussloch, Germany). The slices were then transferred to a pre-chamber containing artificial cerebrospinal fluid (aCSF) (124 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose, pH 7.4), maintained for 30 min at 35°C with constant oxygenation. After recovery for at least 1 h at RT, individual slices were transferred to a submerged recording chamber where they were continuously superfused at a rate of 3 ml/min with oxygenated aCSF at 32°C. A bipolar concentric wire stimulation electrode was placed in layer 2/3 of the mPFC (Figure 2(a)), delivering rectangular pulses of 0.1 ms duration every 20 s. The evoked responses were recorded through a microelectrode filled with aCSF (2–6 MΩ resistance) placed in the layer 5 of the mPFC, using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, United States). Responses were digitized and continuously monitored on a computer using WinLTP 2.20b software (Anderson and Collingridge, 2007). Individual responses were monitored, and averages of six consecutive responses were used to calculate the slope of field excitatory postsynaptic potentials (fEPSPs). Due to difficulties experienced in inducing LTP in mPFC slices, multiple protocols were extensively tested and the protocol employed, which has been used before (Gemperle et al., 2003; Kerkhofs et al., 2018), was found to be the most reliable one in our hands. LTP was induced in the PFC by delivering a train of 100 Hz (50 pulses, 0.5 s duration) stimuli for priming, followed 15 min later by four trains of 100 Hz (50 pulses, 0.5 s duration, 1 every 10 s) stimuli. LTP was quantified as the % change in the average slope of the fEPSPs taken from 50 to 65 min after LTP induction relatively to the average fEPSP slope measured during the 10 min prior to LTP induction. The effect of BDNF on LTP was evaluated by comparing the LTP magnitude in a control (aCSF superfusion) and test slice (BDNF superfusion) from the same animal. BDNF remained in the bath until the end of the recording period. The order of testing BDNF and control

treated slices was counterbalanced, to avoid any potential influence of dissection bias or time of day on the measurements.

To make sure that, in our experimental conditions, we were assessing a synaptic signal, mostly glutamatergic in nature, we performed a control experiment with 10 µM of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA/kainate glutamate receptor antagonist. CNQX was added to the aCSF superfusion solution of the slices containing mPFC taken from a saline-treated animal. After 10 min, CNQX was washed out from the system by superfusion aCSF and individual responses were monitored, and averages of six consecutive responses were used to calculate the slope of fEPSPs (Supplementary Figure 1).

Ex Vivo Electrophysiology Recordings in hippocampal slices

Animals treated with saline or PCP were sacrificed after being deeply anesthetized with isoflurane, 3 to 5 days after the final treatment (days 15-17). The brain was quickly removed and placed in ice-cold continuously oxygenated (O_2/CO_2: 95%/5%) aCSF and the hippocampi were dissected free. The hippocampal slices (400 µm thick) were cut perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper (Campden Instruments, Leicestershire, UK). After recovering functionally and energetically for at least 1 h in a resting chamber filled with oxygenated aCSF at RT, hippocampal slices were transferred to a recording chamber continuously superfused with oxygenated aCSF at 32°C (flow rate of 3 mL/min). fEPSPs were recorded extracellularly through a microelectrode filled with aCSF (2–6 MΩ) placed in the stratum radiatum of the CA1 hippocampal subfield (Figure 3(a)). The Schaffer collaterals were stimulated (rectangular pulses of 0.1 ms duration) every 10 s using a bipolar concentric wire electrode positioned at the CA3-CA1 border. Recordings were obtained with an Axoclamp 2B

amplifier (Axon Instruments, Foster City, CA, United States), digitized and continuously stored on a computer using WinLTP 2.20b software (Anderson and Collingridge, 2007). Individual responses were monitored, and averages of six consecutive responses were obtained to calculate the slope of the initial phase of the fEPSP. LTP induction and quantification were performed as previously described (Diógenes et al., 2011) using a stimulation paradigm optimised to detect the effect of BDNF upon LTP (θ-burst protocol; three trains of 100 Hz, three stimuli, separated by 200 ms) (Fontinha et al., 2008). This stimulus protocol is considered to be closer to physiological stimulations that occur in the hippocampus during episodes of learning and memory *in vivo* (Albensi et al., 2007). After a stable fEPSP slope was achieved, LTP was induced. In an independent slice from the same animal, LTP was induced after 20 min of BDNF perfusion (20 ng/mL). BDNF remained in the bath until the end of the experiment. Again, the order of testing BDNF and control treated slices was counterbalanced, to avoid any potential influence of dissection bias or time of day on the measurements.

LTP was quantified as % change in the average slope of the fEPSP taken from 50 to 60 min after LTP induction relatively to the average slope of the fEPSP measured during the 10 min prior to LTP induction. The effect of BDNF on LTP was evaluated by comparing the LTP magnitude in the first (control, aCSF superfusion) and second hippocampal slices (test, BDNF superfusion) from the same animal, as previously described (Figurov et al., 1996; Fontinha et al., 2008).

BDNF, TrkB Receptor and NMDA-R subunits quantification

Brain tissue samples (PFC and hippocampus) were isolated and washed with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$, and 1.5 mM KH_2PO_4, pH 7.4) and lysed with Radio Immuno Precipitation Assay buffer (RIPA, 50 mM Tris-HCl

(pH 7.5), 150 mM NaCl, 5mM ethylenediamine tetra-acetic acid, 0.1% sodium dodecyl sulfate 145 (SDS) and 1% Triton X-100) containing protease inhibitors (Roche, Penzberg, Germany). Tissue homogenates were clarified by centrifugation (13 000 *g*, 10 min, 4°C), and the amount of protein in the supernatant determined by Bio-Rad DC reagent assay (Bio-Rad Laboratories, Berkeley, USA).

BDNF protein levels were quantified using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Cat. No.: G7611, Promega, WI, USA). Samples were analysed in duplicate.

