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Parvalbumin-containing GABA cells and schizophrenia: experimental model based on targeted gene delivery through AAVs

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Corresponding Author:	Gernot Riedel, Assistant Editor - Reviews, PhD University of Aberdeen Aberdeen, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Aberdeen
Corresponding Author's Secondary Institution:	
First Author:	Gernot Riedel, Assistant Editor - Reviews, PhD
First Author Secondary Information:	
Order of Authors:	Gernot Riedel, Assistant Editor - Reviews, PhD
	Peer Wulff
	Marta U Woloszynowska-Fraser
Order of Authors Secondary Information:	
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Parvalbumin-containing GABA cells and schizophrenia: experimental model based on targeted gene delivery through AAVs

Marta U. Woloszynowska-Fraser[1,2], Peer Wulff[3], Gernot Riedel[1]

1. Institute of Medical Sciences, University of Aberdeen, AB25 2ZD, Scotland
2. Laboratory of Behavioral Neuroscience, National Institute on Aging, Biomedical Research Center, National Institutes of Health, Baltimore, Maryland 21224. USA
3. Institute of Physiology, Christian Albrechts University Kiel, 24098 Kiel, Germany

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Corresponding Author:
Gernot Riedel
Institute of Medical Sciences
Foresterhill
University of Aberdeen
Aberdeen
AB25 2ZD
SCOTLAND
Email: g.riedel@abdn.ac.uk

Abstract

Understanding the contribution of transmitter systems in behavioural pharmacology has a long tradition. Multiple techniques such as transmitter specific lesions, but also localised administration of pharmacological toxins including agonists and antagonists of selected receptors have been applied.
More recently, modern genetic tools have permitted cell-type selective interferences, for example by expression of light sensitive channels followed by optogenetic stimulation in behaviourally meaningful settings or by engineered channels termed DREADDS which respond to peripherally administered drugs. We here took a similar approach and employed a Cre recombinase dependent viral delivery system (adeno-associated virus, AAV) to express tetanus toxin light chain (TeLc) and thus block neural transmission specifically in parvalbumin-positive neurons of the limbic and infralimbic prefrontal circuitry.

PV-TeLc cohorts presented with normal circadian activity recorded in PhenoTyper home cages, but a reproducible increase in anxiety extracted in both the open field and light dark box~~elevated plus maze~~. Interestingly, working memory assessed in a spontaneous alternation Y-maze task was impaired in PV-TeLc mice. We also recorded local field potentials from a separate cohort and found no global changes in brain activity, but a behaviourally relevant lack of modulation in the gamma spectral band. These anomalies are reminiscent of endophenotypes of schizophrenia and appear to be critically dependent on ~~establish that failure in~~ GABAergic signalling through PV neurones ~~is sufficient for cognitive symptoms in psychosis~~. At the same time, these observations validate the use of viral vector delivery and its expression in Cre-lines as a useful tool for understanding the role of selective components of the brain in behaviour and the underpinning physiology.

Introduction

Specific neurotransmitters and the cell populations that release them fulfil highly specific functions in organizing brain activity and behaviour. While their role in normal brain activity is typically difficult to extract in humans , disease states with selective transmitter loss enable insights into their function. Schizophrenia (SZ) is an example of such a disease and affects around 1% of the world population (Perãlã *et al*., 2007). With an onset during adolescence/early adulthood (Lewis, 1997) its chronic and sever psychiatric state affects many aspects of life, including education, employment and family (Konopaske & Coyle, 2015; Volk & Lewis, 2015). Symptoms have been classified as positive, negative and cognitive, with the former two being of primary diagnostic value (American Psychiatric Association, 2013). Positive symptoms have the most dramatic manifestation and include hallucinations, delusions and hypersensitivity to psychostimulants (van Os & Kapur, 2009); negative symptoms such as anhedonia, social withdrawal and blunted affect, are closely linked to the morbidity associated with SZ, they are persistent and progressive (Barnes *et al*., 2014; Laruelle, 2014; Rømer Thomsen, 2015). Despite its lesser recognition, impairment of cognitive function constitutes an early onset and core feature of the illness (Carter, 2005; Keefe & Fenton, 2007), and covers domains such as working memory (Gooding & Tallent, 2004; Galletly *et al*., 2007; Barch & Caesar, 2012), attention (Cornblatt *et al*., 1997; Morris *et al*., 2013), executive function, problem solving and verbal memory (Heinrichs & Zakzanis, 1998). The proposed physiological underpinnings of these cognitive defects arise from altered brain circuitries, excitation/inhibition imbalance, and changes in neuronal oscillations (Haenschel *et al*., 2009; Andreou *et al*., 2015; Pittman-Polletta *et al*., 2015). Based on extensive neuropsychology and functional imaging, dysfunction of the dorsolateral prefrontal cortex (dlPFC) is critically associated with cognitive endophenotypes in SZ (Sagrado-Pineda *et al*., 2004; Lewis *et al*., 2011). Anomalies in neuronal activity are frequently observed in prefrontal cortex of SZ patients (Basar-Eroglu *et al*., 2007; Uhlhaas *et al*., 2008; Lodge *et al*., 2009) resulting in a lack of network activation between prefrontal cortex and hippocampus during cognitive tasks (Perlstein *et al*., 2001; Barch *et al*., 2003; Lodge *et al*., 2009) and working memory (Perlstein *et al*., 2001; Lewis *et al*., 2008).

The pathophysiology of SZ is dominated by strongly reduced Impaired GABAergic neurotransmission in dlPFC (Lodge *et al*., 2009; Fung *et al*., 2010; McNally *et al*., 2013) and levels of GAD67 mRNA (glutamic acid decarboxylase responsible for GABA synthesis) are reduced in post-mortem SZ brains (Straub *et al*., 2007). Most notable is this reduction or absence of GAD67 mRNA in ~50% of parvalbumin-positive (PV+) GABAergic cells (Hashimoto *et al*., 2003; Gonzales-Burgos *et al*., 2010; Curley *et al*., 2011), although there is no frank cell loss and the overall density of PV+ neurones in PFC is not different to that of healthy controls (Eyles *et al*., 2002; Hashimoto *et al*., 2003; Tooney & Chahl, 2004). Dysfunction of PV+ neurones is confirmed by lowered PV mRNA per neuron (Woo *et al*., 1998; Beasley *et al*., 2002) and more specifically in chandelier cells of the dlPFC in layers 3 and 4, but not in 2 or 5/6 (Beasley and Reynolds, 1997; Hashimoto *et al*., 2003). PV cells are critical for the control of network activity and by targeting somata and dendrites of principal cells execute powerful

modulation on the cell's discharge patterns, especially the highly synchronous oscillation in the gamma frequency band (>20Hz) that emerge as a signature during working memory (Basar-Eroglu *et al.*, 2007; Sohal *et al.*, 2009; McNally *et al.*, 2013).

We therefore proposed (Murray et al., 2015) that a logical approach for mimicking the pathology in dlPFC of SZ patients in rodents is the selective inactivation of PV+ GABAergic interneurons in the dlPFC homolog, i.e. the infralimbic and prelimbic prefrontal cortex of mouse. For our approach however, which has a strong translational component, we aimed for a long-lasting or constant lowering of GABAergic transmission from PV interneurons, conceptually similar to previous approaches used for glutamatergic transmission (Riedel *et al.,* 1999; Morris *et al.,* 2003; Micheau *et al.,* 2004).

To this end we generated AAVs carrying an inverted GFP-tagged tetanus toxin light chain (TeLc; or GFP alone as control) reading frame in a flip-excision cassette (AAV-FLEX-TeLc and AAV-FLEX-GFP) (Murray et al., 2011) by transfection of human embryonic kidney cells (HEK293) with the AAV backbone plasmids (see Murray et al., 2011; McClure et al., 2011; Murray *et al.,* 2015). TeLc is a bacterial neurotoxin that acts intracellularly (Gaisano *et al.*, 1994), by cleaving the vesicle-associated membrane protein-2 (VAMP-2). The VAMP (vesicle associated membrane protein) family is comprised by 7 members involved in vesicle fusion and thus form an integral part of the core of the SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors) complex (for review, see Cupertino *et al.,* 2016). Therefore, TeLc affected neurones are unable to complete vesicle docking for transmitter release and our viral transfection tool constitutes a permanent inactivation of transmission of the cell whereas the external inputs remain intact. While AAVs will infect all cells close to the injection sites, gene expression is limited to Cre recombinase expressing cells (see Murray *et al.,* 2011; McClure *et al.,* 2011 for description). Consequently, Cre expressing neurones, which also express TeLc are the only cells with reduced neurotransmitter release (Yamamoto *et al.*, 2003; Atasoy *et al.*, 2008). As Cre expressing line for the experiments reported here, we selected PV-Cre mice and targeted the medial prefrontal cortex below motor cortex and cingulate for viral injection. Animals were tested behaviourally and/or implanted with twisted gold wire electrodes for local field potential recordings in the prefrontal cortex and ipsilateral input structures in the dorsal hippocampus. Data clearly conform that PV cells are critically involved in locomotor activity, working memory and the generation of gamma oscillations of prefrontal cortex.

