Cortical layer 6 control of sensory responses in higher-order thalamus

Josephine Ansorge¹, Desire Humanes-Valera¹, François P. Pauzin¹, Martin K. Schwarz² and Patrik Krieger¹

¹Faculty of Medicine, Department of Systems Neuroscience, Ruhr University Bochum, Bochum, Germany
²Institute of Experimental Epileptology and Cognition Research, University of Bonn Medical School, Bonn, Germany

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Key points

- Thalamic activity is regulated by corticothalamic feedback from layers 5B and 6.
- To selectively study the importance of the layer 6 corticothalamic (L6 CT) projection, a transgenic mouse line was used in which layer 6 cells projecting to posterior medial thalamus (POm) were targeted for expression of channelrhodopsin-2.
- Repetitive optogenetic stimulation of this sub-type of L6 cells caused a rapid adaptation in POm spiking output, but had little effect on the spiking activity in the other cortical layers.
- L6 photoactivation increased POm spiking to the first, but not to subsequent whisker deflections in a 4 Hz train.
- A sub-population of L6 CT cells that can cause an initial increase in POm activity, that is not sustained with repetitive stimulation, could indicate that this L6 projection does not modulate ongoing sensory processing, but rather serves to briefly increase POm activity in specific behavioural contexts.

Abstract Thalamic activity is regulated by corticothalamic feedback from layers 5B and 6. The nature of these feedback systems differs, one difference being that whereas layer 5 provides ‘driver’ input, the layer 6 input is thought to be ‘modulatory’. To selectively study the importance of the layer 6 corticothalamic (L6 CT) projection, a transgenic mouse line was used in which layer 6 cells projecting to posterior medial thalamus (POm) were targeted for expression of channelrhodopsin-2 and in vivo electrophysiology recordings were done in urethane-anaesthetized mice. Pre- and postsynaptic targets were identified using tracing techniques and light-sheet microscopy in cleared intact brains. We find that optogenetic activation of this subtype of L6 CT cells (L6-Drd1) has little effect on cortical activity, but activates POm. Repetitive photoactivation of L6-Drd1 cells evoked a reliable response following every photoactivation, whereas in the connected POm area spiking was only initially increased. The

Josephine Ansorge received her Master of Science from the Faculty of Biology at Ruhr University Bochum in 2016. Currently, she is a PhD student in the Faculty of Medicine at Ruhr University Bochum working on topics related to sensory processing using optogenetics and in vivo electrophysiology.
response to repetitive whisker stimulation showed a similar pattern with only an initial increase in whisker-evoked spiking. Furthermore, the increase in whisker-evoked spiking with optogenetic activation of L6-Drd1 cells is additive, rather than multiplicative, causing even cells that in the absence of L6 activation produce relatively few spikes to increase their spiking substantially. We show that layer 6 corticothalamic cells can provide a strong, albeit rapidly depressing, input to POm. This type of cortical L6 activity could be important for rapid gain control in POm, rather than providing a modulation in phase with the whisking cycle.

**Introduction**

The sense organs detect information from the outside world, relaying this information to the brain for further processing. The rodent whisker system is well suited to investigate sensory processing because sensory touch stimuli can be clearly defined, and the resulting behaviour and responses can be quantified (Kleinfeld et al. 2006; Fox, 2008; Krieger & Groh, 2015; de Kock et al. 2018). Before reaching cortex the sensory evoked spiking activity travels via subcortical nuclei, including the thalamus. The lemniscal ventral posteromedial nucleus of thalamus (VPM) and the paralemniscal posterior medial thalamus (POm) are two thalamic nuclei that participate in the processing of ascending information from the rodent whiskers. Whereas most VPM neurons respond strongly with a short latency to single whisker deflections, the POm neurons respond with longer latencies and also have a multi-whisker receptive field (Diamond et al. 1992; Veinante & Deschenes, 1999). Thalamus not only projects to the cortex, but also receives information from it (Alitto & Usrey, 2003). Main sources of corticothalamic projections are layer 5B and layer 6 neurons (Hoogland et al. 1987; Bourassa et al. 1995; Rouiller & Welker, 2000; Feldmeyer, 2012; Groh et al. 2014; Sherman, 2016; Sumser et al. 2017). Whereas the layer 6 corticothalamic feedback to VPM has been studied in some detail in the whisker system (Deschenes et al. 1998; Mease et al. 2014; Crandall et al. 2015; Li & Ebner, 2016), less is known regarding the interaction between cortex and POm in shaping information processing of whisker inputs (chiaia et al. 1991; Diamond et al. 1992; Moore et al. 2015; Casas-Torremocha et al. 2017; Sumser et al. 2017). VPM is referred to as a ‘first’ order relay. First in the sense that afferent information first reaches VPM for further relay to cortex. POm in contrast is a ‘higher order’ relay because it receives input from one part of cortex that can be passed on to other cortices (Sherman et al. 2006). In the rodent somatosensory cortex POm receives strong inputs (large amplitude EPSPs) from cortical layer 5B (Groh et al. 2008, 2014; Mease et al. 2016b) whereas L6 inputs have been found to be of the ‘modulatory’ type with small facilitating EPSPs (Reichova & Sherman, 2004). Layer 6 can be divided into an upper and lower part referred to as L6A and L6B, respectively. Although the border is ill defined there are general differences in their cortical and thalamic projections (Radnikow et al. 2015), with the upper layer 6 cells projecting to both VPM and POm, whereas lower L6 cells preferentially target POm (Zhang & Deschenes, 1997; Chevee et al. 2018). Layer 5 corticothalamic cells project to POm with large driver synapses, whereas cortical terminals originating from layer 6 are smaller in size (Groh et al. 2014). In light of these difference between layer 5 and 6 inputs to POm, it is proposed that layer 6 inputs modify the ascending sensory information. In the current study we investigated the properties of L6B projections to the POm sensory thalamus using in vivo electrophysiology in anaesthetized mice, and used monosynaptic retrograde labelling, and light sheet microscopy to show the thalamic projection specificity. To control the activity of this projection using optogenetics, we used a transgenic mouse line (GENSAT, FK164Gsat Drd1-Cre) in which L6B neurons in somatosensory cortex that project to POm, are under the control of Cre recombinase (Gong et al. 2007; Gerfen et al. 2013; Hoerder-Suabedissen et al. 2018). We find that photoactivation of this sub-population of L6 corticothalamic cells (L6-Drd1 CT cells) provides a strong initial increase in POm spiking, which, however, rapidly adapts. The rapid adaptation in POm spiking output suggests that this sub-type of L6 cells provides a stronger initial input, which then decreases. If the L6-Drd1 input to POm was purely of the ‘modulatory’ kind, a facilitation would instead have been expected (Sherman & Guillery, 2013). In contrast to genetically identified L6 corticothalamic cells that project to both VPM and POm (the L6-Ntsr1 subtype; Pauzin & Krieger, 2018; Frandolig et al. 2019), photoactivation of L6-Drd1 cells did not alter whisker-evoked responses in other excitatory cortical cells.

**Methods**

**Ethical approval**

All experiments were done in accordance with the local government ethics committee (Landesamt für Natur, 2007; Geffen et al. 2013; Hoerder-Suabedissen et al. 2018). We find that photoactivation of this sub-population of L6 corticothalamic cells (L6-Drd1 CT cells) provides a strong initial increase in POm spiking, which, however, rapidly adapts. The rapid adaptation in POm spiking output suggests that this sub-type of L6 cells provides a stronger initial input, which then decreases. If the L6-Drd1 input to POm was purely of the ‘modulatory’ kind, a facilitation would instead have been expected (Sherman & Guillery, 2013). In contrast to genetically identified L6 corticothalamic cells that project to both VPM and POm (the L6-Ntsr1 subtype; Pauzin & Krieger, 2018; Frandolig et al. 2019), photoactivation of L6-Drd1 cells did not alter whisker-evoked responses in other excitatory cortical cells.

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**Corresponding author P. Krieger: Department of Systems Neuroscience, Ruhr University Bochum, Universitatsstraße 150, D-44780 Bochum, Germany. Email: patrik.krieger@rub.de**

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Animals and experimental methods

Extracellular recordings in both somatosensory cortex and posterior medial thalamus (POM) were performed in sixteen (10 males and 6 females) Drd1-cre mice (Tg(Drd1-cre)FK164Gsat) injected with AAV-channelrhodopsin and five (2 males and 3 females) Drd1-cre/RosaChr mice (a cross between Tg(Drd1-cre)FK164Gsat and Gt(Rosa)26 Sortm27.1(CAG-COP4+H134R/ttdTomato)Hze (female)). Animals were housed in ventilated animal cabinets on a 12-h reversed light/dark cycle. Food and water were provided ad libitum. The expression of channelrhodopsin-2 was similar between the Drd1a-cre mouse injected with AAV and the Drd1-cre/RosaChr mice (Fig. 1), and thus in vivo electrophysiology data from all animals were pooled. In the Drd1-cre mouse line used in this study, the coding sequence for Cre recombinase was inserted into the mouse genomic bacterial artificial chromosome (BAC) at the transcription initiation codon of the dopamine receptor D1A gene. As shown in previous studies (Gong et al. 2007; Gerfen et al. 2013) and here, Cre recombinase expression is localized to layer 6 pyramidal cells in neocortex. The Gt(Rosa)26SoR mouse line expresses a channelrhodopsin-2/ttdTomato fusion protein following exposure to Cre recombinase (Madisen et al. 2012), and thus when crossing these mice with the Drd1-cre mice, channelrhodopsin-2 is expressed in layer 6 Drd1 cells. Furthermore, three Drd1-cre mice and three Ntsr1-cre mice (Tg(Ntsr1-cre)GN220Gsat) were used in transsynaptic tracing experiments, two mice from each mouse strain were used to visualize projection targets with light-sheet microscopy, and three Drd1-cre/Rosa-tdTomato Drd1-cre and one Ntsr1-cre/Rosa-tdTomato were used in retrograde tracing experiments with cholera toxin.