For western blot, a loading buffer (350 mM Tris pH 6.8, 10% SDS, 30% glycerol, 600 mM Dithiothreitol, 0.06% bromophenol blue) was added and the mixture was heated to 95-100°C for 5 min. Each sample (70 µg of total protein) and the molecular weight marker (NZYTech, Lisbon, PT) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) within a standard migration buffer (25 mM Tris pH 8.3, 192 mM Glycine, 10% SDS), at a constant voltage between 80-120 mV. Proteins were then transferred onto Polyvinylidene Difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK), previously soaked in methanol for 5 min, in the standard buffer (25 mM Tris pH= 8.3, 192 mM 157 Glycine, 15% methanol) for 90 min. Membranes were then stained with Ponceau S solution to determine protein transference efficacy and then blocked with a 3% (w/v) bovine serum albumin in Tris-buffered saline solution containing 0.1% Tween-20 (20 mM Tris base, 137 mM NaCl and 0.1% Tween-20). Membranes were incubated with the primary antibody (overnight at 4°C). These antibodies were: C-14 - the C-terminal of Trk-FL rabbit polyclonal antibody (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), which was used to quantify TrkB-FL levels, and the pan-TrkB mouse monoclonal antibody (1:1500), raised against the extracellular domain of

human TrkB (aa. 156–322; BD Bioscience), used to quantify the levels of TrkB-TC protein. We also quantified NMDA receptor expression in the PFC and hippocampus (Supplementary Figure 2) using an GluN1 mouse monoclonal antibody (1:500; BD Bioscience, Franklin Lakes, NJ, USA), GluN2A mouse monoclonal antibody (1:200; Santa Cruz Biotechnology) and GluN2B rabbit polyclonal antibody (1:1000, Abcam, Cambridge, UK). Membranes were then incubated for 1h at RT with goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibodies (1:10000, Santa Cruz Biotechnology). Immunoreactivity was visualized using the Enhanced Chemiluminescence (ECL) detection system (GE Healthcare). Band intensity was recorded using the ChemiDoc system (Bio-Rad Laboratories) and quantified by digital densitometry software in ImageJ 1.45 (Bethesda, MD, USA). The intensity of GAPDH (mouse anti-GAPDH; 1:5000, Thermo Fischer Scientific) was used as the loading control.

All Western Blot experiments were performed by the same researcher and with the same equipment.

Behavioural Testing

Y-Maze spontaneous alternation test

Spontaneous alternation was tested in a Y-shaped maze with three white, opaque plastic arms (each with 15 x 5 x 12 cm) at a 120° angle from each other, as previously described (Hughes, 2004). The animal was placed in an arm, facing the center of the maze, and allowed to freely explore the maze for 8 min. A correct alternation occurred when the animal sequentially moved to the other two arms of the maze without retracing its steps (*i.e.,* from arm A to B to C). Movement sequences involving the animal re-visiting a previously visited arm, such as ABA or ACA, were recorded as being incorrect alternations. Based on all movements made over the entire session, the percentage of correct alternations was calculated. An arm entry was

considered to occur when all four of the animal's limbs were within the given arm. The percentage of correct alternations was calculated by dividing the number of correct alternations by the number of alternation opportunities minus one x 100.

Open field test

The OFT was used to determine any impact of PCP on locomotor activity or anxiety-like behaviour that might affect performance on the other behavioural tests. The arena used (white, square, 40 x 40 x 40 cm) was the same used for the NORT. The OFT was carried out on the first time that animals had contact with the environment, *i.e.*, on the first 5 min of the first day of NOR habituation phase, indicating the animals locomotor activity in response to a novel environment. The arena was virtually divided into three different square zones, namely a peripheral zone, an intermediate zone and a central zone. The amount of time spent in the central zone was used as an inverse indicator of anxiety-like behaviour. Mean velocity and distance travelled were quantified to compare locomotor activity. Movements were recorded and analysed using the videotracking software SMART® (PanLab, Barcelona, Spain).

Novel object recognition test

The NORT was conducted in an open field arena (40 X 40 X 40 cm), to which the animals had previously been exposed, in accordance with published protocols (Mouro et al., 2018). Animals were habituated to the arena context in the absence of any stimulus or object, under the same lighting and environmental conditions and every day around the same hour, for 15 min on 2 consecutive days (habituation phase). The objects used in the training and test days were wooden dolls (7 cm height × 6 cm width). The role of the object, as either familiar or novel, and the location of their presentation were randomized across animals. Between every trial, the arena and the objects were cleaned with a 30% ethanol solution to erase any olfactory clues.

The objects were placed in symmetric and opposed corners of the arena, as previously described (Antunes and Biala, 2012). Animals were placed inside the arena facing away from the objects. During the acquisition phase the animals were exposed to two identical objects (familiar objects) and were allowed to freely explore the environment and the objects for 5 min. After a retention interval of 24 h, to test long-term memory (Antunes and Biala, 2012; Clarke et al., 2008), animals were placed as before inside the arena, this time containing one of the familiar objects and one novel object (test phase). Animals were allowed to explore the objects for 5 min, after which they were removed from the arena. The habituation phase coincided with days 11 and 12 of the subchronic treatment regimen, with PCP being administered to animals after behavioural testing. Immediately after being removed from the arena, an i.p. injection of a saline solution or PCP was administrated to the animals and then they returned to their home cage. The acquisition (day 15) and test phases of NORT (day 16) were performed after the injections had stopped, to prevent any impact of acute PCP administration on NORT performance.

Exploratory behaviour was quantified as the amount of time animals spent sniffing the object, rearing towards the object or touching the object (Mouro et al., 2018). Object preference was quantified as the percentage of exploration time spent exploring each object over the total time spent exploring both objects. The Novelty Preference Index (NPI) was also calculated as (N-F)/(N + F), where N corresponds to the time spent exploring the novel object and F the time spent exploring the familiar object, during the test phase of NORT. Thus, this index ranges from -1 to 1, where 0 represents the absence of discrimination between novel and familiar objects, 1 corresponds to exploration of the novel object only and -1 corresponds to exploration of the familiar object only.