Methods:

Ethical Statement

All experiments were performed under the United Kingdom Animals (Scientific Procedures) Act 1986 and EU directive 63/2010EC and approved by the local Ethical Review Committee.

Animals

Mice expressing Cre recombinase in PV+ cells were purchased from The Jackson Laboratory (Maine, USA; Pvalb/B6;129P2-*Pvalb*$^{tm1(cre)Arbr}$/J; Repository number PV 008069), and a colony established at a commercial breeder (Charles Rivers Laboratories UK). The colony was maintained in isolator cages running on positive pressure and was expanded for provision of subjects for experiments. Both male and female mice were used in this study with similar numbers of each gender. They were genotyped for expression of Cre and then delivered at an age of 8-12 weeks to our local facility and habituated for 1-2 weeks prior to experiments. Mice were housed in same gender groups in Macrolon type II cages at a 12-hour light/dark cycle (lights on at 7am, simulated dawn and dusk), in temperature (18.7˚C; ±0.4˚C) and humidity (63.3%; ±5%) controlled holding rooms (3-4m) with 20-30 air exchanges per hour. At no time were animals food or water restricted and groups were maintained even following surgery and electrode implantation (for details, see below).

Virus, Viral infusion and electrode implantation

The AAV vector was generated as previously described (Murray *et al.*, 2011). . Green fluorescent protein (GFP) or GFP fused to tetanus TeLc was cloned in the AAV vector in a reverse reading frame (3'-5') between two sets of anti-parallel heterotypic *loxP* sites. In the presence of Cre-recombinase the reading frame flipped into the 5'-3' orientation allowing the expression of the toxin and GFP reporter gene only in Cre positive cells (Murray *et al*., 2011). AAVs were produced at Penn Vector Core (University of Pennsylvania, USA).

Glass micropipettes prepared on a pipette puller (~8 µm bore) were filled with 3.5µl of AAV (titre ≥6x10^6 infectious particles per µl) using 20ml Hamilton syringe avoiding bubbles. They were kept on ice until use. Depth electrodes were made of double stranded and twisted insulated gold wires (ADVENT Research Materials, Oxford, UK) soldered onto a gold plated pin connector. Gold plated watchmaker screws (TSE Systems GmbH, Bad Homburg, Germany) were used as reference electrodes and soldered onto Kynar wire (ProPower 100-30B, 0.05mm^2, copper; A Premier Farnell, UK) whereas the distal end attached to a gold plated pin connector. Pins were assembled into a connector board to form the head-stage.

Mice were anaesthetised using isoflurane (IsoFlo, AbbVie, Germany; 3% for induction, 1-1.5% maintenance; Murray *et al*., 2011). Viral delivery to the brain was performed as previously described by Cetin *et al*. (2006) with the animal's head positioned in a stereotaxic frame (Stoelting, USA) with the skull levelled. The

skull was exposed through a longitudinal incision to the skin, Bregma was identified and burr holes were drilled bilaterally for medial PFC (+2.0mm anterior/posterior (AP) and ±1.0mm lateral (L)) and for CA1 (-2.0mm AP and ±2.0mm L), according to the mouse stereotaxic brain atlas (Paxinos & Franklin, 2001). Two screw ground/indifferent electrodes were inserted in holes above the posterior part of the parietal lobes and an additional screw purely for anchoring purposes was placed into the anterior part of the frontal plates. Glass micropipettes were advanced into the PFC at an angle of +/- 17° to the depth (D) of -2.0mm from Bregma. AAVs were pushed through into tissue through micro-tube-connected to a 60ml Hamilton syringes and 3µl AAV was infused over the 5-10min. Pipettes were left in place for further 2 minutes to avoid backflow, removed and replaced by twisted LFP gold wire electrodes. Dental cement (Kemdent™ simplex rapid liquid and powder) fixed the electrodes into place, and the procedure was repeated for hippocampal placement of electrodes. Finally, a head cap was modelled with wires assembled into a head stage and pins protected by a light weight dummy connector. Surgery was completed by intraperitoneal injection of sterile saline (1ml 0.9% sodium chloride; Baxter, UK) and subcutaneous injection of analgesic (0.3ml Temgesic, 300µg buprenorphine, RB Pharmaceuticals Limited, UK). For immediate recovery, animals were singly housed and placed on a heated plate to mitigate hypothermia. Animals from individual cages were operated in sequence and randomly assigned to virus groups TeLc or GFP. The same animals were re-united after surgery and maintained at ad libitum water and food in this group until completion of experiments.

Three cohorts of animals were used in total, from which cohort A (n=10 each) was equipped with recording devices (Fig. 1). All other cohorts (B: n=29 each; C: n=12 GFP, n=15 TeLc) were virus infused only.

Behavioural tests, apparatuses, protocols and analyses

A series of behavioural tests were conducted starting 10-14 days post-surgery. The timeline for the different cohorts and their assignment to the test is summarised in Fig. 1. All behavioural observations were carried out during the light phase (7am-7pm), recorded and analysed through overhead cameras using Ethovision 3.1 software (Noldus IT, Wageningen, Netherlands) and cohorts were run in several replications. After week 5, animals were perfused intracardially and tissue was harvested for further ex vivo analyses (not shown here).

Open Field (OF): The OF (custom-made square white Perspex arena 60x60x60cm) was performed as described in Murray *et al.,* 2015. Individual test mice were placed in the centre of the arena and allowed to explore it freely for 10mins in a single trial. The arena was cleaned with 70% ethanol (EtOH) after testing. Overall path length was recorded as a proxy of locomotion and an inner zone was contrasted to the outer zone (equiarea) as an index of anxiety.

Y-maze and EEG recording during behaviour: A spontaneous alternation test was conducted in the Y-maze to assess working memory (WM). This exploits the natural tendency of mice to visit alternate arms and we followed our previous protocol (Murray et al., 2015). The maze consists of 3 equally sized arms (A, B and C;

each 60cm long, 10cm wide, 10cm high) configured in a Y shape (angled at 120˚). A CCTV camera connected to the Ethovision software captured the movement of the animal during the first (10days post-surgery) and a second (21 days) Y-maze exposure (see Fig. 1 for cohort A and B). The first exposure was treated as a habituation session and data are not shown. After release into A, animals freely explored the maze for 10 mins and entries were recorded once all 4 paws had left the previous arm. Each arm entry was catalogued to derive the number of alternations (consecutive entry into three different arms) and the alternation index = number of alternations/(arm entry-2) x 100. Additional proxies included velocity; time spent in proximal or distal segments of each arm, and overall number of arm entries as an additional index of activity.

For cohort A (Fig. 1) local field potentials (LFPs) were recorded from PFC (and CA1; not shown) while animals were freely performing the spontaneous alternation task in the Y-maze. Their dummy protector was removed and replaced by the wireless EEG recording devices (NeuroLogger; TSE Systems) with all 4 channels sampling at 200Hz and band pass limited (1-70Hz). Transitions between arms and proximal/distal zones (Fig. 1D) were registered through the Ethovision software and TTL pulse fed into an infrared (IR) generator. We developed unique pulse sequence signatures for each transition and these were event stamped by an IR sensor on the NeuroLogger (transitor-transitor logic pulse activated IR transmitted to the logger). Only entries into zones were registered; yet the direction of movement was deduced from the sequence of entries and could be inwards (towards the centre with behavioural decision) or outwards (after entry into new arm running towards end of arm). This segregation enabled recording and analysis of behaviourally relevant EEG patterns during varying stages of spontaneous alternation responding. NeuroLoggers were removed after the experiment and data downloaded and extracted into txt format using a customised MatLAB script (MatLAB 8.3, The MathWorks Inc., Natick, USA).