Stereotaxic injections

Stereotaxic virus injections for in vivo electrophysiology experiments were performed in 10 males and 6 females Drd1-cre mice between 3 and 5 months old. The animals were anaesthetized with ketamine (97 mg kg\(^{-1}\)) and the body temperature was kept constant at 37°C using a heating pad (5 × 12.5 cm, 40-90-2-07, FHC, Bowdoin, ME, USA) connected to a temperature controller (DC Temperature Controller 40-90-8D, FHC). Animals were placed in a stereotaxic frame (Model 1900, David Kopf Instruments, CA, USA). The skin over the head was opened, the skull exposed, and 1 μl of the Adeno-associated virus (AAV-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-pA, floxed Channelrhodopsin-mCherry; titre range from 1–8 × 10\(^{12}\) vg ml\(^{-1}\); UNC Vector Core) was injected (anterior-posterior −1.7 mm (from bregma); lateral to midline 3.1 mm) at a depth of 0.9 mm under the dura. Analgesia (4–5 mg kg\(^{-1}\) of carprofen) was administered subcutaneously after viral injections.

Trans-synaptic tracing experiments (in 3 Drd1-cre and in 3 Ntsr1-cre animals) were performed as previously described (Niedworok et al. 2012). Briefly, 200 nl of virus containing solution (rAAV EF1-Df-mCherry: rAAV EF1-Df-RG ired TVA in a ratio 1:3) was injected into the primary somatosensory cortex (antero-posterior: −1.5 mm; lateral to midline: 2.9 mm; depth 0.9 mm). Six days later the mice were injected with 500 nl RAVVAG-EGFP(EnvA) using the same coordinates. After additional 12 days the mice were deeply anaesthetized with urethane (1.5 g kg\(^{-1}\) I.P.) and transcardially perfused. After perfusion the brains were removed from the skull, postfixed overnight in 4% PFA and further processed for immunohistochemistry.

For the retrograde tracing experiments with cholera toxin subunit B (CT-B), Drd1-cre/Rosa-tdTomato mice (n = 3 male; cross between male Tg(Drd1-cre)FK164Gsat and female Gt(Rosa)26Sortm14(CAG-tdTomato)Hze) and Ntsr1-cre/Rosa-tdTomato mice (n = 1 female; cross between male Tg(Ntsr1-cre)GN220Gsat and female Gt(Rosa)26Sortm14(CAG-tdTomato)Hze) were used. Two craniotomies were made to target both VPM and POM (Mease et al. 2016b) in the same animal. Then 200 nl of cholera toxin (conjugated Alexa Fluor 647, Invitrogen) were injected in VPM (antero-posterior (to bregma) −1.6 mm; lateral (to midline) 1.8 mm; depth: 3.5 mm) and 200 nl of cholera toxin (conjugated Alexa Fluor 488, Invitrogen) were injected in POM (antero-posterior (to bregma) −1.6 mm; lateral (to midline) 1.2 mm; depth: 3.0 mm). After 4 days, mice were deeply anaesthetized with urethane (1.5 g kg\(^{-1}\) I.P.) and transcardially perfused. After perfusion the brains were removed from the skull, postfixed overnight in 4% PFA and sliced to 100 μm coronal slices.

In vivo electrophysiology

Animals were anaesthetized with urethane (1.5 g kg\(^{-1}\) I.P.). The body temperature was kept constant (37°C) using an automatically controlled heating pad (FHC). Animals were placed in a stereotaxic frame (SR-6M-HT; Narishige Scientific Instruments, Japan). The skin of the head was removed and the craniotomy made over the left vibrissal primary somatosensory cortex for viral injections was enlarged to facilitate electrophysiology
recordings (anterior-posterior −1.7 mm (from bregma); lateral to midline 3.1 mm (Franklin & Paxinos, 2008); these coordinates were also used for recordings in the Drd1-cre/RosaChr mice in which no viral injections preceded the in vivo electrophysiology recordings). In addition, a second craniotomy was made on the left hemisphere over the POm thalamus: anterior-posterior −1.8 mm (from bregma) and lateral to midline 1.25 mm. Extracellular recordings were obtained with 32 channels silicon electrode arrays (for cortex: A1 × 32–6 mm-50-177; for POm A2 × 16–10 mm-50-500-177; NeuroNexus Technologies, MI, USA) using a multichannel acquisition processor (MAP, Plexon Inc., TX, USA). Recordings were done simultaneously in both cortex and POm using two separate multi-channels electrodes. The silicon probe used for recording POm responses was placed in an area where optogenetic light stimulation caused a direct, short latency, response. The S1 and POm regions were thus aligned according to corticothalamic projections. The raw signal (acquired at 40 kHz) was bandpass filtered between 0.1–10 kHz, and the signals were amplified by a ×1000 amplification factor.
300–3000 Hz and signals that exceed the background noise with at least 4 SDs were detected as spikes. To build peristimulus time histograms (PSTHs) the spikes were summed over 60 sweeps (Fig. 4B and C). Spike sorting was done with Offline Sorter (Plexon Inc, TX, USA), using the T-Dist E-M method (outlier Threshold 1.00; D.O.F. 2.00). Units were considered single-units when \( P < 0.05 \) in the 3D principal component analysis (Fig. 4F and G). Single-units in layer 6 responding with an increased averaged activity in the first 5.5 ms following light that was higher than the mean \( \pm 2 \) SD, calculated from 200 ms preceding the light stimulation, were classified as units recorded from L6-Drd1 cells \((n = 128)\). This selection criterion was corroborated by analysing the first spike latency in these spike-sorted units with the first-spike latency in other layer 6 cells (data from 60 light pulses in each cell). The average first-spike latencies were 5.3 ms \( \pm 0.06 \) ms (mean \( \pm \) SD) and 48.7 ms \( \pm 4.25 \) ms for units \((n = 128)\) classified as L6-Drd1 cells and L6 non-Drd1 units \((n = 22)\), respectively. Furthermore, spike-sorted units were analysed with cross- and autocorrelation to ensure that they were separate units (Fig. 4F and G). The refractory period in the autocorrelogram (estimated as the peak from the hazard function (Bar-Gad et al. 2001) and calculated with Neuroexplorer (Nex Technologies, USA)) was 12.1 ms \( \pm 2.6 \) ms (mean \( \pm \) SD; \( n = 128 \) spike sorted units). The multi-unit activity (MUA) from each cortical layer and POM was normalized to the number of channels (for each experiment) recorded in each respective layer/area in a given experiment. Furthermore, the single-unit activity (SUAs) and MUA were divided by the number of recorded sweeps (60 sweeps) (Figs 4A, 7C and F, and 8–12). Thus if, for example, a total of 100 spikes were calculated in sixty sweeps, 1 s time windows, from 2 channels, and a total of 180 spikes from 3 channels, the result would be 0.83 spikes s\(^{-1}\) (100 spikes/(2 channels \( \times \) 60 sweeps)) and 1 spike s\(^{-1}\) (180 spikes/(3 channels \( \times \) 60 sweeps)), respectively.

With increased whisker deflection frequency, the response latency increased (Juczewski et al. 2020). The following borders were used: for L2/3: 65–319 \( \mu \)m; for L4: 320–539 \( \mu \)m; for L5: 540–774 \( \mu \)m and for L6: \( > 775 \mu \)m. In addition, the layer borders were confirmed by the characteristic shape of the local field potential (LFP) (Di et al. 1990). For the LFP amplitude analysis, the raw signal was averaged over 60 sweeps and the response was calculated as the negative peak amplitude. The LFP amplitude measured in L6-Drd1 cells (Fig. 9A), is the LFP from channels with single-units identified as L6-Drd1 cells. Results were analysed by routines written in Matlab (The Mathworks, USA), Neuroexplorer (Nex Technologies, USA), pClamp (Axon Instruments) and Plexon (Offline Sorter, Plexon, USA). Data are presented as means \( \pm \) SD.

All electrophysiology data are pooled data from Drd1/RosaChR animals and from Drd1-Cre animals injected with AAV. The rational for summarizing the data was that the increase in spike rate and dynamics of the optogenetically induced activity in L6-Drd1 cells was the same (Drd1/RosaChR \((n = 36 \) spike sorted units); spont. spike rate 5.0 \( \pm \) 7.1; opto. spike rate 17.5 \( \pm \) 12.4 Drd1-cre/AAV \((n = 52 \) spike sorted units); spont. 6.8 \( \pm \) 7.0, opto 19.6 \( \pm \) 13.4 mean \( \pm \) SD; two-way ANOVA repeated on one factor (spont. – opto.); \( P = 0.3054 \) for the factor: Drd1/RosaChR vs. Drd1/AAV).

**Production of pseudotyped rabies virus**

BHK cells (~1.5 \( \times \) 10\(^7\); ATCC CCL-10) were plated in a 15 cm Petri dish. The following day, cells were transfected with 15 \( \mu \)g plasmid DNA (pCAGG/SAD-G) by calcium phosphate transfection. Twenty-four hours later RABV\(\Delta\)G-EGFP was added at a multiplicity of infection (MOI) of 3. After an additional 48 h incubation the RABV\(\Delta\)G-EGFP containing supernatant was equally distributed onto four 15 cm plates containing pCAGGs/SAD-G (15 \( \mu \)g/plate) transfected BHK cells (~1.5 \( \times \) 10\(^7\) cells/plate). Two days later the virus-containing supernatant was applied onto four 15 cm plates containing BHK-EnvARGCD cells.