Morris Water Maze Test

Hippocampal-dependent spatial learning and memory was evaluated by the MWMT, as previously described (Batalha et al., 2013). The water maze consisted of a circular pool (100 cm diameter, 60 cm height) filled with white opaque water at RT. The pool was virtually divided into 4 quadrants. An escape platform (8 cm diameter) was submerged beneath the water surface in the centre of the target quadrant. Animals were pre-trained in a one-day session to be familiarized with the maze and the presence of the platform, with no extra-maze visual cues presented. In the first trial the platform was placed at 1 cm above the water surface at the centre of the maze. Animals were placed directly on the platform being allowed to remain there for 20 s before being removed from the maze. In the next trial, the platform was submerged 1 cm beneath the water surface, and the same protocol repeated. On the next day, extra-maze visual cues were positioned on the walls of the testing room. During the learning phase, animals were tested for 4 consecutive days (acquisition phase) with 4 trials per day and a 30 min inter-trial interval. Animals were released into the pool, facing the wall of the maze. If the animal located the platform in less than 60 s, it was allowed to remain on the platform for 10 s. If the animal failed to locate the platform within 60 s, the animal was guided or placed on the platform and allowed to remain there for 20 s. The start position (1, 2, 3, or 4) was randomized across trials to ensure that the animals were using extra-maze spatial cues to determine the platform location during the learning phase. For any given animal the platform location was maintained in the same target quadrant of the maze, but the target quadrant was randomized across animals to avoid bias. After successful completion of a trial, mice were removed from the maze and placed back into their home cages. One day after the final session of the acquisition phase (day 5), a probe trial was conducted. During this probe trial (60 s), the platform was removed from the maze, and the search pattern of the mice within the maze tracked. The proportion of

time spent in the target quadrant (the quadrant where the platform was previously located) was determined. The distance travelled to locate the platform (cm) and swim speed (velocity (cm/s), as a measure of possible motor defects that could interfere with an animal abilities to perform the task) were determined using videotracking software (SMART®).

Statistical Analyses

Data were analysed in GraphPad Prism 8 (San Diego, CA) using ANOVA or Student's t-test, where appropriate. Where significant interactions were detected in ANOVAs, *post-hoc* t-tests with Bonferroni correction were performed. Outliers were identified by Grubbs' test. Statistical significance was set at $p<0.05$.

A post-hoc power analysis based on the actual group sizes included in our manuscript was undertaken and included in the Supplementary material.

Results

The facilitatory effect of BDNF upon LTP is attenuated in the PFC, but not in the hippocampus, of PCP-treated mice

High Frequency Stimulation (HFS) induced LTP in L5 neurons of the mPFC in control mice as previously reported (Gemperle et al., 2003; Hempel et al., 2000; Fénelon et al., 2011). Subchronic PCP-treatment impaired the induction of LTP in the mPFC (main PCP treatment effect, $F_{(1, 31)}$=10.17, p=0.003, two-way ANOVA) (Figure 2(d)). The half-life of PCP in the rodent brain after *i.p*. injection under repeated treatment conditions is approximately 30 min (Nabeshima et al., 1987). Therefore, at the time of recording, 72-120h after the final PCP administration, the PCP-induced impairment of LTP cannot be attributed to the acute blockade of NMDA-Rs. However, changes in the expression of the GluN2B NMDA-R subunit, known to be critical for LTP in the PFC (Zhao et al., 2005), may be involved as we found decreased GluN2B expression levels in the PFC of PCP-treated animals (Supplementary Figure 2).

There was strong trend towards a significant interaction between subchronic PCP treatment and BDNF treatment on mPFC LTP ($F_{(1,31)=}$4.12, p=0.051, two-way ANOVA), while the main effect of BDNF itself was found to be non-significant ($F_{(1, 31)}$=2.42, p=0.130, two-way ANOVA). Given that the significance of the interaction was found to be very close to the significance threshold in this case, conservative *post-hoc* testing was applied to determine if there was any difference in the response to BDNF between the two treatment groups. We found that exogenous BDNF (20 ng/mL) significantly enhanced LTP magnitude ($LTP_{CTR+BDNF}$: 92 ± 16 % vs. LTP_{CTR}: 37 ± 13 %, n=8-10, p=0.031, t-test with Bonferroni correction) (Figure 2(d)) in the mPFC of control animals. However, BDNF failed to enhance LTP in mPFC slices from PCP-treated

mice ($LTP_{PCP+BDNF}$: 12 ± 19 % vs. LTP_{PCP}: 19 ± 12 %, n=8-9, p>0.999, t-test with Bonferroni correction).

Given our functional data showing attenuated BDNF signalling in the mPFC of PCP-treated mice, we measured BDNF, TrkB-FL and TrkB-TC expression levels in the PFC (Figure 2(e-g)). While BDNF levels (n=6-9, p=0.482, t-test) were not significantly altered, there was a marked decrease in TrkB-FL receptor expression in the PFC of PCP-treated animals (n=11, p=0.008, t-test). By contrast, there was no difference in TrkB-TC levels in the PFC of PCP-treated animals (n=11, p=0.117, t-test). This suggests that decreased BDNF signalling in the PFC of PCP-treated animals is mediated by decreased TrkB-FL receptor expression rather than through increased negative modulation (TrkB-TC) or alterations in the levels of the endogenous ligand.

[insert Figure 2.]

In the hippocampus, we did not find evidence for a significant PCP treatment effect on LTP ($F_{(1,32)}$=0.77, p=0.386, two-way ANOVA) or for a significant PCP-treatment x BDNF-treatment interaction ($F_{(1,32)}$=0.06, p=0.806, two-way ANOVA). However, BDNF was found to significantly enhance hippocampal LTP ($F_{(1, 32)}$=14.83, p=0.0005, two-way ANOVA). Thus, as previously reported (Fontinha et al., 2008; Tanqueiro et al., 2018), BDNF (20 ng/mL) facilitated LTP in hippocampal slices from both control animals. Interestingly, in clear contrast with PFC, the facilitatory action of BDNF on hippocampal LTP was also present in PCP-treated mice (Figure 3(d)). Our molecular data also showed that subchronic PCP administration did not alter BDNF levels (n=9-10, p=0.610, t-test), TrkB-FL or TrkB-TC (n=11, p=0.106 and p=0.134, respectively, t-test) expression in the hippocampus (Figure 3(e-g)). Additionally, GluN2A/B subunit expression

was unaltered and only GluN1 subunit was increased in the hippocampus of PCP-treated animals (Supplementary Figure 2).

Overall, the data suggest that the ability of BDNF to enhance LTP is selectively compromised in mPFC. This is consistent with decreased TrkB-FL receptor expression seen in the PFC, but not the hippocampus, of PCP-treated mice.

[insert Figure 3.]

