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

Marta U. Woloszynowska-Fraser^{a,b}, Peer Wulff^c, and Gernot Riedel^a

^aInstitute of Medical Sciences, University of Aberdeen, Aberdeen, Scotland, ^bLaboratory of Behavioural Neuroscience, National Institute on Aging, Biomedical Research Centre, National Institutes of Health, Baltimore, Maryland, USA ^cInstitute of Physiology, Christian Albrechts University Kiel, Kiel, Germany

Abstract

Understanding the contribution of transmitter systems in behavioural pharmacology has a long tradition. Multiple techniques such as transmitter-specific lesions, and also localized 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 that respond to peripherally administered drugs. We here took a similar approach and employed a Cre recombinase-dependent viral delivery system (adeno-associated virus) to express tetanus toxin light chain (TeLc) and thus, block neural transmission specifically in parvalbumin-positive (PV +) neurons of the limbic and infralimbic prefrontal circuitry. PV-TeLc cohorts presented with normal circadian activity as recorded in PhenoTyper home cages, but a reproducible increase in anxiety was extracted in both the open field and light–dark box. 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 found 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 GABAergic signalling through PV neurones. 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.

Keywords

adeno-associated virus; cortex; Cre mouse; electroencephalogram; GABA; working memory

Correspondence to Gernot Riedel, PhD, Institute of Medical Sciences, Foresterhill, University of Aberdeen, Aberdeen AB25 2ZD, Scotland, g.riedel@abdn.ac.uk.

Conflicts of interest

There are no conflicts of interest.

Introduction

Specific neurotransmitters and the cell populations that release them fulfil highly specific functions in organizing brain activity and behaviour. Although 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 (Perälä *et al.*, 2007). With an onset during adolescence/early adulthood (Lewis, 1997) its chronic and severe psychiatric state affects many aspects of life, including education, employment and family (Konopaske and Coyle, 2015; Volk and 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 and Kapur, 2009); negative symptoms such as anhedonia, social withdrawal and blunted affect, are closely linked to the morbidity associated with SZ, and 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 and Fenton, 2007), and involves domains such as working memory (Gooding and Tallent, 2004; Galletly *et al.*, 2007; Barch and Caesar, 2012), attention (Cornblatt *et al.*, 1997; Morris *et al.*, 2013), executive function, problem solving and verbal memory (Heinrichs and 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). On the basis of extensive neuropsychology and functional imaging, dysfunction of the dorsolateral pre-frontal cortex (dlPFC) is critically associated with cognitive endophenotypes in SZ (Salgado-Pineda *et al.*, 2004; Lewis *et al.*, 2011). Anomalies in neuronal activity are frequently observed in prefrontal cortex (PFC) of SZ patients (Basar-Eroglu *et al.*, 2007; Uhlhaas *et al.*, 2008; Lodge *et al.*, 2009) resulting in a lack of network activation between PFC 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 GABAergic neurotransmission in dlPFC (Lodge *et al.*, 2009; Fung *et al.*, 2010; McNally *et al.*, 2013) and levels of GAD67 mRNA (the 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; Gonzalez-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 and 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 layers 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 (>20 Hz) 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 in rodents the pathology in dlPFC of SZ patients is the selective inactivation of PV + GABAergic interneurons in the dlPFC homologue, that is the infralimbic and prelimbic PFC of the mouse. This approach 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 adeno-associated viruses (AAVs) carrying an inverted green fluorescent protein (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 (McClure *et al.*, 2011; Murray *et al.*, 2011, 2015). TeLc is a bacterial neurotoxin that acts intracellularly (Gaisano *et al.*, 1994), by cleaving the vesicle-associated membrane protein-2 (VAMP-2). The vesicle-associated membrane protein (VAMP) family is comprised of seven members involved in vesicle fusion and thus forms an integral part of the core of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) 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, while the external inputs remain intact. Although AAVs will infect all cells close to the injection sites, gene expression is limited to Cre recombinase-expressing cells (see McClure *et al.*, 2011; Murray *et al.*, 2011 for description). Consequently, Cre-expressing neurones, which also express TeLc are the only cells with reduced neuro-transmitter release (Yamamoto *et al.*, 2003; Atasoy *et al.*, 2008).

As the Cre-expressing line for the experiments reported here, we selected PV-Cre mice and targeted the medial PFC 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 PFC and ipsilateral input structures in the dorsal hippocampus. The data clearly confirm that PV cells are critically involved in locomotor activity, working memory and the generation of gamma oscillations of PFC.