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Anterograde tracing, tissue clearing and light sheet fluorescence microscopy

The long-range projections from L6-Ntsr1 and L6-Drd1 cells were studied in intact cleared brains prepared as previously described (Schwarz et al. 2015). First, 800 nl of virus containing solution (rAAV-CAG-FLEX-GFP (UNC Vector Core)) was stereotaxically injected in Ntsr1-cre (n = 2; 1 male and 1 female) and Drd1-cre transgenic mice (n = 2; 1 male and 1 female) (somatosensory cortex; antero-posterior: −1.65 mm; lateral: 3.2 mm; dorso-ventral: 0.9 mm). After 14 days the mice were deeply anaesthetized with urethane (1.5 g/kg i.p.) and transcardially perfused using a peristaltic pump. After perfusion the brains were removed from the skull, post-fixed overnight in 4% PFA, cleared and imaged according to Schwarz et al. 2015. Briefly, brains were dehydrated using a tert-butanol-based ascending alcohol series at 30°C (pH 9.5) and mounted in a triethylamine pH-adjusted benzyl alcohol/benzyl benzoate mixture (BABB, pH 9.5). A purpose-built light sheet fluorescence microscope was used to visualize the samples at a xyz resolution of around 1.6 × 1.6 × 3.2 μm voxel⁻¹, resulting in ~60 GB of stitched 16-bit raw data sets per channel and hemisphere. Images were processed using Imaris (Bitplane).

Immunohistochemistry

To differentiate between presynaptic pyramidal cells and interneurons, labelled in the transsynaptic tracing experiments, the cell morphology (apical dendrite, soma) was analysed, and a GAD67 staining was performed (Fig. 3E and F). Animals injected using the virus-based strategy for transsynaptic tracing were transcardially perfused as described above. The brains were postfixed in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.2) for 72 h. Brains were cut coronally in phosphate buffer saline (0.1 M PBS, pH 7.2) to 40 μM on a vibrating microtome. Tissue that included the primary somatosensory cortex was selected and permeabilised for 15 min with 0.3% Triton-X100 (Sigma) in 0.1 M PBS (PBST). The slices were blocked for 1 h with 10% normal goat serum (NGS; Thermo Fisher Scientific, Catalogue number: 50062Z)) and then incubated with the primary antibody solution (mouse anti GAD67 (Merck, MAB5406); 1:500; in 1.5% NGS in PBST) overnight at room temperature. The next day slices were washed three times with PBS and incubated with the second antibody DyLight405 goat anti mouse (Thermo Fisher Scientific, 35501BID) in blocking solution (1:200) for 1 h at room temperature. Slices were analysed with a ZEISS ApoTome microscope (Zeiss, Germany) and Neurolucida software (MBF Bioscience, VT, USA).

Optogenetic stimulation

To activate the L6-Drd1 CT cells via channelrhodopsin, 8.7 mW (69 mW mm⁻²) blue light pulses at 465 nm were used. The output power of the LED driver (PlexBright LD-1, Plexon Inc., TX, USA) was regulated via the current adjust knob. The power output at the fibre tip was measured with a power meter (PM100D; Thorlabs, NJ, USA). The estimated onset of the light induced spiking in POm cells was calculated based on a sample of 10 spike-sorted units. The first bin after light onset with above-chance spiking (in 2 ms bins) was defined as the first bin with at least 3 spikes, followed by a bin with at least 1 spike. The rational for this criterion is that for a Poisson distribution the Chi-square 95% confidence interval, for an average spiking rate of 1 spike (the average POm spike rate was 0.8 spikes/2 ms bin, summed over 60 sweeps), is approximately 3. With this criterion, the light-induced spike increase was at 13.6 ± 3.5 ms after light onset, and hence light induced responses in POm were calculated by adding ~10 ms from the start of light stimulation.

Whisker stimulation

To stimulate the whiskers, an air puff device (PDES-O2DX, npi electronic, Germany) triggered by Master 8 (AMPI) was used. Pulses 20 ms long at 1.12 bar were applied to the whiskers in two different conditions: 0.5 Hz low frequency and 4 Hz high frequency. The dose-response curve (Fig. 8A and B) indicates that the 1.12 bar air puff stimuli was at the bottom plateau for detecting a whisker-evoked increase in spiking. The time...
Figure 2. Comparison of L6-Drd1 and L6-Ntsr1 projection areas

A and B, cleared brains showing the different corticothalamic projections in a Drd1-cre mouse (A) and a Ntsr1-cre mouse (B). The projections of both cell populations were visualized in cleared, intact mouse brains using AAV-FLEX-GFP. L6-Drd1 cells project exclusively to POm whereas L6-Ntsr1 cells project to VPM, POm and nRT. The dashed line marks the axonal projection area in the thalamic region. See also Videos S1 and S2 in Supporting information. C–F, projections to thalamus from the L6-Drd1 CT (C and D) and L6-Ntsr1 CT (E and F) cell populations. Cholera toxin subunit B (CT-B) conjugated to a blue or green fluorophore was injected in VPM and POm, respectively. Cells were counted in somatosensory barrel cortex (see Methods for further details). A relatively small volume of CT-B was injected to ensure that VPM and POm are targeted separately; thus it is not expected that all genetically labelled L6-cells (L6-Ntsr1 and L6-Drd1) will be doubled-labelled, even if they all do project to the target areas. The cells can thus project to the nucleus but not to the region of it that has not been
from onset of the trigger pulse until the air flow reached the whiskers was measured with a piezo pressure sensor (Zehendner et al. 2013) and estimated at approximately 10 ms. The neuron response latency was calculated by adding this delay to the estimated whisker deflection onset time. Air puff deflections of multiple whiskers (10–13 whiskers; estimated from video recordings (Pike F-032B, ALLIED Vision Technologies GmbH)) were used since the whisker map in POm is less distinct compared to that in cerebral cortex (Veinante & Deschenes, 1999), and thus it is difficult to target the POm recording to an area with its major input from one given whisker.

**Results**

**Anatomical characterization of L6-Drd1 corticothalamic cells**

The distribution of L6-Drd1 cells and their projection area was determined. Furthermore, this genetically labelled sub-population was compared to a previously described L6-subpopulation, the L6-Ntsr1 corticothalamic cells (Crandall et al. 2015; Pauzin & Krieger, 2018). To characterize the L6-Drd1 cells used in the *in vivo* electrophysiology experiments their cell distribution and projection target were analysed in Drd1-cre mice injected with CT-B. The percentage labelling thus only shows a relative proportion of cells projecting to VPM and POm. C, double-labelling of L6-Drd1 Cre-cells (red, 2340 cells) and VPM projecting cells (blue). L6-Drd1 Cre-cells, which project to VPM, are thus labelled in pink (105 cells). D, double-labelling of L6-Drd1-cre cells (red) and POm projecting cells (green). L6-Drd1 Cre-cells which project to POm are labelled in yellow (695 cells). This means that 5% (105/2340) of the L6-Drd1 project to VPM and 30% to (695/2340) POm. E, double-labelling of L6-Ntsr1-cre cells (red, 4462 cells) and VPM projecting cells (blue). L6-Ntsr1 cells, which project to VPM, are double-labelled (red and blue) and visualized in pink (2914 cells). F, double-labelling of L6-Ntsr1 Cre-cells (red) and POm projecting cells (green). L6-Ntsr1 cells projecting to POm are double-labelled (red and green) and visualized in yellow (809 cells). This means that 65% (2914/4462) of the L6-Ntsr1 cells project to VPM and 18% (809/4462) to POm.

**Photoactivation of L6-Drd1 cells increases spiking in POm, but has little effect on activity in cortical layers 2–5**

To investigate the role of the L6-Drd1 corticothalamic (CT) cells in the whisker system, we firstly studied the effect of L6-Drd1 photoactivation on the spontaneous firing rate of cortical and POm cells. Note that to simplify the terminology 'spontaneous' activity is here used to refer to spiking recorded either with or without photoactivation and in the absence of whisker deflections. In the former case it is L6-Drd1 activation that affects spiking activity, and in the latter it is intrinsically generated neuronal activity, and not stimulus driven activity. *In vivo* electrophysiology recordings were done in anaesthetized
Figure 3. Retrograde transsynaptic tracing of pre-synaptic input to L6-Drd1 and to L6-Ntrs1 cells

A and C, an example of a coronal brain slice showing labelled cells. Red cells are L6-Drd1 or L6-Ntrs1 cells (rAAV with tdTomato), respectively. Yellow (red + green labelled) cells are the source cells, i.e. L6-cre cells that receive inputs from monosynaptically labelled green (EGFP) cells. B, distribution of presynaptic cells that project to L6-Drd1 cells (373 EGFP (green) labelled presynaptic cells; 15 of which were identified as putative interneurons, data from 3 animals; only cells in somatosensory barrel cortex counted). Most pyramidal cells which project to L6-Drd1 were found in layers 5 and 6. Interneurons were found in all layers (black and green dots; green dots represents interneurons that in one tested animal, in addition to morphological criteria, were GAD67 positive). D, distribution of presynaptic cells that project to L6-Ntrs1 cells (1561 EGFP (green) labelled presynaptic cells; 43 of these identified as interneurons). These presynaptic cells appear to show a more homogeneous distribution in comparison to those presynaptic cells which project to L6-Drd1. Note: dots for interneurons in (B and D) show only the distribution of those cells, and not their frequency distribution (i.e they are not plotted against the y-axis). E and F, examples of a GAD67 positive presynaptic cell (green: EGFP, blue: GAD67 and DyLight405 labelled cell).
mice and channelrhodopsin-2 expressing L6-Drd1 cells were activated with light. Using spike-sorting methods, single-units were recorded in layer 6 from cells classified as L6-Drd1 cells \((n = 128);\) see Methods, and multi-unit activity (MUA) from the other cortical layers and non-L6 Drd1 cells, and POm. To obtain a broad level of activation strengths, different light intensities, three different light durations (20 ms, 70 ms and 120 ms) and two different frequencies were used (0.5 Hz and 4 Hz).