Subchronic PCP administration impairs spontaneous alternation and object recognition memory

The YMT provides an index of working memory, reflecting the preference of control mice to explore less recently visited arms of the maze (Hughes, 2004; Kraeuter et al., 2019), that is regulated by the PFC (Lalonde, 2002). We found that PCP-treated animals performed less correct spontaneous alternations in the YMT (n=9-12, p=0.005, t-test) (Figure 4(a)), while motor performance and activity levels were not significantly altered in PCP-treated animals (Supplementary Figure 3).

In the training phase of the NORT, control and PCP-treated animals explored each of the two identical objects for a similar proportion of time (Figure 4(b)). There was no evidence for a significant PCP treatment effect ($F_{(1, 36)}$=3.17e-008, p=0,999, two-way ANOVA), object effect ($F_{(1,36)}$=0.98, p=0.328, two-way ANOVA) or a PCP treatment x object interaction ($F_{(1, 36)}$=1.13, p=0.294, two-way ANOVA) on object exploration during the training phase. By contrast, on the test day (Figure 4(c)) a significant PCP treatment x object interaction on object exploration time was found ($F_{(1, 36)}$=11.65, p=0.0016, two-way ANOVA). *Post-hoc* tests showed that while control animals showed a significant preference for exploring the novel object (n=9, p<0.0001, t-test

with Bonferroni correction), PCP-treated animals did not (n=11, p=0.088, t-test with Bonferroni correction). In addition, a significant effect of object (familiar v novel) was also found ($F_{(1, 36)}$=38.65, p<0.0001, two-way ANOVA) while the effect of PCP treatment on overall object interaction time was found to be non-significant ($F_{(1, 36)}$=8.57e-008, p=0.999, two-way ANOVA).

A similar effect was seen for the Novelty Preference Index (NPI) with PCP-treated animals showing a decreased preference for the novel object when compared to controls (n=11, p=0.026, t-test) (Figure 4(d)). Moreover, while NPI values in control animals were significantly different from zero (n=9, p=0.003, t-test), indicating novel object preference, this was not significant in PCP-treated animals (n=11, p=0.131, t-test). Total object exploration time (Supplementary Figure 4) and locomotor activity (Supplementary Figure 5) were not different between groups in either the training or test phase.

Hippocampal-dependent spatial learning and memory was assessed using the MWMT. The path length to the platform (distance travelled) was used as an indicator of learning during the acquisition phase of the MWMT. A significant effect of training day ($F_{(3,24)}$=28.78, p<0.0001, two-way ANOVA) on distance travelled was found, with animals travelling shorter distances as training days progressed, indicating that animals had learned the location of the platform. In this case we also found a significant PCP treatment x training day interaction ($F_{(3, 24)}$=3.27, p=0.039, two-way ANOVA) and a significant effect of PCP treatment ($F_{(1,24)}$=6.50, p=0.018, two-way ANOVA). To further understand the basis of the PCP treatment x day interaction *post-hoc* tests were applied. *Post-hoc* tests showed that PCP-treated animals travelled significantly further distances to reach the platform than controls on day 1 (p=0.008), but not on days 2, 3 or 4 (p=0.287, p>0.999, p>0.999, respectively (Figure 4(e)) during the acquisition phase. This suggests that learning may be significantly impaired in PCP-treated animals on day 1 of acquisition, but

this is not seen on the other acquisition days. We also recorded the swim speed (mean velocity) and latency to reach the platform during the acquisition stage of the MWMT, and these data are reported in the supplementary material (Supplementary Figures 6 and 7).

Importantly, during the probe trial, 72h after the final PCP administration, there was no significant difference in the percentage time spent in the target quadrant between groups (n=10, p=0.097, t-test) (Figure 4(f)) or in the number of passes over the target area (Supplementary Figure 8(a)), indicating that long-term spatial memory was not disrupted in PCP-treated animals. In addition, motor abilities in the probe test of MWMT were not altered in PCP-treated mice during this phase of the MWMT (Supplementary Figure 8(b,c)).

[insert Figure 4.]

Discussion

In this work, we have shown that PCP-treated mice display decreased TrkB-FL receptor levels and an impaired facilitatory effect of BDNF on LTP in the mPFC. By contrast, in the hippocampus, the ability of BDNF to facilitate LTP and TrkB-FL receptor expression are unaltered. This shows that prolonged NMDA-R hypofunction, induced by PCP-treatment, does not cause a global and unspecific loss of BDNF signalling in the brain, but that it selectively affects the PFC. PCP-treated mice also show impaired performance in PFC-dependent behavioural tests (YMT and NORT), but not in a hippocampus-dependent long-term spatial memory test (MWMT). This suggests that disturbed PFC BDNF signalling may contribute to PFC-dependent cognitive deficits induced by subchronic PCP administration (Cochran et al., 2003; Dawson et al., 2012). The data also suggest that therapies augmenting TrkB-FL receptor signalling may be useful in reversing the PFC-dependent cognitive deficits seen in the model (Dawson et al., 2012), and potentially in treating patients with schizophrenia. Future work directly accessing this hypothesis is required.

Sustained NMDA-R hypofunction induces deficits in PFC synaptic plasticity that may contribute to the cognitive deficits seen in the subchronic PCP model

LTP, the cellular substrate of learning and memory (Bliss and Collingridge, 1993), was used to evaluate the impact of subchronic PCP and BDNF on synaptic plasticity. In the hippocampus, BDNF increases LTP magnitude through TrkB-FL activation (Korte et al., 1995; Figurov et al., 1996; Minichiello et al., 2002), and we confirmed its facilitatory effect upon LTP (Figure 3(d)). While there are no published data regarding the ability of BDNF to facilitate LTP in the PFC, our data clearly show this effect (Figure 2(d)). We also found that subchronic PCP administration attenuated LTP induction in the mPFC. These findings align with the deficits in

PFC-dependent behavioural tests, including deficits in the YMT and NORT (Antunes and Biala, 2012; Lalonde, 2002)(Figure 4), cognitive flexibility (Dawson et al., 2012) and working memory (Marquis et al., 2007), and PFC metabolism ("hypofrontality") (Cochran et al., 2003; Dawson et al., 2012), that result from subchronic PCP administration. In addition, decreased GluN2B protein levels were found in the PFC of PCP-treated mice, which may contribute to the impact of PCP treatment on PFC LTP, given the crucial role of this NMDA-R subunit in LTP in this brain area (Zhao et al., 2005). Contrasting with previous data (Nomura et al., 2016), we did not find impaired LTP in the hippocampus of PCP-treated animals in our work. Furthermore, GluN1 levels were found increased and GluN2A/GluN2B levels unchanged in the hippocampus of PCP-treated animals (Supplementary Figure 2). Others have found altered GluN1, GluN2A and GluN2B subunits levels in the PFC and hippocampus after subchronic PCP administration, however there are inconsistencies in the data (Anastasio and Johnson, 2008; Lindahl and Keifer, 2004).