Methods

Ethical statement

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

Subjects

Mice expressing Cre recombinase in PV + cells were purchased from The Jackson Laboratory (Pvalb/B6;129P2-*Pvalb*^{tm1(cre)Arbr}/J; Repository number PV 008069; Bar

Harbour, Maine, USA), and a colony established at a commercial breeder (Charles Rivers Laboratories, Tranent, 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 sex. 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 before experiments. Mice were housed in same-sex groups in Macrolon type II cages under a 12-h light/dark cycle (lights on at 07.00 h, simulated dawn and dusk), in temperature ($18.7 \pm 0.4^\circ\text{C}$) and humidity ($63.3 \pm 5\%$) controlled holding rooms (3–4 m) with 20–30 air exchanges per hour. At no time, animals' food or water was restricted and even groups were maintained 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). GFP or GFP fused to tetanus TeLc was cloned in the AAV vector in a reverse reading frame (3'–5') between two sets of antiparallel 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, Philadelphia, Pennsylvania, USA).

Glass micropipettes prepared on a pipette puller (~8 μm bore) were filled with 3.5 μl of AAV (titre 6×10^6 infectious particles/ μl) using a 20 ml Hamilton syringe (Cole Palmer, London, UK), avoiding bubbles and 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, 0.05 mm^2 , copper; A Premier Farnell, Leeds, UK), whereas the distal end was attached to a gold-plated pin connector. Pins were assembled into a connector board to form the head stage.

Mice were anaesthetized using isoflurane (3% for induction, 1–1.5% maintenance; IsoFlo, AbbVie, Germany; 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 Co., Chicago, 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 (+ 2.0 mm anterior/posterior and ± 1.0 mm lateral) and for CA1 (– 2.0 mm anterior/posterior and ± 2.0 mm lateral), according to the mouse stereotaxic brain atlas (Paxinos and 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 $\pm 17^\circ$ to the depth of – 2.0 mm from bregma. AAVs were pushed into tissue through microtube-connected to a 60 ml Hamilton syringe and 3 μl AAV was infused over the 5–10 mins; pipettes were left in place for further 2 min to avoid backflow, removed and replaced by twisted local field potential (LFP) gold wire electrodes. Dental cement (Kemdent simplex rapid liquid and powder,

Kemdent, Swindon, UK) fixed the electrodes into place. The procedure was then repeated for hippocampal placement of electrodes. Finally, a head cap was modelled with wires assembled into a head stage and pins were protected by a light-weight dummy connector. Surgery was completed by intraperitoneal injection of sterile saline (1 ml 0.9% sodium chloride; Baxter, Newbury, UK) and sub-cutaneous injection of analgesic (0.3 ml Temgesic, 300 µg buprenorphine; RB Pharmaceuticals Limited, Sleuth, 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 reunited after surgery and maintained in this group with free access to water and food until completion of experiments.

Three cohorts of animals were used in total, from which cohort A ($n = 10/\text{group}$) was equipped with recording devices (Fig. 1). All other cohorts (B: $n = 29/\text{group}$; 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 postsurgery. The timeline for the different cohorts and their assignment to the test is summarized in Fig. 1. All behavioural observations were carried out during the light phase (07.00–19.00 h), recorded and analyzed through overhead cameras using Ethovision 3.1 software (Noldus IT, Wageningen, the Netherlands) and cohorts were run in several replications. After week 5, animals were perfused intracardially and tissue was harvested for further ex-vivo analyses (data not shown).

Open field—The open field (OF) (custom-made square white Perspex arena $60 \times 60 \times 60$ cm) was performed as described in the study by Murray *et al.* (2015). Individual test mice were placed in the centre of the arena and allowed to explore it freely for 10 min 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 (equi-area) as an index of anxiety.