Optogenetic stimulation of L6-Drd1 cells at a low frequency (0.5 Hz) increased spontaneous firing in both L6-Drd1 cells and POm cells for all three light durations. In barrel cortex, spontaneous spiking in L6-Drd1 cells was, in control conditions (averaged over 20, 70 and 120 ms time windows; see Table 1) \(4.49 \pm 4.18\) spikes s\(^{-1}\), and increased with optogenetic activation (Fig. 4A, upper panel) to: (i) \(58.29 \pm 37.16\) spikes s\(^{-1}\) (spikes calculated during the 20 ms duration of the light pulse), (ii) \(28.31 \pm 22.62\) spikes s\(^{-1}\) (70 ms) and (iii) \(22.14 \pm 15.01\) spikes s\(^{-1}\) (120 ms) (two-way ANOVA repeated on the row factor (with or without light pulse), and column factor (pulse duration (20, 70, 120 ms)); row factor Treatment \(P < 0.0001,\) Table 1). In POm, spontaneous MUA (Fig. 4A, lower panel) was \(12.75 \pm 8.78\) spikes s\(^{-1}\) in control conditions and increased with photoactivation of the L6-Drd1 cells: (i) \(29.62 \pm 14.81\) spikes s\(^{-1}\) (20 ms light pulse), (ii) \(39.45 \pm 26.68\) spikes s\(^{-1}\) (70 ms) and (iii) \(40.55 \pm 21.18\) spikes s\(^{-1}\) (120 ms) (two-way ANOVA repeated on both factors, row factor Treatment (spiking with and without optogenetic stimulation), \(P < 0.0001,\) Table 1). Averaging the number of spikes over the pulse duration (20, 70 or 120 ms) for L6-Drd1 (17 animals) and MUA for the other cortical layers (17 animals) and POm (19 animals).

#### Table 1. (related to Figs 4 and 6). Effect of optogenetic activation of L6-Drd1 cells on spontaneous activity

|               | L2/3 (spikes s\(^{-1}\)) | L4 (spikes s\(^{-1}\)) | L5 (spikes s\(^{-1}\)) | L6 non-Drd1 (spikes s\(^{-1}\)) | L6-Drd1 (spikes s\(^{-1}\)) | POm (spikes s\(^{-1}\)) |
|---------------|--------------------------|------------------------|------------------------|-------------------------------|-----------------------------|------------------------|
| All Ctrl spont averaged | 1.51 ± 1.43              | 3.56 ± 3.56            | 6.67 ± 7.43            | 4.47 ± 2.64                   | 4.49 ± 3.96                 | 12.75 ± 8.78           |
| Ctrl 20 ms    | 1.56 ± 1.10              | 3.61 ± 3.09            | 6.04 ± 6.10            | 4.46 ± 2.68                   | 5.53 ± 4.12\(^{a}\)         | 11.95 ± 6.63           |
| Ctrl 70 ms    | 1.66 ± 1.87              | 3.46 ± 3.32            | 6.61 ± 7.34            | 4.81 ± 2.66                   | 3.90 ± 3.92\(^{b}\)         | 13.21 ± 10.12          |
| Ctrl 120 ms   | 1.32 ± 1.33              | 3.61 ± 4.27            | 7.35 ± 8.86            | 4.13 ± 2.58                   | 4.05 ± 3.83\(^{c}\)         | 13.11 ± 9.58           |
| Opto 20 ms    | 1.87 ± 1.90              | 4.46 ± 4.20            | 7.29 ± 8.41            | 5.12 ± 2.28                   | 58.29 ± 37.16\(^{a}\)       | 29.62 ± 14.81          |
| Opto 70 ms    | 2.24 ± 1.96              | 4.99 ± 5.76            | 7.38 ± 8.47            | 6.23 ± 3.56                   | 28.31 ± 22.62\(^{b}\)       | 39.45 ± 26.68          |
| Opto          | 1.52 ± 1.60              | 5.41 ± 7.71            | 10.79 ± 13.29          | 4.68 ± 2.78                   | 22.14 ± 15.01\(^{c}\)       | 40.55 ± 21.18          |

Light pulses were given every 2 s, and data were the average from 60 pulses. Two-way ANOVAs repeated on both factors, and post hoc Bonferroni multiple comparison test (‘Ctrl vs. opto 20/70/120 ms’). Statistics done separately on data from each recorded area (cortical layers and POm). The relevant comparison is between control and opto in the three different conditions (20, 70, 120 ms), respectively. Data are SUA \((n = 128)\) for L6-Drd1 (17 animals) and MUA for the other cortical layers (17 animals) and POm (19 animals). ‘all Ctrl spont averaged’ is the average of all recordings from the 20, 70 and 120 ms data. Main factors: row factor Treatment (all spontaneous activity in control compared to all ‘spontaneous’ activity with photoactivation of L6-Drd1 cells); column factor Pulse (light pulse duration; 20, 70 or 120 ms). The significant main factor Pulse means that, normalized to pulse duration, the increase in spiking was not the same. From the data in the table, and as illustrated with an example in Fig. 3, this is because the increase in spiking decreased when normalized to light pulse duration. The superscripts a, b and c refer to the respective comparison and the associated \(P\) value. \(P\)-values are highlighted in bold. Grey shading is used to highlight when the \(p\)-value was < 0.05 (i.e. effect was statistically significant).
Figure 4. Photoactivation of L6-Drd1 cells increased spiking in L6-Drd1 cells and in POm

A, upper panel: the L6-Drd1 cells were activated with three different light pulse durations (20, 70, 120 ms). Spike rate was normalized to light pulse duration (i.e. for the 20, 70, 120 ms light pulses, spikes were counted for 20, 70, 120 ms, respectively). The shortest duration led to the highest spike rate, thus the L6-Drd1 firing was not constant over the whole stimulus duration (Table 1). Lower panel: photoactivation of L6-Drd1 cells caused an increased spiking in POm. (Data are SUA for L6-Drd1 (n = 128 single-units, 17 animals) and MUA for POm (19 animals) (mean ± SD; Table 1); asterisks represent differences between activity in control and with photoactivation of L6-Drd1 cells (‘opto’) (*P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.001; ******P ≤ 0.0001; two-way ANOVAs repeated on both factors, P values from post hoc Bonferroni multiple comparison test). Each light pulse duration was tested 60 times, with 2 s between pulses. B and C, averaged PSTHs from single-units recorded in L6 (n = 128) and single-units in POm (n = 20). (Data for each unit are the sum of 60 sweeps, plotted as the average sum). Blue bar shows the light stimulation. D and E, examples of raw traces recorded in vivo using multichannel electrodes. F, left panel: 3D principal component analysis of spiking units (with their waveforms) in L6, in this example, classified as two different units. Right panel: auto- and crosscorrelation between the two clusters.
cross-correlograms of the two spike-sorted single-units. Single-units reliably recorded with a short latency to light pulses were classified as L6-Drd1 cells (see Methods). G, left panel: 3D principal component analysis showing a single unit activity recorded in POM and the waveform of this unit. Right panel, autocorrelogram of this POM single unit. H, the spike-sorted unit waveform did not change during light stimulation. Upper panel, unit amplitude during light stimulation (y-axis) plotted against unit amplitude without light stimulation (x-axis; ‘amplitude spont.’). Lower panel, example waveforms recorded with (‘opto’) and without (‘spont’) optogenetic stimulation. Numbers and statistics in Table 1.

120 ms, respectively) shows that in the L6-Drd1 cells, normalized to light pulse duration, the shortest pulse gave the strongest increase (main factor light pulse duration: Pulse, $P = 0.0004$, Table 1). The spike rate decreased with pulse duration thus the spike rate was not sustained during the entire light pulse duration (as evident from the averaged PSTHs shown in Fig. 4B). In POM the average increase in spiking was not dependent on the light pulse duration (main factor light pulse duration: Pulse, $P = 0.1539$, Table 1). Furthermore, the photo-induced increase in spontaneous spiking activity in L6-Drd1 cells and POM cells was dependent on the light intensity and in the L6-Drd1 cells it was transient over a range of light intensities (Fig. 5, Table 2). As evident from both the stimulation with different pulse durations and different light intensities, the L6-Drd1 driven activity in POM quickly reaches a plateau such that neither longer pulse duration nor higher light intensity increased POM activity (Fig. 5, Table 2). The light intensity used in the experiments was ~85% of maximum light power. POM activity had already reached a maximum at ~30% maximum light intensity (Fig. 5), and thus reported effects on POM activity are not likely to be attributed to too weak an activation of the POM layer.

In contrast to the increase in spiking L6-Drd1 cells and in POM, there was no significant difference between spontaneous MUA spiking activity and light-induced MUA spiking activity in the other cortical layers or in L6 non-Drd1 cells (Fig. 6; Table 1; L2/3: control 1.66 ± 1.87 spikes s$^{-1}$, 70 ms optogenetic stimulation (opto) 2.24 ± 1.96 spikes s$^{-1}$, $P = 0.3435/0.0534$ (1st $P$ value is from post hoc test, 2nd is main factor Treatment (opto or not)); L4: control 3.46 ± 3.32 spikes s$^{-1}$, opto 4.99 ± 5.76 spikes s$^{-1}$, $P = 0.1718/0.0169$; L5: control 6.61 ± 7.34 spikes s$^{-1}$, opto 7.38 ± 8.47 spikes s$^{-1}$, $P > 0.9999/0.0569$; L6 non-Drd1: control 4.81 ± 2.66 spikes s$^{-1}$, opto 6.23 ± 3.56 spikes s$^{-1}$, $P = 0.0465/0.0978$; two-way ANOVA repeated on both factors with post hoc test Bonferroni for multiple comparisons; n = 17 (19 for POM data) animals; data for 20 and 120 ms optogenetic stimulation reported in Table 1). Interestingly, these results differ from those reported when activating a different type of L6-CT cells (the L6-Ntsr1 cells), which when activated decreased the cortical activity (Pauzin & Krieger, 2018).