Effective performance in the NORT is thought to involve both the hippocampus and PFC (Barker et al., 2007; DeVito and Eichenbaum, 2010; Barker and Warburton, 2011; Cohen and Stackman Jr., 2015), whereas performance in the MWMT is thought to predominantly involve the hippocampus (Morris et al., 1982) but not the mPFC (de Bruin et al., 1994). Our data show that PCP-treated mice have deficits in the NORT, corroborating previous work (Hashimoto et al., 2005; Horiguchi and Meltzer, 2013; Redrobe et al., 2010), but not in the MWMT, suggesting that the PFC rather than hippocampal dysfunction may be contributing to the NORT deficit seen in PCP-treated mice.

Interestingly, we found that BDNF enhanced LTP in the hippocampus of PCP-treated animals (Figure 3), suggesting that BDNF signalling is not disrupted in this brain area. This is also consistent with our observation that BDNF, TrkB-FL and TrkB-TC levels were not altered in the

hippocampus of PCP-treated animals. In the hippocampus, the effect of BDNF upon LTP is most well characterised under a θ-burst protocol (Diógenes et al., 2011; Fontinha et al., 2008) However, the effects of BDNF upon synaptic plasticity in the mPFC have not been previously reported. Therefore, we used a published stimulation protocols validated for inducing LTP in the mPFC (Gemperle et al., 2003; Kerkhofs et al., 2018). Having tested several LTP protocols in preliminary experiments (not shown), we opted for the protocol that most reliably induced LTP in the mPFC and, importantly, that induced LTP of a similar magnitude to that induced in the hippocampus with the canonical θ protocol. To our knowledge, the effects of subchronic PCP administration upon mPFC synaptic plasticity have not previously been reported. However, previous work has shown impaired hippocampal LTP induced by subchronic PCP treatment (Nomura et al., 2016). In the PFC, the induction of LTP is more variable than in the hippocampus and requires activation of the NMDA-R and metabotropic glutamate receptors (Vickery et al., 1997), whereas in the hippocampal CA1 LTP is mostly NMDA-R dependent (Nicoll and Malenka, 1999). It was previously reported that the induction protocol used in this present work can induce LTP in approximately one third of attempts (Meunier et al., 2017). We also experienced this variability (Figure 2). Remarkably, in the presence of BDNF LTP in the mPFC did occur in most of the attempts, indicating that BDNF increases the probability of LTP induction in the mPFC.

While behavioural measurement was carried out ~20 hours after the previous PCP administration for some of the measurements taken, and these are unlikely to be influenced by the acute effects of PCP, the drug was given shortly after training in some tests. This was the case for the habituation phase of NORT and after the initial training on days 1 and 2 of the MWMT acquisition phase. Given that NMDAR antagonists can disrupt memory consolidation in test such as the MWMT (Liang et al., 1994; Bye and McDonald, 2019) we might have expected

to see a deficit in performance across days in the acquisition phase of the test in PCP-treated mice. However, while we did see a deficit on day 1 of the MWMT acquisition, we found that PCP-treated mice performed similar to control mice on days 2-4 (Figure 4(e)), suggesting that the consolidation of long-term spatial memory had not been disrupted by PCP treatment following training on day 1. It is also important to note that any potential disruption of consolidation during the initial stages of the MWMT training phase is unlikely to influence the primary outcome measures of the probe trial when performance is similar in the later stages of the acquisition training, as seen in this study (Figure 4(e)). Furthermore, we find no evidence of a performance deficit in PCP-treated mice in our key primary outcome measure; performance during the MWMT probe trial (Figure 4(f)). Therefore, even if PCP-induced consolidation deficits were present during the initial phases of MWMT training, which does not seem to be the case, they would apparently not be sufficient to induce deficits in long-term spatial memory, as evidenced by a deficit in performance at the probe trial. One limitation to our study is that we did not perform a visible platform during day 1 of acquisition of the MWMT, and so we can not rule out that deficits in visual acuity may contribute to the performance deficit seen at this time in PCP-treated mice.

Sustained NMDA-R hypofunction may contribute to decreased BDNF signalling in the PFC and PFC-dependent cognitive deficits in schizophrenia

Our data show that the subchronic PCP model reproduces the reduced PFC TrkB-FL expression seen in patients with schizophrenia (Takahashi et al., 2000; Weickert et al., 2005), supporting a potential role for NMDA-R hypofunction in this impairment. This also highlights the

translational relevance of the subchronic PCP model for this deficit, which will be useful in testing the potential therapeutic value of targeting BDNF signalling to enhance PFC-dependent executive dysfunction in patients (Lesh et al., 2011; Peng et al., 2018). Furthermore, our data also suggest that prolonged NMDA-R hypofunction may contribute to the deficit in TrkB-FL expression seen in the PFC of patients (Takahashi et al., 2000; Weickert et al., 2005), although more work is needed to firmly establish this. Recent findings have also shown that an increased TrkB-FL/TrkB-TC ratio, in peripheral blood mononuclear cells, is predictive of better global cognition and working memory in patients during their first episode of psychosis (Cengotitabengoa et al., 2019), further highlighting the potential role of TrkB-FL signalling in modulating the cognitive deficits seen in patients.

There are important limitations to consider. For example, PCP targets additional receptors to the NMDA-R (Kapur and Seeman, 2002; Seeman et al., 2005) and other mechanisms are likely to be involved in the dysfunctional BDNF signalling seen in patients (McLean et al., 2017; Santini et al., 2013). Moreover, we found that subchronic PCP does not completely reproduce the alterations in BDNF signalling reported in the PFC of patients, such as reduced BDNF (Weickert et al., 2005) and increased TrkB-TC (Wong et al., 2013) levels, potentially limiting translational relevance. However, our molecular observations do align with previous work (Snigdha et al., 2011) showing that subchronic PCP did not alter BDNF levels in the PFC or hippocampus of male rats. Nevertheless, the authors did show that PCP decreased levels of BDNF in female animals.