Y-maze and electroencephalogram recording during behavior—A spontaneous alternation test was conducted in the Y-maze to assess working memory. This exploits the natural tendency of mice to visit alternate arms and we followed our previous protocol (Murray *et al.*, 2015). The maze consisted of three equally sized arms (A, B and C; each 60 cm long, 10 cm wide and 10 cm 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 (10 days postsurgery) and a second (21 days postsurgery) Y-maze exposure (see Fig. 1 for cohorts A and B). The first exposure was treated as a habituation session and data are not shown. After release into arm A, animals freely explored the maze for 10 min and entries were recorded once all four 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 = $\text{number of alternations} / (\text{total arm entries} - 2) \times 100$. 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) LFPs were recorded from PFC (and CA1; data 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 electroencephalogram recording devices (NeuroLogger; TSE Systems) with all four channels sampling at 200 Hz and band pass limited (1–70 Hz). Transitions between arms and proximal/distal zones were registered through the Ethovision software and TTL pulses 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 (transistor–transistor logic pulse activated IR transmitted to the logger). Only entries into zones were registered; the direction of movement was deduced from the sequence of entries and could be inward (towards the centre with behavioural decision) or outward (after entry into new arm running towards end of arm). This segregation enabled recording and analysis of behaviourally relevant electroencephalogram patterns during varying stages of spontaneous alternation responding. NeuroLoggers were removed after the experiment and data downloaded and extracted into txt format using a customized MatLAB script (MatLAB 8.3; The MathWorks Inc., Natick, Massachusetts, USA).

PhenoTyper—Circadian rhythms were recorded in PhenoTyper home cages (Noldus IT, Wageningen, the Netherlands) as described previously (Robinson *et al.*, 2013; Robinson and Riedel, 2014). PhenoTyper boxes (30 cm × 30 cm floor) were equipped with an overhead IR sensitive camera and food and water dispensers. Horizontal movements (path length) were recorded over 72 h and analyzed using Ethovision software, with no food or water restrictions. The first 3 h of the recording were treated as the habituation period in which animals adapted to their new environment; the remainder was averaged in 1-h bins to attain a day/night plot. Diurnal activity of the animals during dark (19.00–0700 h) and light (07.00–19.00 h) phases was also analyzed (mean of 1-h bins during dark and light phases).

Light/dark box—The light/dark box (LDB) consisted of two compartments – an open-top light box (30 × 30 × 30 cm) and a closed dark box (30 × 20 × 25 cm), connected by a small aperture. Animals were placed into the light compartment and allowed to freely explore the apparatus for 10 min. 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—American standard code for information interchange (ASCII) data files with multiplexed orientation, time domain and sampling interval of 5000 μ s (200 Hz) were uploaded and analyzed using BrainVision Analyzer 2.0 software (BrainProducts GmbH, Gilching, Germany). Initially, we considered the whole trial and a power spectral analysis using Fast-Fourier Transform from 4-s bins. Next, data were segmented according to the start/end of the trial and positioning in the maze (distal and proximal zones), on the basis of identified IR event stamps. Event strips of the same category (for example all distal zones) were averaged for each animal and pooled across treatment groups. Spectra are presented as absolute power (μ V²) between 4.2 and 79.8 Hz with the following bandwidth: 4–9 Hz (theta), 9–14 Hz (alpha), 14–20 Hz (beta), 20–50 Hz (gamma low) and 50–80 Hz (gamma high).

Statistical analysis

All statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., La Jolla, California, USA). Group comparisons utilized either unpaired two-tailed Student *t*-tests or factorial two-way analysis of variance, with group as between and time as repeated measures, followed by Bonferroni multiple comparison post-hoc tests. α was set to 5%. Only significant terms are reported.

Results

Normal global activity, but increased anxiety in prefrontal cortex parvalbumin-tetanus toxin light chain 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 PVTeLc, $n = 10$). As for the spatial distribution, both groups spent significantly more time in the proximity of the walls, relative to the centre [$F_{(1,18)} = 273.4$, $P < 0.001$]. However, there was a significant interaction between zone and virus [$F_{(1,18)} = 5.578$, $P < 0.05$]: PV-TeLc animals spent significantly less time in the centre than PVGFP animals [$t_{(18)} = 2.37$, $P < 0.05$] and substantially more time in the outer zone of the OF [$t_{(18)} = 2.36$, $P < 0.05$]. 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, has been widely used in rodents since its first description in 1958 (see Dember and 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), whereas 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 and b). Both groups covered a similar distance during the 10-min test and moved at the same velocity. Time in proximal versus distal zones revealed that more time was spent in 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 tests, see Fig. 3c]. As an additional measure of activity, we recorded the number of arm entries (Fig. 3d) that was not significantly different between groups. An obvious, yet unreliable, reduction was observed in the number of alternations by the PV-TeLc treatment (Fig. 3e), suggesting a small impairment in working memory performance. This was confirmed in the alternation index (Fig. 3f) that was significantly lower in PVTeLc mice relative to PV-GFP controls [$t_{(56)} = 2.79$, $P < 0.01$]. Both PV-GFP and PV-TeLc animals performed significantly above the chance level of 50% [$t_{(28)} = 6.82$, $P < 0.001$ and 2.81 , $P < 0.01$, respectively].