To investigate the synaptic dynamics in the L6-Drd1 to POM connection, the L6-Drd1 cells were activated with repetitive stimuli at a higher frequency (4 Hz). In the L6-Drd1 cells the repetitive photoactivation with 4 stimuli at 4 Hz caused an increased spiking activity, compared to spiking without light, with each light pulse (Fig. 7A, C and E). In POM cells, however, the response adapted and thus decreased to successive light stimulations (Fig. 7B, D and F; Table 3). Sustained increased spiking in
Figure 5. Optogenetic stimulation of L6-Drd1 cells at 0.5 Hz with different light intensities and light pulse durations

A, 70 ms long pulses; B, 120 ms long pulse. The L6-Drd1 cell activity (SUA) did not appear to reach a maximum even with the highest light intensity, whereas the POm cells (MUA) reached a plateau already at ~30% of maximum light intensity. C, PSTHs of a L6-Drd1 single unit at different light intensities. D, PSTHs of a POm single unit at different light intensities. Statistics in Table 2; Data are represented as mean ± SD from 4 animals. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 6. Optogenetic activation of L6-Drd1 at 0.5 Hz

The L6-Drd1 cells were activated with three different illumination durations. The L6-Drd1 activation led to an activation of POm, but to no significant changes in the other cortical layers (data are SUA for L6-Drd1 and MUA for the other cortical layers and POm; cortical recordings from 17 animals and POm recordings from 19 animals). Asterisks (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001; Table 1) represent differences between spontaneous activity in control and with photoactivation of L6-Drd1 cells ('opto'); outliers were marked with coloured dots. Outliers were identified using the ROUT method (with Q = 1%) as implemented in GraphPad Prism (GraphPad Software, CA, USA).
Figure 7. Repetitive photoactivation (4 Hz) caused a repetitive activation of L6-Drd1 cells, whereas in POm the light-induced increase in spiking rapidly decreased.

A and B, examples PSTHs of spike-sorted units from one L6-Drd1 cell, and one POm cell, simultaneously recorded in the same experiment, during light stimulation (blue bars; 4 pulses at 4 Hz; sum from 60 sweeps). C and D, data from L6-Drd1 and POm units, respectively, simultaneously recorded in a different experiment. E and F, summary plots of the 4 light pulses (trials 1–4, 70 and 120 ms light pulses) at 4 Hz caused an increased firing in L6-Drd1 cells.
response to repetitive light-stimulation in the L6-Drd1 cells thus failed to induce an increase in POm spiking in response to each light pulse (L6-Drd1 cells: spontaneous activity (spont act) without light: 6.46 ± 3.72 spikes s⁻¹; 1st opto (70 ms light pulse): 20.41 ± 14.69 spikes s⁻¹, 4th opto 18.09 ± 9.79 spikes s⁻¹; spont act vs. opto: P < 0.001 for all light pulses, post hoc Bonferroni multiple comparison test), (POm: spont act without light: 11.88 ± 8.47 spikes s⁻¹; 1st opto (70 ms light pulse): 23.18 ± 13.08 spikes s⁻¹, 4th opto 14.86 ± 11.44 spikes s⁻¹; spont act vs. opto: P < 0.05 only for spont act vs. 1st light pulse, post hoc Bonferroni multiple comparison test; Table 3). On average, spiking in POm returned to baseline spontaneous level after the first light-pulse, suggesting that this connection is depressing and/or that network mechanism contribute to a depression.

**Low-frequency whisker-evoked responses**

After demonstrating that photoactivation of L6-Drd1 cells that express channelrhodopsin leads to a significant increase in the spiking rate in these cells and in POm, we investigated the influence of this activation on the whisker-evoked response. The L6-Drd1 cells were...
Figure 8. Activation of L6-Drd1 cells during whisker stimulation

A and B, the graphs show a dose-response curve for whisker-evoked activity in L6 (A) and in POm (B). The whiskers were deflected with different air puff strengths. An air puff strength of 1.12 bar (1 bar = 100 kPa) (close to the value at the blue rectangle; notably this air-puff strength is at the bottom plateau) was chosen for the experiments. The data are MUA from 11 animals for L6 and 9 animals for POm. Ca, in L6-Drd1 cells the optogenetic activation did not change the absolute number of whisker-evoked spikes (data from 128 spike sorted units; *significant differences between the spontaneous and whisker-evoked activity). Cd and Cc show the temporal dynamics of the WER of all the 128 L6-Drd1 units (mean and SD). In Cc the optogenetic stimulation started 100 ms before the
activated 50 or 100 ms before the whiskers on the contralateral side were deflected with 20 ms air puffs (air puff stimuli used was in the bottom plateau of the dose-response curve for the whisker stimulation, Fig. 8A and B). The whisker-evoked responses (WER) were compared with those under control conditions (without L6-Drd1 photoactivation). Different latencies (50 or 100 ms) were chosen to achieve different levels of activation (Groh et al. 2014). L6-Drd1 spiking decreased during the duration of the light pulse (see Fig. 4), thus with a relatively longer delay between onset L6-Drd1 activation and whisker stimulation (100 ms compared to 50 ms), L6-Drd1 spiking will be smaller at the time of the whisker deflection.

In the L6-Drd1 cells, photoactivation alone (as already shown in Fig. 4), increased spiking (50 ms delay from start of light pulse to onset of whisker deflection) from 5.06 ± 4.04 to 25.89 ± 16.60 spikes s⁻¹, P < 0.0001, and with 100 ms delay from 5.06 ± 4.04 to 16.52 ± 12.51 spikes s⁻¹ (P = 0.0015; two-way ANOVA repeated on both factors and P value from post hoc test Bonferroni for multiple comparisons, Table 4). However, there was no increase in the absolute number of spikes recorded during the whisker stimulation (WER control: 46.74 ± 40.58; WER + 50 ms opto 49.65 ± 39.98 spikes s⁻¹; P > 0.9999; Fig. 8C). The increase in spiking activity with only photoactivation, and no whisker deflection, was thus not ‘added’ to the WER.

In POM there was, however, an increase in whisker-evoked activity (control: 65.57 ± 38.26, opto (50 ms) 84.58 ± 43.58 spikes s⁻¹, P = 0.0003; opto (100 ms) 80.02 ± 42.53 spikes s⁻¹, P = 0.0051; two-way ANOVA repeated on both factors, P value from post hoc test Bonferroni for multiple comparisons, Table 4; Fig. 8D; data are MUA). Interestingly, in POM the L6-Drd1 photoactivation caused a multiplicative increase in spontaneous spiking and an additive shift in the WER (Fig. 8E). Spiking activity recorded in the absence of light stimulation increased on average with a multiplicative factor (2.3) with L6-Drd1 activation, whereas there was a constant additive increase (20 spikes s⁻¹) to the WER with photoactivation. In other words, with photoactivation the spiking ratio (WER/spiking activity preceding the whisker deflection) decreased.

In addition to the increased spiking in POM, the peak amplitude of the local field potential (LFP) also increased (Fig. 9). The increase was slightly dependent on the temporal relationship between cortical photostimulation of L6-Drd1 cells and whisker stimulation. With a short latency (opto 50 ms before the whisker deflection onset) the increase in the LFP amplitude was significant (one-way paired ANOVA with Bonferroni multiple comparisons test, P = 0.0419), but not with a longer delay (100 ms) (Bonferroni post hoc test, P = 0.6593). The reason presumably being that with the longer latency (100 ms compared to 50 ms) between light pulse onset and whisker deflection, the L6-Drd1 activity has decreased when the whiskers are deflected (see Fig. 4) and consequently the POM activation is reduced.

In cortical layers L2/3–L5 and in L6 non-Drd1 cells, optogenetic activation of L6-Drd1 cells did not affect the WER (Fig. 10). Thus activation of L6-Drd1 cells in layer L2/3–L5 and in L6 non-Drd1 cells affected neither spontaneous spiking nor the WER (Table 4). Again this result is in contrast to another L6 CT cell population (L6-Nts1 CT cells), which when activated reduced the WER in the other cortical layers (Pauzin & Krieger, 2018).