Indeed, previous work has been reported sex differences in schizophrenia rodent models regarding cognitive impairments (Sutcliffe et al., 2007; Li et al., 2016) and in the effects of PCP/NMDA-R antagonists on cognition and BDNF (Snigdha et al., 2010; Leger and Neill, 2016). As females were not included in our study, we cannot conclude how generalisable our

observations might be to female mice. Thus, future work undertaking a systematic characterising of our observations in female animals would be of interest.

Discrepancies between our observations and others in terms of the impact of subchronic PCP treatment on BDNF may be due to differences in the species or strains used, the developmental time at which PCP is administered, the treatment regimen used, and the washout period employed.

Indeed, published works that have commonly reported the application of lower doses of PCP (2-5 mg/kg) are those conducted in rats (Bruins Slot et al., 2005; Lee et al., 2005; Egerton et al., 2008; Snigdha and Neill, 2008; Dawson et al., 2012), whereas in mice higher doses, typically around 10 mg/kg once per day, as applied here, have most commonly been used (Fujita et al., 2007; Hashimoto et al., 2007; 2008; Tanibuchi et al., 2009; Santini et al., 2013). There are also a limited number of subchronic PCP studies that have utilised a 10 mg/kg dosing regimen in adult rats (Martinez et al., 1999; Audet et al., 2009) and this dose has been applied in developmental rat models employing early postnatal administration (Andrews et al., 2018; Shan et al., 2018), albeit with less frequency. Our own experience with using PCP in both species indicates that mice are somewhat less sensitive to the acute effects of PCP than rats, although the field is currently lacking a systematic comparison between the two species, which would be of great benefit. However, the behavioural and neurobiological consequences of the different doses are generally reported to be similar between the two species when using these different doses (Jones et al., 2011; Pratt et al., 2012) and a 10 mg/kg dose has been shown to be optimal in inducing spatial learning and memory deficits in mice, whereas the doses need to induce this deficit in rats is lower (Zain et al., 2018).

The PCP dosing regimen used in our study is the same as that used in a number of previous studies (Hashimoto et al., 2007; Fujita et al., 2008; Hashimoto et al., 2008; Tanibuchi et al., 2009; Santini et al., 2013). All of these studies were conducted in mice, focusing on behavioural and neurochemical changes present during the 24h-72h after the final PCP treatment. As this is one of the most widely reported PCP mouse models, which also appears to show important reproducibility between labs, we chose to focus on this well-defined time point after subchronic PCP administration. There are studies that have looked at longer time points following on from PCP administration. However, these have usually been conducted in rats and can often involve the use of maintenance schedules of intermittent PCP administration in order to maintain the deficits.

Importantly, the persistence of PCP-induced effects is one question that would benefit from more detailed systematic characterisation in terms of the observations we have made in the present work. This is particularly the case for mice, where the deficits are somewhat less well-defined in comparison to those seen in rats (Neill et al., 2016; Asif-Malik et al., 2017; Grayson et al., 2007; McLean et al., 2017; Doostdar et al., 2019; Mitsadali et al., 2020). Thus, it would also be interesting to characterise the persistence of PCP-induced deficits on BDNF signalling in mice with longer PCP timeframes. Similarly, determining whether the reported changes are reproduced in the neonatal PCP model, a model with a more neurodevelopmental focus (Amani et al., 2013; Gaskin et al., 2014; Nakatani-Pawlak et al., 2009), would also be particularly relevant.

If indeed the primary change in BDNF signalling occurs in the frontal cortex as our data suggest, then changes reported in hippocampus by other authors (Semba et al., 2006; Harte et al., 2007; Snigdha et al., 2011; Mouri et al., 2012), whose measurements have been taken

following a longer 'washout' period, could be a downstream adaptive change in the hippocampus in response to the primary change in frontal cortex. This is an interesting suggestion that certainly warrants further systematic characterisation.

There are also reports showing decreased BDNF levels in the hippocampus of patients with schizophrenia (Durany et al., 2001; Iritani et al., 2003) contrary to our observations in PCP-treated animals, again suggesting that there are translational limits of the model in relation to the altered BDNF signalling seen in the disorder, and that mechanisms other than prolonged NMDA-R hypofunction may be important in patients. Alternatively, the observations in patients may relate to the impact of antipsychotic medication, with these drugs shown to influence brain BDNF expression levels, although these effects seem to be facilitatory in most rodent models (de Bartolomeis et al., 2017; Park et al., 2011; Yu et al., 2019). More work is needed to more fully characterise the relationship between antipsychotics and BDNF signalling.

While our study identified BDNF dysfunction as a key mechanism that contributes to the impact of subchronic PCP administration on PFC function, other mechanisms are also involved. This includes, for example, dysfunction of the dopaminergic (Jentsch, 1997; Balla et al., 2003; McLean et al., 2017), GABAergic (Gong et al., 2009; Amitai et al., 2012; Dawson et al., 2014) and serotonergic (Hori et al., 2000; Santini et al., 2013; Yamazaki et al., 2018) systems. Interestingly, there is also some evidence that these neurotransmitter systems regulate BDNF function (Fumagalli et al., 2006; Porcher et al., 2018), therefore future characterisation of the inter-related nature of these mechanisms in the PCP mouse model would be of interest.

Conclusion

In conclusion, our data suggest that reduced BDNF signalling in the PFC is a key mechanism contributing to the PFC dysfunction and the corresponding cognitive deficits in the PCP model, and suggest that NMDA-R hypofunction may be an important mechanism contribution to PFC BDNF dysfunction in schizophrenia. While these deficits have some translational relevance to the disturbed BDNF signalling reported in schizophrenia, the model does not fully recapitulate the BDNF signalling deficits seen in the disorder. Nevertheless, the subchronic PCP model offers an established range of biomarkers and phenotypes that can be used in future studies to further validate the potential of drugs targeting BDNF signalling in the PFC as a novel therapeutic strategy for the treatment of the executive cognitive deficits seen in schizophrenia.

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Declaration of conflicting of interests

The Authors declare that there is no conflict of interest.

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Figures

Figure 1.