PhenoTyper: Circadian rhythms were recorded in PhenoTyper home cages (Noldus IT, Wageningen, Netherlands) as described previously (Robinson *et al.*, 2013; Robinson & Riedel, 2014). PhenoTyper boxes (30cm x 30cm floor) are equipped with an overhead IR sensitive camera and food and water dispensers. Horizontal movements (path length) were recorded over 72 hours and analysed using Ethovision software with no food or water restrictions. The first three hours of the recording were treated as the habituation period in which animals adapted to their new environment; the remainder was averaged in 1hr bins to attain a day/night plot. Diurnal activity of the animals during dark (7pm-7am) and light (7am-7pm) phases was also analysed (mean of 1hr bins during dark and light phases).

Light/dark box (LDB): The LDB consists of two compartments – open-top light box (30x30x30cm) and closed dark box (30x20x25cm) connected by a small aperture. Animals were placed into the light compartment and allowed to freely explore the apparatus for 10 minutes. A CCTV camera recorded horizontal activity in the illuminated compartment only and time in each compartment was used as a proxy for anxiety (more in dark).

Analysis of Local field potentials (LFPs): ASCII (American standard code for information interchange) data files with multiplexed orientation, time domain, and sampling interval of 5000µs (200Hz) where uploaded and

analysed using BrainVision Analyzer 2.0 software (BrainProducts GmbH, Germany). Initially, we considered the whole trial and a power spectral analysis using Fast-Fourier Transform (FFT) from 4 second bins. Next, data was segmented according to the start/end of trial and positioning in the maze (distal and proximal zones) based on identified IR event stamps. Event strips of the same category (for example all distal zones) were averaged for each animals and pooled across treatment groups. Spectra are presented as absolute power (μV^2) between 4.2Hz and 79.8Hz with the following bad-width: 4-9Hz (theta), 9-14Hz (alpha), 14-20Hz (beta), 20-50Hz (gamma low) and 50-80Hz (gamma high).

Statistical analysis

All statistical analysis was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). Group comparisons utilised either unpaired 2-tailed Student t-tests or factorial 2-way Analysis Of Variance (2-way ANOVA) with group as between and time as repeated measure followed by Bonferroni multiple comparisons post-hoc tests. Alpha was set to 5%. Only significant terms are given.

Results:

Normal global activity, but increased anxiety in PFC PV-TeLc mice

General motor activity was assessed in a square OF during a single exploration trial (Fig. 2). Neither the overall distance moved nor velocity were different between groups, suggesting that the locomotor activity of TeLc-injected mice was intact (PV-GFP n=10 and PV-TeLc n=10). As for the spatial distribution, both groups spent significantly more time in the proximity of the walls relative to the centre ($F_{(1,18)}$=273.4, p<0.0001). However, there was a significant interaction between zone and virus ($F_{(1,18)}$=5.578, p<0.05), which was confirmed as significantly less time of PV-TeLc animals spent in the centre than PV-GFP subjects (t=2.365, df=18, p<0.05) and substantially more time in the outer zone of the OF (t=2.359, df=18, p<0.05). These data suggest a role of PFC PV cells in either trait or state anxiety.

Impaired spontaneous alternation in Y-maze

The behavioural analysis of working memory measured in the Y-maze using spatial alternation is widely used in rodents since its first description in 1958 (Dember & Fowler; 1958; Lalonde 2002 for review). We ran two cohorts (A and B) in this paradigm but could not identify behavioural differences between the two cohorts. The behavioural analysis is based on cohort B (n=29 each) while the physiological account is based on cohort A. We assumed that owing to the lack of differences between the two cohorts, implanted subjects seem to truthfully represent the normal group behaviour. Locomotor activity of PV-GFP and PV-TeLc animals did not differ (Fig. 3A,B). Both groups covered a similar distance during the 10-minute test and moved at the same velocity. Time in proximal versus distal zones revealed that more time was spent distal zones of each arm despite the fact that zones were equal in size ($F_{(56,1)}$=14.58, p<0.001 for main effect of zones; for post-hoc, see Fig. 3C). As an additional measure for activity, we recorded the number of arm entries (Fig. 3D) which was not different. An obvious, yet unreliable, reduction was observed in the number of alternations for the PV-TeLc treatment (Fig. 3E) suggesting a small impairment in working memory performance. This was confirmed in the alternation index (Fig. 3F) which was significantly lower in PV-TeLc mice relative to PV-GFP controls (t=2.785, df=56, p<0.01). Both PV-GFP and PV-TeLc animals performed significantly above the chance level of 50% (t=6.819, df=28, p<0.0001 and t=2.807, df=28, p<0.01, respectively).

Spectral power of prefrontal LFPs was extracted for i) the whole 10 minute session and ii) for the IR-identified segments corresponding to behavioural entries into pre-defined zones. For the entire session (Fig. 4), there was no global effect of the virus on prefrontal power at low or high frequency bands. Despite some small reductions in the Theta and Alpha frequency bands (Fig. 4A), the power was highly variable and not significantly different (Fig. 4A-C). In addition, we did not obtain an interaction between frequency and virus if individual bands were considered. In behavioural terms, we have rationalised a segmentation of the

behavioural response in terms of inward and outward movement, but also more importantly in terms of distal and proximal zones with the latter having important valence as a decision for entry into a new arm is imminent. FFT from segments recorded when animals traversed this decision zone are depicted in Fig. 5. A global lowering in power in Theta, Alpha and Gamma bands became more pronounced for the PV-TeLc cohort and this was significant for Alpha and Gamma bands (see asterisks in Fig. 5A-C). In the context of different alternation performance, it is noteworthy that a higher alternation of PV-GFP mice is paralleled by significantly higher Gamma band power compared with PV-TeLc.

Circadian rhythms are not affected by inactivation of prefrontal PV cells

The circadian rhythm of the animals was measured using PhenoTyper home cages (Fig. 6). Upon first introduction into the new home cages, animals were allowed to habituate, which was complete after about 2.5-3 hours. All test animals showed heightened exploratory activity at the beginning of the recording and their path length gradually declined over time. Neither peaks nor the time course of the decline differed between treatment groups. However, there was a significant effect of time on the movement of these animals ($F_{(17,425)}$=67.65, p<0.0001), but time did not interact with viral treatment (Fig. 6 A).

General locomotor activity of the animals was analysed during dark (7pm-7am) and light (7am-7pm) phases. Both PV-GFP and PV-TeLc animals showed increased activity during hours of darkness (Fig. 6B) and no difference between the groups was detected. There was a significant change in activity of both PV groups depending on the time of the day (main effect of time: $F_{(23,575)}$=45.8, p<0.0001), with both groups increasing their movement during the dark phase. No interaction between time and viral treatment was detected. There was a strong effect of phase ($F_{(25,1)}$=136.8, p<0.0001) when light and dark period were contrasted (Fig. 6C), but this happened to a similar degree in both groups.

Heightened anxiety in PV-TeLc mice in the light-dark box

The light/dark box (LDB) is an anxiety test that relies on the natural aversions of rodents towards bright and open places (Kulesskaya & Voikar, 2014). We exposed our mice to the LDB for 10 minutes and recorded the distribution between illuminated and darkened compartment (in seconds) for PV-GFP (n=11) and PV-TeLc mice (n=10). 2-way ANOVA revealed no differences between place (light or dark box) and viral treatment. PV-GFP animals spent similar times in both compartments (chance level of 300s, t<1). PV-TeLc animals on the other hand spent significantly more time in dark compartment of the apparatus as confirmed Student's t-test (t=5.716, df=18, p<0.0001). Time spent by these mice in light box was substantially below chance level (t=4.042, df=9, p<0.01). There were no differences between the groups in time spent in light and dark boxes during whole 10min test and in 5min bins. These results indicate that lack of prefrontal PV+ cell discharges induced anxiogenic effects.

Discussion

In this study, we show that region- and cell-type-specific interference with transmitter systems using AAV delivery of gene constructs to cause transmitter silencing can have pronounced effects on both behaviour and physiology, in which the network under scrutiny is normally involved. This has been achieved by a single surgical treatment of a Cre-expressing mouse line and lead, as hypothesises at the outset, to anomalies reminiscent of a schizophrenic phenotype. Here we first consider the technique of viral cell silencing and contrast it to alternative viral methods. Then, we discuss the results in the context of our hypothesis, i.e. the role of the PV-GABAergic network in SZ.