High-frequency whisker-evoked responses – adaptation

We used two different experimental protocols to investigate how cortical activity affects repetitive (4 Hz) whisker responses in POM. The rationale behind the different protocols is that they provide different temporal patterns between on the one hand cortical and thalamic activation, and on the other hand sensory input (i.e. whisker deflection). Furthermore, previous experiments
Table 4. (related to Figs 8 and 10). Effect of the optogenetic activation of L6-Drd1 cells on whisker-evoked activity at 0.5 Hz

|                     | L2/3 (spikes s⁻¹) | L4 (spikes s⁻¹) | L5 (spikes s⁻¹) | L6 non-Drd1 (spikes s⁻¹) | L6-Drd1 (spikes s⁻¹) | POm (spikes s⁻¹) |
|---------------------|------------------|-----------------|-----------------|---------------------------|---------------------|-----------------|
| Treatment           |                  |                 |                 |                           |                     |                 |
| Spont control       | 2.29 ± 3.18      | 7.68 ± 8.52     | 9.94 ± 7.49     | 5.19 ± 6.71               | 5.06 ± 4.04         | 13.07 ± 5.47    |
| Spont opto 50 ms    | 3.82 ± 5.40      | 10.26 ± 12.47   | 14.85 ± 15.98   | 6.01 ± 8.03               | 25.89 ± 16.60       | 28.29 ± 19.27   |
| Spont opto 100 ms   | 2.37 ± 2.21      | 8.39 ± 7.13     | 14.37 ± 12.32   | 6.80 ± 8.72               | 16.52 ± 12.51       | 27.31 ± 16.65   |
| WER control         | 26.71 ± 35.12    | 70.80 ± 53.17   | 90.03 ± 60.68   | 39.18 ± 30.20             | 46.74 ± 40.58       | 65.57 ± 38.26   |
| WER + 50 ms opto    | 28.11 ± 37.62    | 71.01 ± 49.80   | 94.12 ± 61.03   | 38.82 ± 30.77             | 49.65 ± 39.98       | 84.58 ± 43.58   |
| WER + 100 ms opto   | 22.50 ± 28.12    | 63.67 ± 47.14   | 82.77 ± 56.29   | 39.46 ± 28.34             | 47.50 ± 42.13       | 82.77 ± 47.53   |

Two-way ANOVAs repeated on both factors and the post hoc tests Bonferroni multiple comparison test done separately on data from each cortical layer and POm. Data are SUA for L6-Drd1 (n = 128) and MUA for the other cortical layers collected from 21 animals. From 16 of the 21 animals there was also MUA from POm. Main factors: row factor Treatment (spontaneous activity compared to whisker-evoked responses (WER)); Pulse (light pulse duration: zero (no light pulse), 50 or 100 ms). Letter superscripts mark values that were compared and the related P value. P-values are highlighted in bold. Grey shading is used to highlight when the P-value was < 0.05 (i.e. effect was statistically significant).

on a different sub-population of L6 CT cells, the L6-Ntr1 cells, used continuous light stimulation (Mease et al. 2014), and thus this protocol was also repeated for comparison. In both protocols the whiskers were stimulated 4 times at 4 Hz, every 2 s. Each of the four whisker deflections was 20 ms in duration. In one protocol the photostimulation started 50 ms or 100 ms before the first whisker deflection and was continuous for the duration of the four whisker deflections (Fig. 10A). In the other protocol the photostimulation was repeated with every whisker stimulation and started 50 ms or 100 ms before each whisker stimulation (Fig. 12A). In both cases whisker-evoked responses were counted for 100 ms (from the onset of the whisker deflection).

4 Hz whisker stimulation with a continuous light pulse. In all cortical layers and in the L6-Drd1 cells, the continuous optogenetic activation of the L6-Drd1 cells had no effect on the WER (similar to that found for low-frequency whisker deflections; see Figs 8 and 10), and accordingly adaptation was similar to the control condition (Fig. 11 and Table 5: L6-Drd1 and POm; Table 6: data for the other cortical layers). In POm, however, optogenetic stimulation led to a change in the degree of adaptation. Under the experimental condition where the optogenetic activation of the L6-Drd1 cells starts 50 or 100 ms before the first whisker deflection, the 4th WER/1st WER ratio decreased (one-way paired ANOVA: post hoc test (Bonferroni test for multiple comparisons); 50 ms: P = 0.0042; 100 ms: P = 0.0311) (Fig. 11C). A relatively higher 1st response and a smaller 4th response both contribute to a stronger adaptation.

4 Hz whisker stimulation with 4 Hz light pulses. To investigate the effect on sensory adaptation in POm with a different temporal pattern between cortical and thalamic
activation, and sensory input, the 4 Hz stimulation protocol with a continuous light pulse was changed to a 4 Hz light pulse stimulation protocol. In this protocol the L6-Drd1 activity is regulated with short pulses preceding each of the four whisker deflections and ending at the end of each whisker deflection (Fig. 12A). The 4 Hz whisker and 4 Hz light pulse protocol had no effect on WER in the L6-Drd1 cells (Fig. 12B; Table 7). The pulse starting 50 ms, rather than 100 ms, before the whisker deflection gave a clearer increase in the WER in POm. The increase with a 50 ms delay but not with a 100 ms delay is presumably due to the fact that the light induced increase in spiking is not constant (see Fig. 4), and thus when a whisker deflection arrives 100 ms after the onset of the light pulse, L6-Drd1 driven activation of POm is somewhat decreased. The subsequent whisker deflections caused similar WER in all conditions (control and 50 and 100 ms; Fig. 12; Table 7), thus adaptation was largely unaltered (Fig. 12C).

In conclusion, repeating the L6-Drd1 cell activation with each whisker deflection increases the initial WER, but has no effect on subsequent WERs. Continuous L6-Drd1 activation throughout the whisker stimulation train leads, in addition to the increased response to the first whisker deflection, to a small increase in adaptation.

Discussion
We find that photoactivation of L6-Drd1 CT cells in somatosensory barrel cortex has little effect on cortical activity, but activates cells in POm. Repetitive photoactivation evoked a reliable response in L6-Drd1 cells following every light pulse, whereas in the connected POm area spiking was only initially increased. The response to repetitive whisker stimulation in combination with optogenetic stimulation showed a similar pattern with only an initial increase of the first whisker-evoked response (Figs 11 and 12). The increase in whisker-evoked spiking with optogenetic activation of L6-Drd1 cells is additive, rather than multiplicative, causing even cells that in the absence of L6 activation produce relatively few spikes, to increase their spiking substantially. The results thus indicate that L6-Drd1 cells are a sub-population of L6 CT cells that can cause an initial increase in POm activity that is not sustained with repetitive stimulation. This could indicate that L6 projections are a mix of driver and modulatory input. L6-Drd1 inputs to somatosensory thalamus might thus not (only) modify ongoing sensory processing, but rather serve to briefly increase POm activity in specific behavioural contexts. Anatomical tracing techniques (Figs 2 and 3; Supporting information: Videos S1 and S2), including both anterograde and retrograde tracing, showed that Drd1-cell are localized in the lower part of layer 6, project mainly to POm and receive most of their presynaptic inputs from layers 5 and 6 (Zolnik et al. 2020). A comparison with a different

![Figure 9](image_url)

**Figure 9.** The whisker-evoked LFP amplitude did not change in channels with L6-Drd1 single-unit spikes

A, left panel: averaged LFP traces recorded in electrode channels, in layer 6, from which identified L6-Drd1 units were also recorded. The traces are shown with mean amplitude and SD bands. Right panel: the normalized whisker-evoked LFP amplitudes with and without optogenetic stimulation. The LFP amplitude in L6-Drd1 cells was not affected (one-way paired ANOVA; statistics were done on the absolute values, not the normalised data). B, in POm the LFP amplitude increased statistically significant for the 50 ms delay protocol (one-way paired ANOVA with Bonferroni multiple comparisons test, $P = 0.0419$). Left panel, the averaged LFP amplitude in POm. Right panel, the normalized values of the whisker-evoked LFP amplitudes with and without L6-optogenetic stimulation.
sub-population of layer 6 corticothalamic cells, the L6-Ntr1, show that they have more extensive projections including VPM, POM and nRT, and presynaptic cells were found in layers 4–6.

**Effect of L6-Drd1 activation on spontaneous activity**

Optogenetic activation of L6-Drd1 cells increased spiking not only in the L6-Drd1 cells, but also in POM cells (Fig. 4). Interestingly, however, in the cortical layers L2/3–L5 and in L6 non-Drd1 cells, no clear effect of the optogenetic activation was detected (Fig. 6, Table 1). Consequently, it seems that the L6-Drd1 cells have, at most, only very weak projections within the barrel cortex. It was also shown for another L6 corticothalamic cell population (L6-Ntr1) that the photoactivation of those cells leads to a decrease in spiking in cortex (Pauzin & Krieger, 2018). The L6-Drd1 corticothalamic cells thus differ from the L6-Ntr1 cells not only anatomically (Figs 2 and 3; (Chevee et al. 2018; Hoerder-Suabedissen et al. 2018)), but also functionally.

Normalizing to the duration (20, 70, 120 ms; Fig. 4) of the light pulse used to photo-activate the L6-Drd1 cells showed that the spike rate decreased with duration, meaning that the L6-Drd1 cells did not fire at a steady rate during the complete stimulus duration, but rather spiking decreased. The transient light-induced L6-Drd1 spiking was observed over a range of stimulation intensities, and furthermore spiking activity did not appear to plateau even at the highest light intensities used (Fig. 5). Interestingly, in POM, however, a plateau was reached even at medium illumination intensity (Fig. 5). In addition, repetitive photoactivation of the L6-Drd1 cells caused a strong adaptation of the light-evoked POM response, even though the spiking rate of the L6-Drd1 cells remained constant over the four pulses (Fig. 7). Furthermore, intrinsic spiking properties in POM cells and inhibition via nRT, could contribute to the decreased excitability upon repetitive spiking (Landsman & Connors, 2007; Groh et al. 2008; Seol & Kuner, 2015). Taken together, this indicates that the L6-Drd1-POM synapse depresses, such that the POM cells will not get more activated even with stronger activation of the L6-Drd1 population, indicating that the L6-Drd1 cells provide a rapid activation of POM that is not sustained with repetitive whisker deflections. The relative contribution of L6-Drd1 cells and GABAergic inputs on POM activity remains, however, to be clarified (Lavallee et al. 2005).

**Effect of L6-Drd1 activation on low-frequency whisker-evoked responses**

To investigate the function of the L6-Drd1 cells on sensorimotor integration, the optogenetic activation of these cells was combined with whisker stimulation. Spiking activity in the L6-Drd1 cells was not constant during the light stimulus duration (Fig. 4), and therefore the L6-Drd1 cells were activated 50 ms or 100 ms immediately before the onset of whisker stimulation to achieve different levels of cortical drive of POM. Although the photoactivation increased spiking in L6-Drd1 cells, whisker deflections...
evoked the same number of spikes in the L6-Drd1 cells as under control conditions (whisker deflection with no photoactivation, Fig. 8C). This suggests that the WER had already maximized L6-Drd1 spiking such that a further increase in the number of spikes was not possible. Notably, the air puff whisker stimulation used evoked a response in the bottom plateau of the dose-response curve for the whisker-evoked activity (Fig. 8A and B), and thus the lack of an effect with optogenetic stimulation is not likely to be caused by an already saturating whisker stimulation.