Spectral power of prefrontal LFPs was extracted for (i) the whole 10 min session and (ii) for the IR-identified segments corresponding to behavioural entries into predefined 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 between groups (Fig. 4a–c). In addition, there was no significant interaction between frequency and virus if individual bands were considered. In behavioural terms, we rationalized a segmentation of the behavioural response in terms of inward and outward movements, 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. Fast-Fourier Transform 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 parvalbumin cells

The animals' circadian rhythm was measured using PhenoTyper home cages (Fig. 6). Upon first introduction into the new home cages, animals were allowed to habituate, which was completed after about 2.5–3 h. 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 [$F_{(17,425)} = 67.65$, $P < 0.001$], but no significant interaction of time with viral treatment (Fig. 6a).

General locomotor activity of the animals was analyzed during the dark (19.00–07.00 h) and light (07.00–19.00 h) phases (Fig. 6b). There was a significant change in activity of both PV groups depending on the time of the day [main effect of time: $F_{(23,575)} = 45.8$, $P < 0.001$], with both groups increasing their movement during the dark phase. No significant interaction between time and viral treatment was detected. There was a strong effect of phase [$F_{(25,1)} = 136.8$, $P < 0.001$] when light and dark period were contrasted (Fig. 6c), but this happened to a similar degree in both groups.

Heightened anxiety in parvalbumin-tetanus toxin light chain mice in the light–dark box

The LDB is an anxiety test that relies on the natural aversions of rodents towards bright and open places (Kuleshkaya and Voikar, 2014). We exposed our mice to the LDB for 10 min and recorded the distribution of time between illuminated and darkened compartment for PVGFP ($n = 11$) and PV-TeLc mice ($n = 10$). Two-way analysis of variance revealed no significant overall differences between place (light or dark box) and viral treatment. PV-GFP animals spent similar time in both compartments (chance level of 300 s, $t < 1$). PV-TeLc animals, on the other hand, spent significantly more time in the dark compartment [$t_{(18)} = 5.72$, $P < 0.001$]. Time spent by these mice in the light box was substantially below chance level [$t_{(9)} = 4.04$, $P < 0.01$]. There were no differences between the groups in time spent in light and dark boxes both during the whole 10 min test and in 5 min bins. These results indicate that lack of prefrontal PV + cell discharges induced anxiogenic effects (Fig. 7).

Discussion

In this study, we show that region-specific 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 was achieved by a single surgical treatment of a Cre-expressing mouse line and led, as hypothesized at the outset, to anomalies reminiscent of a schizophrenic phenotype. Herein, we first consider the technique of viral cell silencing and contrast it to alternative viral methods. Thereafter, we discuss the results in the context of our hypothesis, that is the role of the PV-GABAergic network in SZ.

Methodical consideration

Techniques alternative to the one used here were considered during the development of these experiments. In the field of chemogenetics, DREADDs are engineered macromolecules that interact with previously unrecognized small molecules and come in different forms. The most widely used are receptors activated solely by synthetic ligands or designer receptors sensitive to the systemic administration of an inactive clozapine analogue clozapine-*N*-oxide (CNO). Although 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; Schnütgen *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 depolarization/hyperpolarization of the neurone and although optogenetic stimulation has a highly controllable and rapid onset and offset, CNO may take between a few minutes and about 2 h to be cleared from plasma, so DREADDs 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 PFC, 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 this might have been expected to become a powerful tool for investigation of the role of different cell populations

in predefined brain regions, the method has been slow to be embraced, possibly owing to the low fidelity in terms of onset of effect and irreversibility. In contrast, utilization 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 the treatment of disease (for review, see McCown, 2011). In terms of its use in 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 (Dvorzhak *et al.*, 2016; Vagner *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 (Hippenmeyer *et al.*, 2005; Murray *et al.*, 2011; 2015). We have utilized the method also to transfect AAV into other GABA-containing interneurons selectively expressing Cre recombinase (Woloszynowska-Fraser MU, Wulff P, Riedel G, unpublished data). The medial PFC, especially prelimbic and infralimbic regions were selected for this study on the basis of their homology to the dlPFC of humans and our interest in the role of GABAergic neurons in SZ.

Parvalbumin-cell inactivation and schizophrenic phenotypes

The manipulation of PV cells with TeLc induced behavioural alterations clearly reminiscent of SZ phenotypes. Our animals presented with anxiety phenotypes in the OF and LDB, and 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 Braga *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. This is consistent with stress models of anxiety that have revealed a lowering of PV-GABA neurones in PFC 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 antidepressive drugs or environmental enrichment remains to be determined.