Methodical consideration

Techniques alternative to the one used here have been considered during the development of these experiments. In the field of chemogenetics, DREADDs are engineered macromolecules that interact with previously unrecognised small molecules and come in different forms. The most widely used are receptors activated solely by synthetic ligands (RASSLs) or designer receptors sensitive to the systemic administration of an inactive clozapine analogue clozapine-N-oxide (CNO). While this in itself does not enable region or cell-type specificity, both characteristics can be achieved in an experimental setting through AAV viral delivery of gene constructs in a flip-excision cassette (FLEx-switch; Schnuetgen *et al.*, 2003; for further details, see below). Viruses containing loxP sites are microinjected into the brain region of interest of Cre-expressing mice under general anaesthesia and after recombination in situ, any systemic administration of CNO would later selectively activate these expressed DREADDs in the region/cell type, in which Cre recombinase is expressed. If mcherry or tdTomato are used as part of the gene sequence, shining light onto the cells expressing these receptors would enable optogenetic activation of the cells. Light activation leads to immediate depolarisation / hyperpolarisation of the neurone and while optogenetic stimulation has a highly controllable and rapid onset and offset, CNO may take a few minutes and about 2 hours to be cleared from plasma so that DRAEDDs can be reactivated repeatedly. (Note that this is not possible with our AAV-TeLc technology.) Many experimental approaches benefit from high fidelity of the optogenetic approach as exact temporal resolution is required. For our approach however, which has a strong translational component, repeated inactivation of a GABAergic cell group through laser or CNO administration is clearly different from the pathological condition of SZ patients. As these patients suffer from a permanently reduced inhibition and a lack of modulation of inhibitory circuits in prefrontal cortex we aimed for a long-lasting or constant lowering of GABAergic transmission from PV interneurons. In addition, the requirement of a tether (optogenetics) frequently hampers natural behavioural responding in the test animal (Deisseroth, 2015).

Moreover, use of genetically modified animals can further enhance the selectivity of the viral construct. The usage of FLEx cassettes make the vectors sensitive to Cre-recombinase and a great number of

mouse lines are now available with cell selective expression of Cre (see Jackson Laboratories website: https://www.jax.org/jax-mice-and-services) and for investigation of GABAergic neurons (Taniguchi *et al.,* 2011). Although one would have expected this to become a powerful tool for the investigation into the role of different cell populations in predefined brain regions, the method has been slow to be embraced by scientist possibly due to the low fidelity in terms of onset of effect and irreversibility. On the other hand, utilisation of AAV delivery to express foreign genes, endogenous genes, antisense RNA or of RNAi has been widely used experimentally and some progress has been made towards its use in disease treatment (for review see McCown, 2011). In terms of its use infecting and activating/inactivating specific cell types recent work has selectively expressed α-synuclein in dopaminergic neurons (Caudal *et al.*, 2015), Kir4.1 sodium channels in astrocytes (Vagner *et al.*, 2016; Dvorzhak *et al.,* 2016); or replacement therapy expressing the FMR1 gene in fragile X syndrome (Gholizadeh *et al.*, 2014).

For the specific scientific questions we sought to answer, our choice was the PV-Cre line described previously in our studies (Murray *et al.,* 2011; 2015; Hippenmeyer *et al.,* 2005). We have utilised the method also to transfect AAV into other GABA containing interneurons selectively expressing Cre-recombinase (unpublished). The medial prefrontal cortex, especially prelimbic and infralimbic regions were selected based on their homology to the dlPFC of humans and our interest in the role of GABAergic neurons in SZ.

PV-cell inactivation and schizophrenic phenotypes

Clearly, the manipulation of PV cells with TeLc induced behavioural alterations reminiscent of schizophrenic phenotypes. Our animals presented with anxiety phenotypes in the open field and light dark box, with impaired working memory measured as a lower alternation index in the Y-maze spontaneous alternation task coincident with a severe deficit in gamma frequency power recorded in PFC. The traits are highly selective as there was no anomaly in terms of home cage activity or circadian rhythms.

Recent evidence suggests that the emergence of anxiety syndromes is a source of morbidity in patients with SZ. According to the recent review of Brada and colleagues (Brada *et al.,* 2013) the epidemiology of anxiety disorders is present in more than 38% of SZ patients, with social phobias being the most frequently observed symptoms. Clearly, these are difficult to mimic in experimental models and we here have used two frequently applied behavioural tests for anxiety in rodents. Consistently, our PV-TeLc mouse model presented with heightened anxiety, which we consider as a trait of the model. It is consistent with stress models of anxiety that have revealed a lowering of PV-GABA neurones in prefrontal cortex of mice; this phenotype can be rescued by environmental enrichment (Sampedro-Piquero *et al.,* 2016). Although we have not mechanistically assessed the expression of this anxiety phenotype in PV-TeLc mice, our method has led to a lasting functional

impairment of parvalbumin neurones in prefrontal regions (Murray *et al.,* 2015). Whether this can be rescued by treatment using anti-depressive drugs or environmental enrichment remains to be determined.

The most profound cognitive deficits in SZ include executive function and working memory (Bach & Ceasar 2010; Goldman-Rakic, 1995; Benchenane *et al*., 2011). Necessary for complex cognitive functions, transient maintenance and modulation of information through the working memory system can be explored across modalities and species allowing translation to the patient. Our approach of back-translation from the human SZ, in which the cellular basis of working memory failures appears to arise from a lack of persistent firing of principle neurones in prefrontal regions (Fuster, 1997; Jones & Wilson, 2005; Benchenane *et al*., 2010), also accounts for the observation that SZ pathology is found as a lowering in cellular markers for PV GABAergic cells (see Introduction for details). A particular role is played by the GABAergic circuitry, which applies inhibition to the otherwise persistent activity of principal cells such that an optimal excitation/inhibition balance is achieved and selective cortical brain rhythms are established (Starc *et al.,* 2017). The rhythmic inhibition by PV-GABA cells entrains prefrontal cortex with highly synchronous gamma oscillations and these may provide a physiological marker for the working memory process (Powell *et al*., 2012). In line with these predictions, our PV-TeLc mice presented with impaired working memory, but no global anomalies in prefrontal brain activity during this task. Our mice also showed normal exploration patterns, normal activity and were specifically reduced in the normalised number of alternations, i.e. working memory. However, when expediting the physiological responses, we realised that a crucial period of the working memory task is the moment in which the animal traverses the proximal zone within each arm leading directly on to the entry into the next arm. At this stage, we have not discriminated between a correct or incorrect alternation as each run through the proximal arm leads to a decision. In terms of behaviour, animals seem to linger in the distal zone longer than the proximal part of each arm. This seems to establish that the transition through the proximal arm is more purposeful and with the aim of exiting arm x and entering arm y, so that tracking prefrontal activity throughout this period would reveal a proxy for this decision process. Indeed, our PV-GFP mice showed high levels of gamma spectral power (both low and high) while this was considerably and significantly reduced in mice lacking PV-GABA activity due to TeLc expression. It is conceivable that the transition of the proximal zone is a period of heightened cognitive load (which arm have I explored last? which direction do I preferentially go? ….) that requires fast spiking cells to drive modulation of neural activity (Cardin *et al*., 2009; Goonawardena *et al.,* 2010a), most likely in the gamma frequency band. Removal of PV cell input onto the pyramidal cells may disturb this oscillatory adaptation and it is behaviourally displayed as a lowering of working memory performance.

It is therefore likely that the disturbance of the excitation/inhibition balance in medial PFC in our PV-TeLc mice is the underlying cause for the failure to execute working memory and the model is consistent with decreased mRNA levels of cortical PV in patients with SZ (Hashimoto *et al*., 2003, Fung *et al*., 2010). It has similarly modelled using pharmacological treatments of ketamine (Jeevakumar & Kroener, 2014; Jeevakumar *et*

al., 2015; Jadi *et al*., 2015) further supporting the notion that the critical pathology for the cognitive symptoms of SZ is the loss of function of the inhibitory system in PFC (Murray *et al.,* 2015). Moreover, patients with SZ cannot synchronise their neuronal activity (Basar-Eroglu *et al*., 2007; Spellman & Gordon, 2015) and display lower power of frontal lobe gamma oscillations during performance in tasks that require cognitive control (Cho *et al*., 2006) confirming the importance of high frequency oscillations in cognitive experiences. Although not yet fully explored in terms of alleviation of all symptoms, it is clear that administration of benzodiazepine derivatives to boost the activity of $GABA_A$ receptors can increase frontal gamma power and reverse working memory deficits in patients with SZ (Williams & Boksa, 2010; Lewis *et al*., 2008).