In the other cortical layers L6-Drd1 photoactivation did not affect the WER (Fig. 10), nor did photoactivation per se substantially increase spiking (Fig. 6). Optogenetic stimulation of POm axons in cortex can, however, increase whisker-evoked responses in cortex (Mease et al. 2016a; Zhang & Bruno, 2019) and in the visual and auditory cortico-thalamic loop layer 6 corticothalamic projections are largely to reciprocally connected thalamic regions (Van Horn & Sherman, 2004; Llano & Sherman, 2008). The lack of a cortical effect in the present experiments, when activating POm via L6-Drd1 cells, could be because the activated L6-Drd1 cells project to an area in POm which does not project back to the area recorded from. Alternatively, weaker subthreshold effects could not be detected in the present experiments since in layers 2–5 only spikes were recorded.

In POm, optogenetic activation led to a complex effect such that L6-photoactivation had a multiplicative effect on spontaneous spiking activity (spiking increased ~2.3-fold) and an additive effect on the whisker-evoked response (increased with ~20 spikes s⁻¹; data from 50 ms delay between photoactivation and whisker deflection; Fig. 8E). There was no correlation (Spearman correlation, P = 0.1480) between the multiplicative and additive effects, meaning that for a given MUA recording one could not predict the whisker-evoked response from the effect of photoactivation of L6-Drd1 cells on spiking activity. The relevance of additive and multiplicative modulation (Thiele et al. 2009; Goris et al. 2014; Lin et al. 2015; Arandia-Romero et al. 2016) of POm spiking remains to be investigated, but could tentatively be interpreted such that an increase in a fixed number of spikes (additive effect) ensures that weakly responding cells also reach a spiking rate threshold at which information is efficiently communicated. A fundamental question is: do the multiplicative or additive gain functions reveal anything interesting regarding the L6 gain control of POm? If, following a short, ‘unexpected’ whisker deflection, the L6-input to POm boosts the activity, then it can be argued that when there is only low spontaneous
L6-Drd1 driven activation of POm is primarily important for brief activation caused by sudden whisker stimulation, rather than for encoding of repetitive tactile stimuli. When spiking activity increases in POm, the additional increase in whisker-evoked spiking that is necessary to reach ‘threshold’ (here interpreted as spike rate; Ahissar et al. 2000) will be lower, giving increased sensitivity at the expense of selectivity. An alternative interpretation is that the importance of L6-Drd1 in modifying POm activity is not the initial increase in POm activity, but rather the reduced response to repetitive whisker deflections (as seen with a continuous light pulse, Fig. 11) mediating a suppression of ongoing behaviour (Smith et al. 2012; Alloway et al. 2017). Reducing whisker-evoked responses to repetitive stimuli could be related to the proposed AND-gate function of POm where input from both brainstem and cortex is necessary to drive POm output (Sosnik et al. 2001; Groh et al. 2014). Further considerations when interpreting the present results is the relative timing between whisker activation and L6 activation. The timing

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**Table 5. (related to Fig. 11). Optogenetic activation (continuous pulse) during high frequency whisker stimulation**

| Treatment | L6-Drd1 (50 ms) (spikes s⁻¹) | L6-Drd1 (100 ms) (spikes s⁻¹) | POM (50 ms) (spikes s⁻¹) | POM (100 ms) (spikes s⁻¹) |
|-----------|----------------------------|----------------------------|--------------------------|--------------------------|
| Spont opto | 8.13 ± 9.77 | 5.78 ± 4.94 | 15.23 ± 7.70 | 15.63 ± 6.69 |
| 1. WER + opto | 25.58 ± 23.62 | 24.25 ± 25.08 | 40.02 ± 21.17 | 43.09 ± 27.62 |
| 2. WER + opto | 14.28 ± 11.70 | 15.13 ± 14.75 | 19.36 ± 10.62 | 21.64 ± 13.19 |
| 3. WER + opto | 13.21 ± 11.41 | 13.49 ± 14.08 | 17.02 ± 9.33 | 18.17 ± 11.40 |
| 4. WER + opto | 11.22 ± 9.22 | 11.36 ± 10.47 | 16.63 ± 11.27 | 18.95 ± 11.63 |
| Spont control | 5.73 ± 5.42 | 24.95 ± 23.93 | 33.48 ± 19.68 | 24.53 ± 18.99 |
| 1. WER | 16.25 ± 14.28 | 21.51 ± 14.62 | 20.71 ± 12.07 |
| 3. WER | 13.72 ± 11.11 | 25.08 ± 19.99 |
| 4. WER | 12.65 ± 10.32 | 21.51 ± 14.62 |
| Pulse | 0.8621 | 0.5986 | 0.5235 |
| Interaction | 0.7938 | 0.0013 | 0.9258 |
| Control-opto: 1. pulse | >0.9999 | >0.9999 | 0.0151 | 0.0003 |
| Control-opto: 2. pulse | 0.9139 | >0.9999 | 0.0665 | 0.4603 |
| Control-opto: 3. pulse | >0.9999 | >0.9999 | 0.1266 | 0.3299 |
| Control-opto: 4. pulse | >0.9999 | >0.9999 | 0.1822 | 0.9258 |

'Spont opto' is spiking activity with only light pulse (50 or 100 ms) but no whisker deflection. 'Spont control' is spiking activity with no light pulse. WER = whisker-evoked response. The table shows the results of the two-way ANOVAs (repeated on both factors) and the post hoc tests (Bonferroni multiple comparison test) in L6-Drd1 cells and in POm. Data are SUA for L6-Drd1 and MUA for POm from 17 animals. Main factors: row factor Treatment (spontaneous activity, 1st WER, 2nd WER, 3rd WER and 4th WER); column factor Pulse (light pulse duration: zero (no light pulse), 50 or 100 ms). P-values are highlighted in bold. Grey shading is used to highlight when the p-value was < 0.05 (i.e. effect was statistically significant).
Table 6. Effect of the optogenetic activation of L6-Drd1 cells on whisker-evoked activity at high frequency (continuous light stimulation)

|               | L2/3 50 ms (spikes s\(^{-1}\)) | L2/3 100 ms (spikes s\(^{-1}\)) | L4 50 ms (spikes s\(^{-1}\)) | L4 100 ms (spikes s\(^{-1}\)) | L5 50 ms (spikes s\(^{-1}\)) | L5 100 ms (spikes s\(^{-1}\)) | L6 non-Drd1 50 ms (spikes s\(^{-1}\)) | L6 non-Drd1 100 ms (spikes s\(^{-1}\)) |
|---------------|---------------------------------|---------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------------|---------------------------------|
| Spont opto    | 1.80 ± 4.64                    | 1.70 ± 3.06                    | 4.17 ± 6.96                 | 4.07 ± 4.98                 | 6.82 ± 7.56                 | 9.55 ± 8.53                 | 4.61 ± 5.91                    | 4.97 ± 5.26                    |
| 1. WER + opto | 15.27 ± 24.19                  | 14.54 ± 21.67                  | 39.53 ± 43.30               | 39.63 ± 45.03               | 49.48 ± 45.15               | 47.76 ± 49.97               | 22.73 ± 25.76                  | 25.27 ± 31.63                  |
| 2. WER + opto | 12.46 ± 22.29                  | 11.26 ± 18.13                  | 33.74 ± 40.68               | 34.15 ± 39.61               | 38.32 ± 38.06               | 38.67 ± 41.06               | 13.08 ± 15.76                  | 18.19 ± 24.78                  |
| 3. WER + opto | 11.18 ± 20.29                  | 10.56 ± 17.55                  | 31.07 ± 38.78               | 31.91 ± 38.44               | 34.34 ± 34.91               | 34.19 ± 39.28               | 11.20 ± 14.47                  | 16.42 ± 24.12                  |
| 4. WER + opto | 9.49 ± 17.29                   | 9.85 ± 16.01                   | 27.95 ± 35.33               | 29.48 ± 35.68               | 30.64 ± 31.13               | 32.34 ± 36.70               | 10.34 ± 12.70                  | 14.45 ± 21.81                  |
| Spont control |                                |                                |                             |                             |                             |                             | 1.91 ± 4.00                    | 3.71 ± 6.01                    |
| 1. WER        | 15.22 ± 24.48                  | 15.22 ± 24.48                  | 37.41 ± 49.78               | 37.41 ± 49.78               | 42.01 ± 41.24               | 42.01 ± 41.24               | 53.13 ± 51.11                  | 24.72 ± 31.45                  |
| 2. WER        | 12.56 ± 21.41                  | 12.56 ± 21.41                  | 32.84 ± 43.65               | 32.84 ± 43.65               | 38.46 ± 37.36               | 38.46 ± 37.36               | 42.01 ± 41.24                  | 16.55 ± 23.43                  |
| 3. WER        | 10.14 ± 16.46                  | 10.14 ± 16.46                  | 29.49 ± 39.12               | 29.49 ± 39.12               | 38.46 ± 37.36               | 38.46 ± 37.36               | 14.70 ± 22.29                  | 14.70 ± 22.29                  |
| 4. WER        | 10.09 ± 15.84                  | 10.09 ± 15.84                  | 28.46 ± 36.86               | 28.46 ± 36.86               | 36.23 ± 35.30               | 36.23 ± 35.30               | 14.11 ± 22.40                  | 14.11 ± 22.40                  |

Control-opto:1. pulse  
Control-opto:2. pulse  
Control-opto:3. pulse  
Control-opto:4. pulse  