The most profound cognitive deficits in SZ include executive function and working memory (Goldman-Rakic, 1995; Bach and Ceasar, 2010; 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 involve a lack of persistent firing of

principle neurones in prefrontal regions (Fuster, 1997; Jones and 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 section 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 (Starck *et al.*, 2017). The rhythmic inhibition by PV-GABA cells entrains PFC 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 PVTeLc mice presented with impaired working memory, but there were no global anomalies in prefrontal brain activity during this task. Our mice also showed normal exploration patterns and locomotor activity, but were specifically reduced in the normalized number of alternations, that is working memory. However, when analyzing the physiological responses, we realized 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 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 suggests that the transition through the proximal arm is more purposeful and has 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), whereas this was considerably and significantly reduced in mice lacking PV-GABA activity because of 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?, etc.) 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, displayed behaviourally 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). This has been similarly modelled using pharmacological treatment with ketamine (Jeevakumar and Kroener, 2014; Jadi *et al.*, 2015; Jeevakumar *et al.*, 2015), further supporting the notion that the critical pathology for the cognitive symptoms of SZ is a loss of function of the inhibitory system in PFC (Murray *et al.*, 2015). Moreover, patients with SZ cannot synchronize their neuronal activity (Basar-Eroglu *et al.*, 2007; Spellman and 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 (Lewis *et al.*, 2008; Williams and Boksa, 2010).

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 three different arms, but each exit and entry constitutes a single 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 here treated them equally for the purpose 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 nonmatching to sample procedures, in which correct responses are rewarded and incorrect responses are punished (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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Fig. 1. Study design, cohorts and sizes. (a–c) Study protocols for the three different cohorts tested in this study. Cohort A underwent adeno-associated virus (AAV) administration and electrode implantation to record local field potentials (LFPs) and was submitted to two Y-maze tests, at the beginning of week 3 and during week 4, and a final open-field exposure. Cohort B followed a similar timeline, 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 section. PV, parvalbumin; GFP, green fluorescent protein; TeLc, tetanus toxin light chain.

Fig. 2.

Locomotion and anxiety measured in the open field (OF). (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 two-tailed Student's *t*-test, **P* < 0.05; *****P* < 0.001, following two-way analysis of variance. PV, parvalbumin; GFP, green fluorescent protein; TeLc, tetanus toxin light chain.

Fig. 3.

Locomotion and working memory in the Y-maze spontaneous alternation task. Segmentation of the Y-maze into three equivalent arms (A, B, C) and distal (A1, B1, C1) and proximal sectors is also presented at the bottom of the figure. (a) Horizontal activity (distance moved) and (b) movement speed in treatment groups did not differ (also see Fig. 2 for comparison). (c) Time spent in proximal or distal segments of the maze: note the 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 mean + SEM; unpaired Student's *t*-test (two-tailed): ** $P < 0.01$, *** $P < 0.001$. PV, parvalbumin; TeLC, tetanus toxin light chain.

Fig. 4.

Spectral analysis of LFPs recorded during spontaneous alternation testing. Recordings from prefrontal cortex (PFC) for the entire 10 min session: group mean $\pm$ 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. PV, parvalbumin; GFP, green fluorescent protein; TeLc, tetanus toxin light chain.

Fig. 5.

Spectral analysis of LFPs recorded during spontaneous alternation testing. Recordings from prefrontal cortex (PFC) for time spent in proximal (decision) zones (see right top corner). Group mean $\pm$ 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 indicate reliable group difference (two-way analysis of variance; $P < 0.05$). Note that there was an overall lowering in power in the PV-TeLc animals, which affected the alpha band (synchronized electroencephalogram) and there was significantly reduced gamma power. PV, parvalbumin; GFP, green fluorescent protein; TeLc, tetanus toxin light chain.

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 h 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-h cycle in hourly bins. The dark section indicates the night hours and shows 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 with the light phase (asterisks) did not differ between groups (mean $\pm$ SEM). **** $P < 0.0001$, Student t-test, paired. PV, parvalbumin; GFP, green fluorescent protein; TeLc, tetanus toxin light chain.

Fig. 7.

Anxiety test in the light/dark box. Time spent in each zone during the 10 min test by PV-GFP ($n = 11$; green columns) and PV-TeLc mice ($n = 9$, blue columns). PV-GFP 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. PV, parvalbumin; GFP, green fluorescent protein; TeLc, tetanus toxin light chain.