Although our data suggest that decision making in working memory tasks is coincident with periods of high-frequency oscillations, our data cannot yet discriminate between correct and incorrect decisions. An inherent problem of spontaneous alternation is the way the alternations are scored. An alternation consists of three visits to 3 different arms, but each exit and entry constitutes a decision; some are correct (and are alternations), some are incorrect (such as visits to the previous arm). It is not clear to us what drives these alternations and how they can be distinguished in terms of motivational responses. From an analytical standpoint, all entries require decision making and we have so far treated them equally for the sake of sampling and event averaging. Differences in their quality are difficult to establish and arbitrary and our recordings may not be able to selectively discriminate between correct/incorrect responding. This would require the administration of different behavioural paradigms, for example delayed matching or non-matching to sample procedures (DMS/DNMS), in which correct responses are rewarded and incorrect ones are punished (see Goonawardena *et al.,* 2011; 2010b). These would present a more tractable task for treatment options and a more direct forward translation into the clinic.

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Figure legends:

Fig. 1: **Study design, cohorts and sizes.** A-C: Study protocols for the three different cohorts tested in this study. Cohort A underwent AAV administration and electrode implantation to record local field potentials (LFPs) and was submitted to two Y-maze tests at the beginning of week three and during week 4 and a final open field exposure. Cohort B followed a similar time line, but had no electrode implants. Cohort C matched B in terms of viral administration but animals were tested in the light/dark box (LDB) and PhenoTyper home cages for measurement of circadian activity. ~~D) Segmentation of the Y maze into 3 equivalent arms (A,B,C) and distal (A1,B1,C1) and proximal sectors.~~ The Table indicates number of animals tested in each behavioural paradigm, for details see Methods).

Fig. 2: **Locomotion and anxiety measured in the Open Field**. **A)** Horizontal activity (distance moved) and **B)** movement speed in treatment groups did not differ. **C)** Time spent in the inner and outer zones of the OF revealed subtle differences related to viral status of the groups. Data are presented as Mean +/- SEM. Unpaired 2-tailed Student's t-test * p<0.05; *** p<0.0001 following 2-way ANOVA (see Results).

Fig. 3: **Locomotion and working memory in the Y-maze spontaneous alternation task.** Segmentation of the Y maze into 3 equivalent arms (A,B,C) and distal (A1,B1,C1) and proximal sectors is also presented at the bottom.~~.~~ **A)** Horizontal activity (distance moved) and **B)** movement speed in treatment groups did not differ (see also Fig. 2 for comparison). **C)** Time spent in proximal or distal segments of the maze. Note preference for distal zones in both groups. **D)** Number of arm entries and **E)** number of correct alternations were not significantly affected by PV blockade. **F)** The alternation index, a measure of working memory, was reliably reduced in PV-TeLc mice. Group means + SEM; unpaired Student's t-test (2-tailed): ** p<0.01, *** p<0.001.

Fig. 4: **Spectral analysis of LFPs recorded during spontaneous alternation testing.** Recordings from PFC for entire 10 minute session. Group mean +/- SEM. **A)** Selected low frequency bands up to 20 Hz; note that absolute spectral power varies in Theta band, but there was little difference in Gamma low **(B)** or Gamma high **(C).** PV-GFP (n=10) and PV-TeLc (n=10). None of the differences reached significance.

Fig. 5: **Spectral analysis of LFPs recorded during spontaneous alternation testing.** Recordings from PFC for time spent in proximal (decision) zones (see right top corner). Group mean +/- SEM. **A)** Selected low frequency bands up to 20 Hz; note that absolute spectral power varies in Theta and Alpha band, but also in Gamma low **(B)** or Gamma high **(C).** PV-GFP (n=10) and PV-TeLc (n=10). Asterisks indicates reliable group difference (2-way ANOVA; p<0.05). Note that there was an overall lowering in power in the PV-TeLc animals, which affected the Alpha band (synchronised EEG) and there was significantly reduced Gamma power.

Fig. 6: **Circadian rhythms in PV-TeLc mice. A)** Habituation to the novel environment of the PhenoTyper measured as activity (distance moved) during the first 3 hours of exposure. Both PV-GFP (n=12) and PV-TeLc (n=15) animals did not differ. **B)** Horizontal activity (distance moved) recorded for 3 days and pooled over a 24 hour cycle in hourly bins. Dark section indicates night hours and show highly increased activity of these nocturnal animals. No difference was observed between treatments. **C)** Mean group activity separated for dark and light phase. Heightened nocturnal activity compared to the light phase (asterisks) did not differ between groups. Mean +/- SEM. **** p<0.0001, Student T-test, paired

Fig. 7: **Anxiety test in the light/dark box.** Time spent in each zone during 10min test by PV-GFP (n=11; green columns) and PV-TeLc mice (n=9, blue columns). PV-GFB spent equal amounts of time in each zone, but PV-

TeLc mice anxiously preferred the dark box. Group mean + SEMs. **** p<0.0001, Student T-test, paired; # p<0.05 against chance level, one sample t-test.

Figure 1 revised
Wołoszynowska-Fraser et al., Fig. 1

Bahavioural paradigm	PV-GFP (n)	PV-TeLC (n)
Open field (OF)	10	10
Y-maze	29	29
Y-maze LFP	10	10
Circadian Rhythm	12	15
LDB	11	10

Fig. 1: **Study design, cohorts and sizes.** A-C: Study protocols for the three different cohorts tested in this study. Cohort A underwent AAV administration and electrode implantation to record local field potentials (LFPs) and was submitted to two Y-maze tests at the beginning of week three and during week 4 and a final open field exposure. Cohort B followed a similar time line, but had no electrode implants. Cohort C matched B in terms of viral administration but animals were tested in the light/dark box (LDB) and PhenoTyper home cages for measurement of circadian activity. The Table indicates number of animals tested in each behavioural paradigm, for details see Methods).