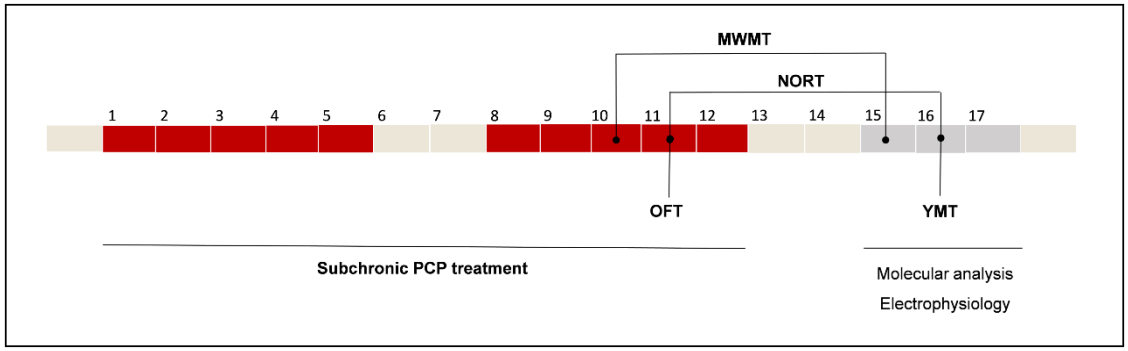


Figure 2.

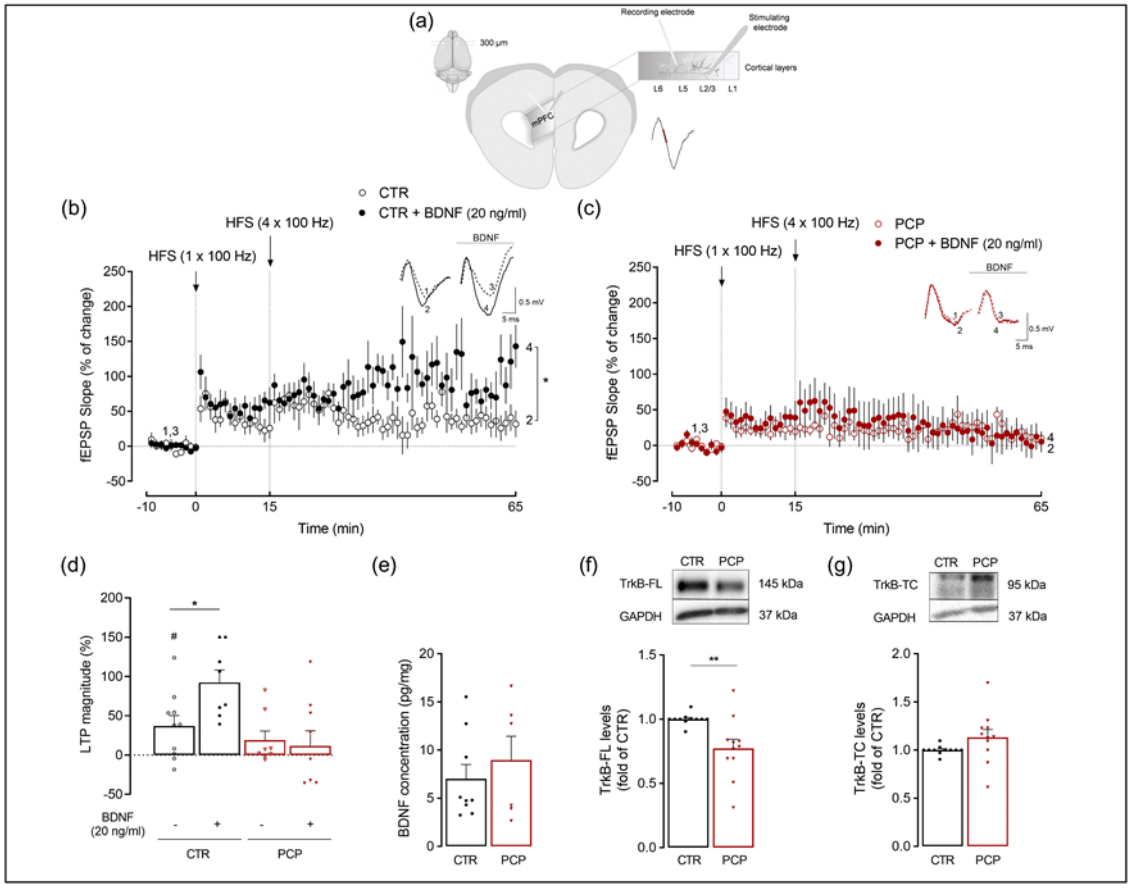


Figure 3.