Two-way ANOVAs repeated on both factors and the post hoc tests Bonferroni multiple comparison test were done separately on data from each cortical layer. WER = whisker-evoked response. Data are MUA from 17 animals. Main factors: row factor Treatment (spontaneous activity, 1st WER, 2nd WER, 3rd WER and 4th WER); column factor Pulse (light pulse duration: without, 50 or 100 ms). P-values are highlighted in bold. Grey shading is used to highlight when the p-value was < 0.05 (i.e. effect was statistically significant).
between L5B activation and whisker stimulation can affect POM activity (Groh et al. 2014), and thus presumably the timing between L6 and whisker activation will also be important. Motor cortex strongly activates deep L6 pyramidal cells (Zolnik et al. 2020); thus when L6 is activated before the whisker deflection (as in the present experiments) this could be representative of a condition where, for example, motor cortex activation has modified somatosensory activity. The present experiments were done in anaesthetized animals, and thus questions pertaining to timing of relative cortical and subcortical areas can only be preliminary (Simons et al. 1992). In rat POM, spontaneous activity increases in awake compared to anaesthetized animals (Masri et al. 2008; Zhang & Bruno, 2019). Notably, however, anaesthetized and awake recordings in POM have been shown to give qualitatively similar results with regards to whisker-evoked responses (Masri et al. 2008), and cortical layer 2/3 activation (Zhang & Bruno, 2019). In the present experiments in urethane-anaesthetized animals, driving POM via L6 activation did not have a strong effect on cortical responses. In awake animals a brief POM activation could, however, have long-lasting effects on cortical L2/3 neurons (Zhang & Bruno, 2019). Thus, in view, of the increased responsiveness of cortical layers 2/3 and 5 upon direct POM activation (Gambino et al. 2014; Mease et al. 2016a; Zhang & Bruno, 2019), and the fact that POM activity is state-dependent (Urbain et al. 2015), it is possible that the cortical effect of driving POM via L6 activity is more complex than presently observed. If, as suggested from the present experiments in anaesthetized mice, the L6-POM interaction is rather one of brief activation, and not a passive transmission of cycle-to-cycle activity, the effect is likely to be important in more complex tasks beyond single-whisker contacts (Sachidhanandam et al. 2013; Groh et al. 2014; Zhang & Bruno, 2019).

In addition to the increased evoked spiking rate in POM, the peak amplitude of the local field potential (LFP) increased (Fig. 9B). With a short delay (50 ms between onset of optogenetic stimulation and whisker stimulation) the increase of the LFP amplitude reached statistical significance but not with a longer delay (100 ms). This result is in line with the effect on the whisker-evoked response (WER), where the 50 ms delay appeared to increase the WER slightly more than the 100 ms delay (Fig. 8D). The reason presumably being that with the longer latency the L6-Drd1 activity decreases and thus contributes less at the time of the WER, and consequently the POM activation decreases. It is thus clear that the timing between the cortically driven increase in POM

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Figure 12. The effect of a 4 Hz L6-Drd1 photoactivation protocol on adaptation to whisker deflections

A, the stimulation protocol. Each whisker deflection was preceded by a light pulse starting 50 ms (blue bar) or 100 ms (pink bar) before the whisker deflection. B and C, there is no change in the adaptation rate in L6-Drd1 cells or in POM cells (black = control; blue = 50 ms and pink = 100 ms). Mean ± SD from 10 animals. *significant differences between the 1st whisker-evoked response and 4th whisker-evoked response. Numbers and statistics in Table 7. [Colour figure can be viewed at wileyonlinelibrary.com]
spiking and a subsequent incoming whisker stimulation will determine the net effect of whisker stimulation of POm activity (Groh et al. 2014).

**Effect of L6-Drd1 activation on high-frequency whisker-evoked responses**

Adaptation is an important feature in the whisker system (Higley & Contreras, 2007; Maravall et al. 2007; Mease et al. 2014, 2017; Musall et al. 2014; Lampl & Katz, 2017; Liu et al. 2017), and consequently the effect of L6-Drd1 activation on the adaptation in POm was investigated. This was done with two different protocols to create two different temporal patterns between cortical and thalamic activation. One protocol used a continuous light pulse, starting 50 or 100 ms before the first whisker deflection, and lasting during the entire 4 Hz whisker stimulation (Fig. 11). The second protocol used repetitive 4 Hz light pulses preceding each of the four whisker stimulations by either 50 or 100 ms (Fig. 12). The 4 Hz whisker stimulation in combination with continuous L6-Drd1 photoactivation resulted in a stronger adaptation in POm. The response to the first whisker stimulation increased, resulting in a decreased ratio (4th WER/1st WER; Fig. 11C). The protocol using a 4 Hz whisker stimulation in combination with repetitive optogenetic activation (Fig. 12), resulted in an increased response in POm to the first whisker deflection, but the responses to the following whisker deflections reached similar levels as under the control condition. This is consistent with the effect of repeated photostimulation (Fig. 7) of L6-Drd1 resulting in sustained spiking in the L6-Drd1 cells, whereas POm activity was only transiently increased. The decreased WER in POm with continuous light could be due to a stronger depression of the L6-POm synapse and/or to other, non-cortical, sources of inhibition being recruited (Tregasus & Keller, 2004; Lavallee et al. 2005; Halassa & Acsady, 2016). These results show that the timing between cortical activation and subsequent whisker inputs, and the duration of the cortical activation determine how whisker inputs are encoded in POm, the interpretation being that it is the first rather than subsequent stimuli that most efficiently increase POm spiking. When the mouse moves freely, and the whiskers suddenly touch an object, it is important to quickly adjust the body and the whiskers to a new position to optimize tactile detection (Hill et al. 2011; Chakrabarti & Schwarz, 2018; Svoboda & Li, 2018). Results suggesting a possible ‘reset’ come from experiments (Casas-Torremocha et al. 2017) showing that POm activation can inhibit motor cortex, thus presumably reducing whisking transiently to adapt to the tactile stimuli.

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**Table 7. (related to Fig. 12). Optogenetic activation (short pulses) during high frequency whisker stimulation**

|                  | L6-Drd1 (50 ms) | L6-Drd1 (100 ms) | POm (50 ms) | POm (100 ms) |
|------------------|----------------|-----------------|-------------|-------------|
|                  | (spikes s⁻¹)   | (spikes s⁻¹)    | (spikes s⁻¹)| (spikes s⁻¹)|
| Spont opto       | 8.19 ± 11.21   | 7.33 ± 5.01     | 12.84 ± 3.96| 12.71 ± 5.91|
| 1. WER + opto    | 17.05 ± 17.73  | 18.19 ± 20.44   | 27.64 ± 10.63| 25.35 ± 5.58|
| 2. WER + opto    | 12.25 ± 12.40  | 11.99 ± 14.61   | 17.43 ± 7.36| 16.84 ± 6.34|
| 3. WER + opto    | 10.61 ± 12.11  | 9.82 ± 12.75    | 16.00 ± 5.99| 18.47 ± 6.73|
| 4. WER + opto    | 10.54 ± 11.49  | 9.98 ± 12.01    | 15.24 ± 4.91| 17.27 ± 6.22|
| Spont control    | 4.00 ± 3.21    | 8.69 ± 4.64     |             |             |
| 1. WER           | 16.96 ± 17.62  | 22.19 ± 9.39    |             |             |
| 2. WER           | 11.41 ± 10.66  | 17.10 ± 5.88    |             |             |
| 3. WER           | 10.87 ± 10.86  | 15.34 ± 5.20    |             |             |
| 4. WER           | 8.69 ± 8.87    | 14.01 ± 5.94    |             |             |

The upper part shows SUA (spikes s⁻¹) recorded from L6-Drd1 cells and MUA from POm cells. ‘spont. opto’ is spiking activity with only light pulse (50 or 100 ms) but no whisker deflection. ‘Spont control’ is spiking activity with no light pulse. WER = whisker-evoked response. Statistics were done using separate two-way ANOVAs (repeated on both factors) and the post hoc tests (Bonferroni multiple comparison test) in L6-Drd1 cells and POm, respectively. Main factors: row factor Treatment (spontaneous activity, 1st WER, 2nd WER, 3rd WER and 4th WER); Pulse (light pulse duration: zero (no pulse), 50 or 100 ms). Data are SUA for L6-Drd1 and MUA for POm from 10 animals. P-values are highlighted in bold. Grey shading is used to highlight when the p-value was < 0.05 (i.e. effect was statistically significant).
Comparing L5B and L6 activation of POm

A complication when determining cortical effects on POm activity is that the area commonly referred to as ‘POm’ appears in fact to consist of different sub-nuclei (Sumser et al. 2017). Based on anatomical characterization, the recordings in the present study were predominately done in the medial part of POm (Fig. 1D). Cortical layer 5B and 6 both provide excitatory inputs to POm (Groh et al. 2014). Whereas the L5B cells provide input to POm (Hoogland et al. 1991; Groh et al. 2008; Mease et al. 2016b), the L6 to thalamus pathway is thought to rather provide a modulatory input (Sherman & Guillery, 2002; Reichova & Sherman, 2004). The exact nature of the L6-Drd1 activation of POm cells needs to be further characterized in awake behaving animals, but at least in anaesthetized animals the spiking output of POm cells adapted with repetitive whisker stimulation, which would not be anticipated if the L6-Drd1 to POm synapse is of the ‘modulatory’ kind that produces facilitation (Sherman, 2016). It is worth noting, however, that previous in vitro investigations of the L6 to POm connection actually have reported that a minority of POm responses might be ‘driver’-like connections (Reichova & Sherman, 2004), and that paralemniscal input to POm can be of both types (Mo et al. 2017). In the present study we provide initial insights to the L6-Drd1 projection to POm, but further analysis of sub-threshold activity will, however, be required to determine the nature of the L6-Drd1 to POm synapse.

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