Figure 2
Wołoszynowska-Fraser et al., Fig. 2

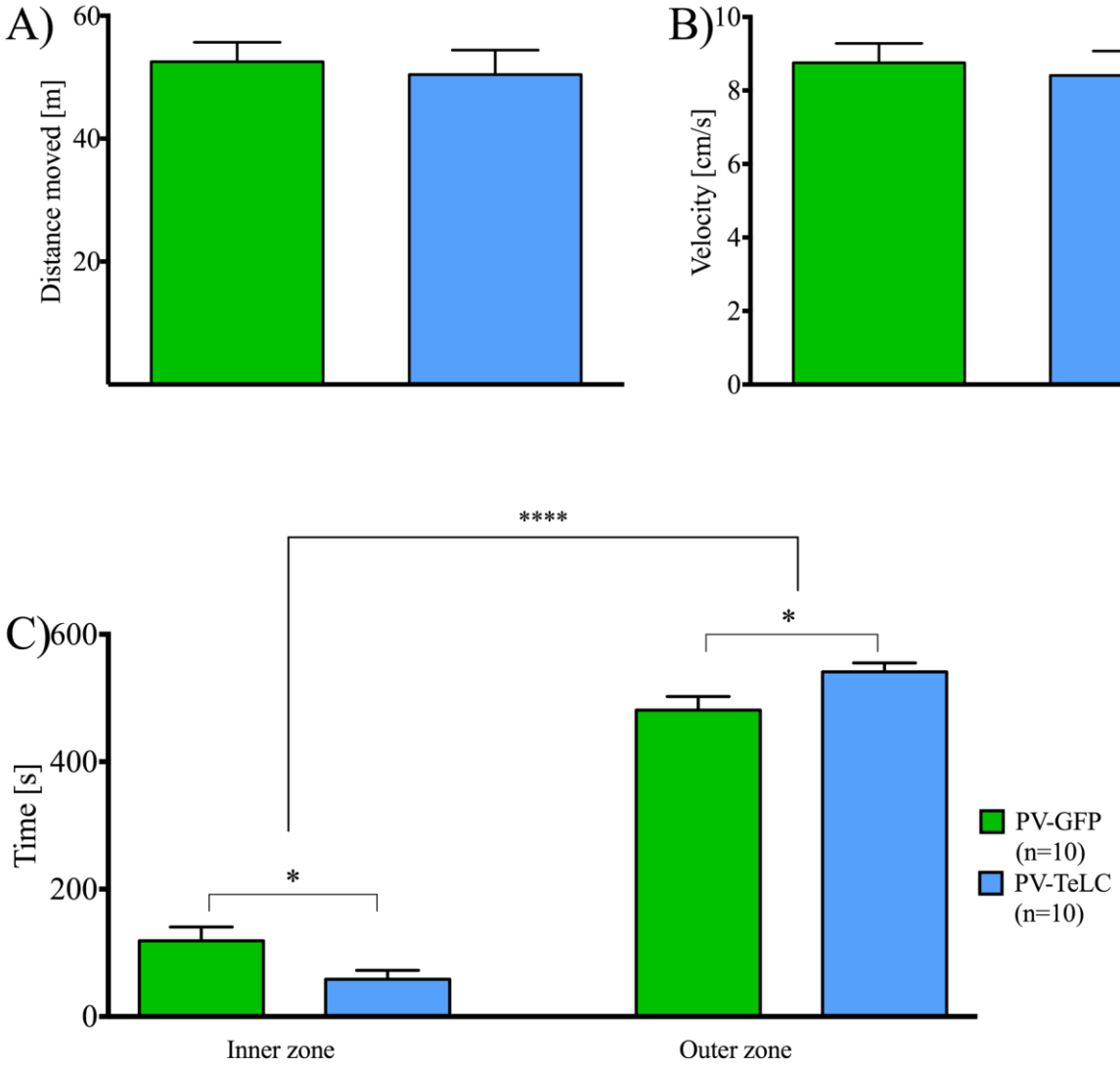


Fig. 2: **Locomotion and anxiety measured in the Open Field**. **A)** Horizontal activity (distance moved) and **B)** movement speed in treatment groups did not differ. **C)** Time spent in the inner and outer zones of the OF revealed subtle differences related to viral status of the groups. Data are presented as Mean +/- SEM. Unpaired 2-tailed Student's t-test * $p<0.05$; *** $p<0.0001$ following 2-way ANOVA (see Results).

Figure 3 revised
Wołoszynowska-Fraser et al., Fig. 3

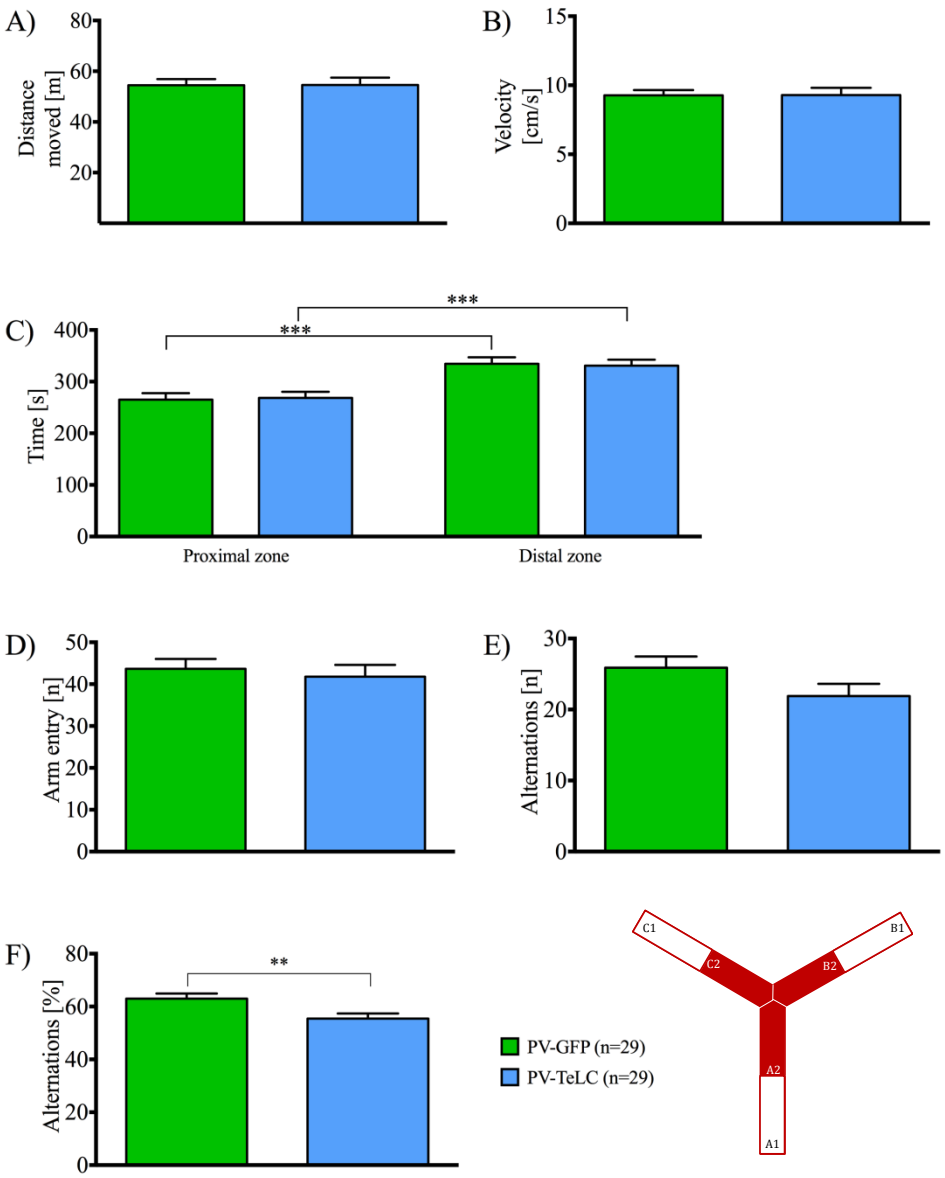


Fig. 3: **Locomotion and working memory in the Y-maze spontaneous alternation task**. Segmentation of the Y maze into 3 equivalent arms (A,B,C) and distal (A1,B1,C1) and proximal sectors is also presented at the bottom. **A)** Horizontal activity (distance moved) and **B)** movement speed in treatment groups did not differ (see also Fig. 2 for comparison). **C)** Time spent in proximal or distal segments of the maze. Note preference for distal zones in both groups. **D)** Number of arm entries and **E)** number of correct alternations were not significantly affected by PV blockade. **F)** The alternation index, a measure of working memory, was reliably reduced in PV-TelC mice. Group means + SEM; unpaired Student's t-test (2-tailed): ** $p<0.01$, *** $p<0.001$.

Figure 4
Wołoszynowska-Fraser et al., Fig. 4

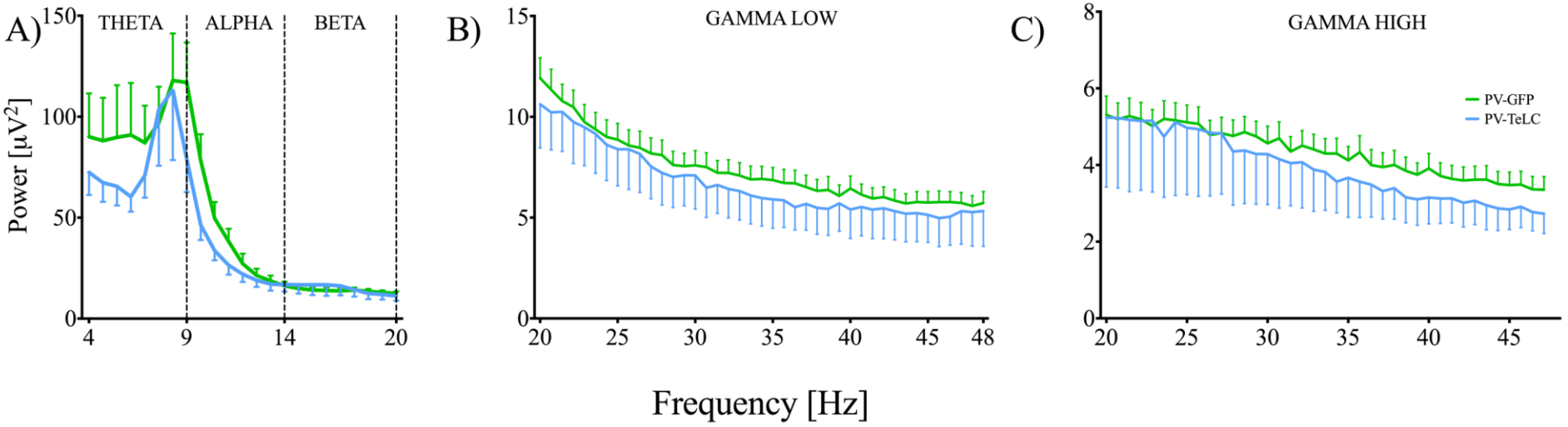


Fig. 4: **Spectral analysis of LFPs recorded during spontaneous alternation testing.** Recordings from PFC for entire 10 minute session. Group mean +/- SEM. **A)** Selected low frequency bands up to 20 Hz; note that absolute spectral power varies in Theta band, but there was little difference in Gamma low **(B)** or Gamma high **(C).** PV-GFP (n=10) and PV-TeLC (n=10). None of the differences reached significance.