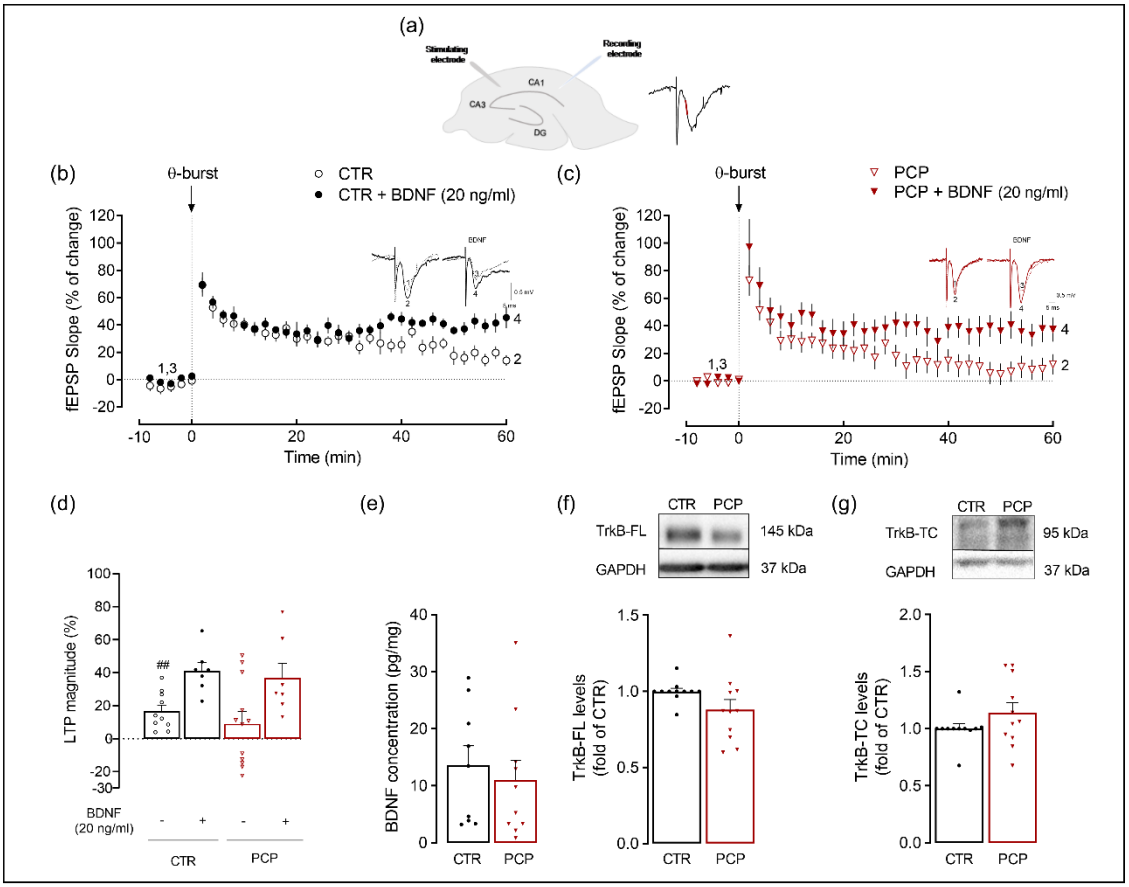
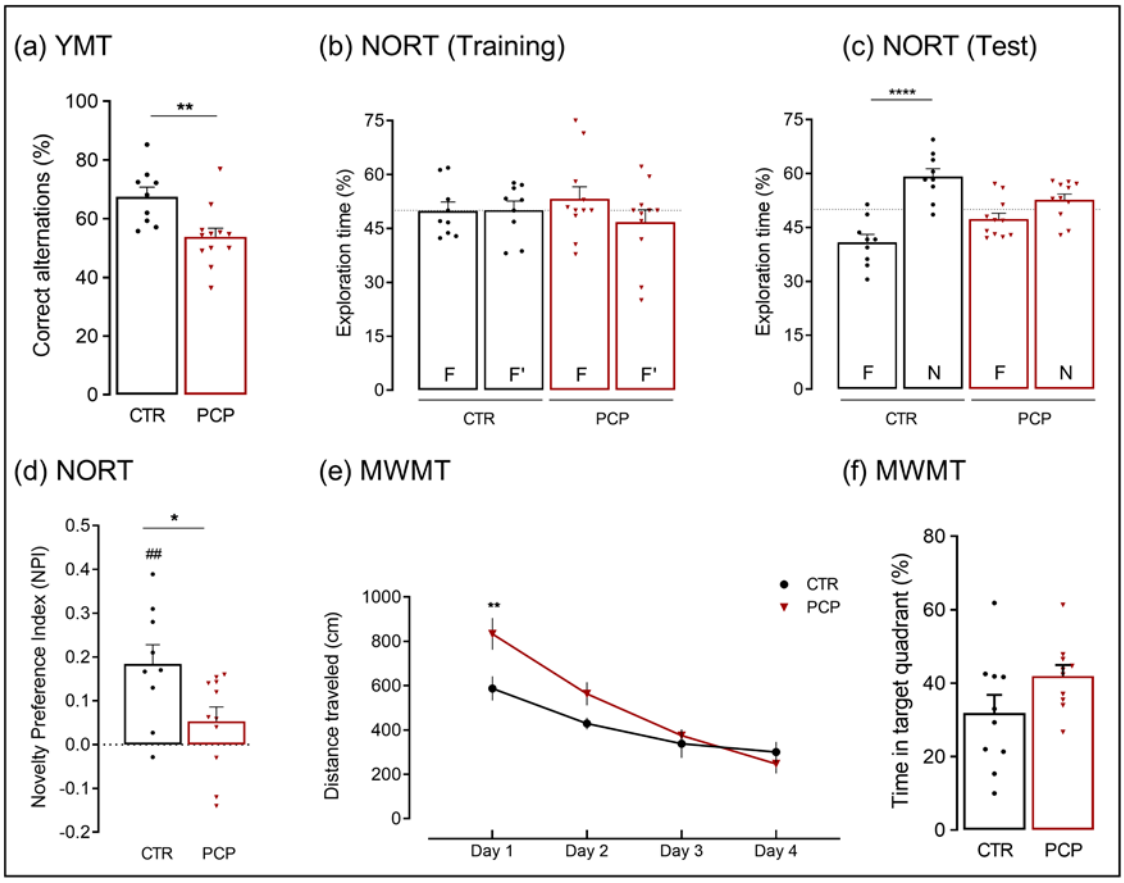

(a)
Stimulating electrode
Recording electrode
CA1
CA3
DG
(b)
θ-burst
CTR
CTR + BDNF (20 ng/ml)
fEPSP Slope (% of change)
Time (min)
BDNF
1,3
2
4
(c)
θ-burst
PCP
PCP + BDNF (20 ng/ml)
fEPSP Slope (% of change)
Time (min)
BDNF
1,3
2
4
(d)
LTP magnitude (%)
##
BDNF (20 ng/ml)
CTR
PCP
(e)
BDNF concentration (pg/mg)
CTR
PCP
(f)
CTR
PCP
TrkB-FL
145 kDa
GAPDH
37 kDa
TrkB-FL levels (fold of CTR)
CTR
PCP
(g)
CTR
PCP
TrkB-TC
95 kDa
GAPDH
37 kDa
TrkB-TC levels (fold of CTR)
CTR
PCP

Figure 4.


(a) YMT
Correct alternations (%)
100
80
60
40
20
0
**
CTR
PCP
(b) NORT (Training)
Exploration time (%)
75
60
45
30
15
0
F
F'
F
F'
CTR
PCP
(c) NORT (Test)
Exploration time (%)
****
F
N
F
N
CTR
PCP
(d) NORT
Novelty Preference Index (NPI)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
*
##
CTR
PCP
(e) MWMT
Distance traveled (cm)
1000
800
600
400
200
0
**
CTR
PCP
Day 1
Day 2
Day 3
Day 4
(f) MWMT
Time in target quadrant (%)
80
60
40
20
0
CTR
PCP