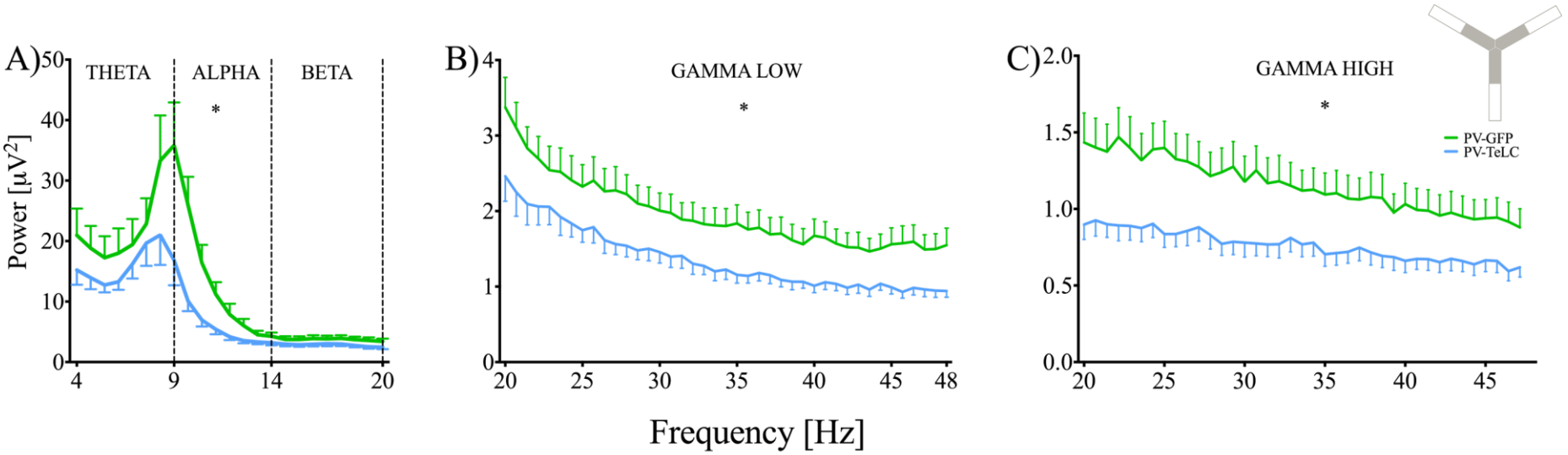
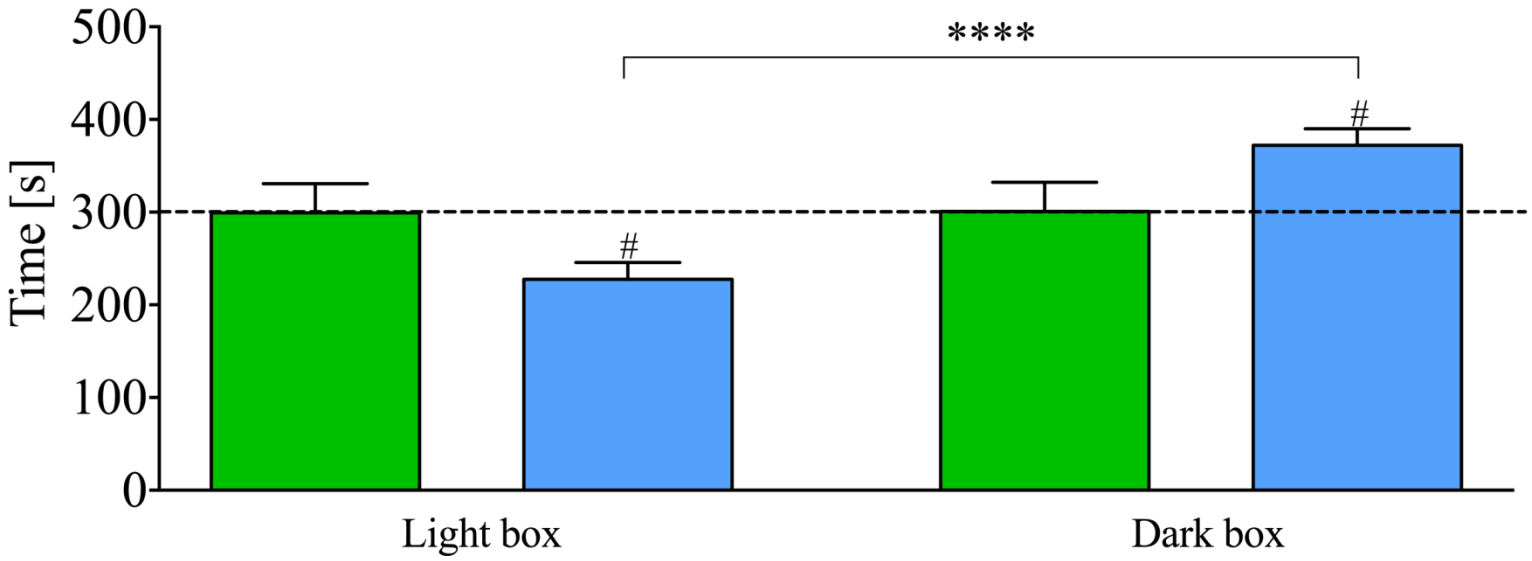


Fig. 5: **Spectral analysis of LFPs recorded during spontaneous alternation testing.** Recordings from PFC for time spent in proximal (decision) zones (see right top corner). Group mean +/- SEM. **A)** Selected low frequency bands up to 20 Hz; note that absolute spectral power varies in Theta and Alpha band, but also in Gamma low **(B)** or Gamma high **(C).** PV-GFP (n=10) and PV-TeLC (n=10). Asterisks indicates reliable group difference (2-way ANOVA; $p<0.05$). Note that there was an overall lowering in power in the PV-TelC animals, which affected the Alpha band (synchronised EEG) and there was significantly reduced Gamma power.

Figure 7
Wołoszynowska-Fraser et al., Fig. 7



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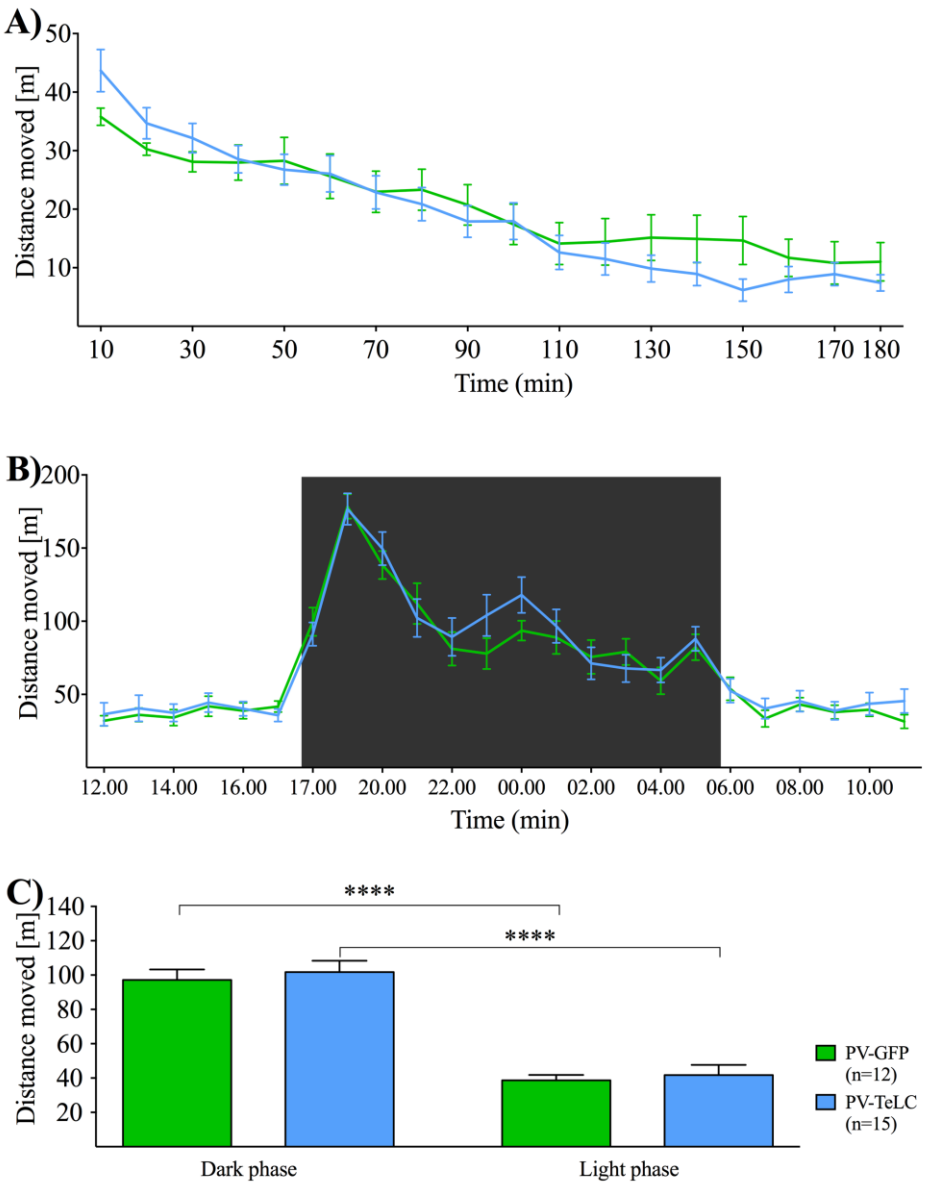

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Gernot Riedel, Ph.D.
Professor
University of Aberdeen
Department of Biomedical Sciences
Institute of Medical Sciences
Foresterhill
Aberdeen AB25 2ZD, UK
Tel - 0044 – (0) 1224 437377
Fax – 0044 – (0) 1224 437465
Email – g.riedel@abdn.ac.uk

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Behavioural Pharmacology

BP-17-127

Dear Paul,

Many thanks for the constructive comments on our paper. Please find below our answers to these comments including some explanations on why changes have or have not been made. All modifications/corrections in the text are highlighted in track-change mode.

If any further information is needed, please don't hesitate to contact me.

Kind regards, Gernot

Reviewer #1

1. **Abstract:**
   It seems to me that the paragraph dedicated to the genetic construct is to long as the technique has already been demonstrated to be relevant.
   I have not seen that the authors have used the elevated plus maze to evaluate anxiety.
   The following sentence is over interpreted: "and establish that failure in GABAergic signalling ….. is sufficient for cognitive symptoms in psychosis."

- We understand the comments and also are aware that the technique has been repeatedly used and thus is validated. However, since the report is specifically tailored for the special issue on Techniques in BP, we would like to maintain this (and the discussion section) for that matter.
- Thanks for the comment. It should read 'light dark box'. This is now corrected.
- The sentence has been changed to account for the comment. It now reads: These anomalies are reminiscent of endophenotypes of schizophrenia and appear to be critically dependent on GABAergic signalling through PV neurones.

2. **Introduction:**
   I don't think this is useful to use the abbreviation SZ for schizophrenia.

- SZ is widely used in the literature and from many colleagues used as a search item. We think it is appropriate.

3. **Methods:**
   - Subjects: both male and female mice were used, which is relevant as a translational approach but we need to know the distribution of each gender in each experiment to avoid bias due to sex differences. This has been well demonstrated that rodent males and females exhibit different sensitivity to anxiety-related situations.
   3 cohorts of mice were used: the third cohort (cohort C) was submitted to the LDB and the phenotyper observation but the animal size is different (see Figure 1). Why?
   - Viral injection: p6 are the AAVs infused through a 60mL Hamilton syringe? More importantly, do we have an idea of the extent of the diffusion? This is critical because although the infralimbic (IL) cortex is adjacent to the prelimbic (PL) cortex, these two regions of the brain have differential functions in the expression of anxiety-like behaviors. In addition, the PL has consistently been found to be necessary for the acquisition of goal-directed actions and the IL is belonging to stimulus-bound, habitual response system.
   - Open Field: because the authors are comparing in the result section the time spent in an inner and outer zone, it is necessary to know how these 2 zones have been

designed. I would have chosen 3 zone for better evaluation of the anxiety status of the mice.
- Y-maze: the presence of the Y-maze in Figure 1 is a bit odd. I would insert this Y-maze representation in the figure depicting the results. I am not very fond of Y-maze to evaluate working memory but I would like to know why the 2 Y-maze sessions are separated by 10 days?
- p7: to homogenise the formal presentation of this section it would be better to put a title for the description of physiological experiment.

- Subjects: We used similar numbers of each gender to avoid this bias (amendment in page 5 of MS). Cohort three only was for behavioural purposes. As described in the Methods, we also conducted EEG recordings in some cohorts and tried to have similar numbers of surgically operated animals and non-operated in each group to be able to compare and define any effect of surgery. Groups were pooled if no effect was detected leading to bigger cohort sizes.
- It is indeed correct that a 60 ml syringe was used to exert pressure in the micro-tubes and push the virus through the glass pipette into brain. The sentence is corrected in the revised MS. As for the area coverage, this has been described previously in Murray et al., 2015 Fig. 1, a paper cited in the manuscript. We achieved about 80% transfection within both structures and cannot distinguish specifically between pre-limbic and infra-limbic regions, both are affected equally.
- Open field: This was corrected in the text. We indeed used equal areas for inner and out zone.
- A Y-maze figure is now given in Fig. 3 together with the behavioural data. At the same time, it is removed from Fig. 1. Figure legends have also been corrected. As we were uncertain about the onset of effect of the virus, we selected two time points for behavioural assessment. The first marked an early time point at which the viral expression may already be effective. As for the second one, literature suggested a by then very well expressed level of viral DNA with an almost guaranteed cellular effect of the construct.
- We now reworded the sub-title of the Y-maze section to also reflect the EEG arm of the experiment.

4. **Results:**
General comment: please check the way the degrees of freedom are presented for the ANOVAs because sometimes we have F(18,1) and sometimes the other way around F(1,18).
Open field: the between zone comparison is relevant if the surface is the same for both zones.
Y-maze alternation: the proximal zone is considered by the authors to be the decision zone. Therefore, I would have compared the number of arm entries completed to the extremity of the arms to the arm entries restricted to the proximal. This would give a suspicion on decision making in the two groups. This is particularly interesting as one analysis of recording of local field potentials has been limited to the proximal zone of the Y-maze.
Prefrontal cortex recording: because of the distinct roles of the IL and the PL a

drawing of the histological control of the tips of the electrodes would be informative. Light-dark box experiment: I don't know what chance level is. The intensity of the light is unknown but we can assume that both compartments are not considered equally in terms of safety by the animals. Therefore, control mice would be more prone to go to the dark box, which is not the case in this experiment. Anyway, the comparison between light and dark boxes is sufficient.

- Degrees of freedom were a misspelling. Corrected. Thanks for spotting this.
- Open field. Indeed, equiarea was used, see also above.
- Y-maze: We have considered this in our original interpretation but found that mice hardly make any arm entry that is not connected with a visit to the distal arm. There seems to be no ambiguity for the decision in a mouse. In the end, we wanted to simplify the data set. Also, trials in which animals did not enter the decision zone from the distal end present an ambiguity of its own right as they meander within the decision zone for quite some time. This discussion reveals therefore that behaviour in the Y maze is considerably more complex than widely assumed.
- EEG recordings: Again, as already commented on for viral deposition, we have not discriminated between pre-limbic and infra-limbic cortex.

5. Discussion: the discussion on methodological consideration is far too long because the authors have already published on the technique with even more details (Murray et al., 2011; 2015). We have no doubt that the technique is relevant to inactivate parvalbumin-containing neurons.
Schizophrenic phenotypes:
- Anxiety: as reported by the authors anxiety is observed only in a fraction of schizophrenia patients. Knowing that PL are IL playing different roles in the expression of anxiety this should be considered in the discussion.
- Circadian rhythm: the absence of disturbance of the circadian rhythm in the TeLc mice is in disagreement of what is observed in schizophrenia patients. This should be a bit discussed.
The other parts of the discussion are appropriate.

- Again, the discussion of methods is relevant due to this being submitted for a special issue.
- As we have not distinguished between the two structures, we avoided debating their respective roles for anxiety and circadian behaviours.