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FRONTIERS IN NEURAL CIRCUITS - EDITORS' PICK 2021

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A Computational Model of Loss of Dopaminergic Cells in Parkinson's Disease Due to Glutamate-Induced Excitotoxicity

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Parkinson's disease (PD) is a neurodegenerative disease associated with progressive
and inexorable loss of dopaminergic cells in Substantia Nigra pars compacta (SNc).
Although many mechanisms have been suggested, a decisive root cause of this cell
loss is unknown. A couple of the proposed mechanisms, however, show potential for
the development of a novel line of PD therapeutics. One of these mechanisms is the
peculiar metabolic vulnerability of SNc cells compared to other dopaminergic clusters;
the other is the SubThalamic Nucleus (STN)-induced excitotoxicity in SNc. To investigate
the latter hypothesis computationally, we developed a spiking neuron network-model of
SNc-STN-GPe system. In the model, prolonged stimulation of SNc cells by an overactive
STN leads to an increase in 'stress' variable; when the stress in a SNc neuron exceeds a
stress threshold, the neuron dies. The model shows that the interaction between SNc and
STN involves a positive-feedback due to which, an initial loss of SNc cells that crosses
a threshold causes a runaway-effect, leading to an inexorable loss of SNc cells, strongly
resembling the process of neurodegeneration. The model further suggests a link between
the two aforementioned mechanisms of SNc cell loss. Our simulation results show that
the excitotoxic cause of SNc cell loss might initiate by weak-excitotoxicity mediated
by energy deficit, followed by strong-excitotoxicity, mediated by a disinhibited STN. A
variety of conventional therapies were simulated to test their efficacy in slowing down SNc
cell loss. Among them, glutamate inhibition, dopamine restoration, subthalamotomy and
deep brain stimulation showed superior neuroprotective-effects in the proposed model.

Keywords: Parkinson's disease, excitotoxicity, deep brain stimulation, Izhikevich neuron model, Substantia Nigra
pars compacta, SubThalamic Nucleus, Globus Pallidus externa, metabolic disorders

1. INTRODUCTION

There is a long tradition of investigation into the etiology and pathogenesis of Parkinson's Disease
(PD) that seeks to link molecular (pesticides, oxidative stress, protein dysfunction etc.) (Hwang,
2013; Ortiz et al., 2016; Chiti and Dobson, 2017; Anselmi et al., 2018; Stykel et al., 2018) and
subcellular (mitochondrial dysfunction etc.) (Henchcliffe and Beal, 2008; Reeve et al., 2018; Tsai
et al., 2018) factors with the disease development. However, recent years see the emergence of two

novel lines of investigation into PD pathogenesis. These approaches, that aim to understand the PD pathology at the cellular and network level, mark a significant deviation from the traditional approaches (Rodriguez et al., 1998; Pissadaki and Bolam, 2013; Pacelli et al., 2015; Chakravarthy and Moustafa, 2018).

The first approach believes the primary factor that causes the degeneration of dopaminergic neurons of Substantia Nigra pars compacta (SNc) is its high metabolic requirements. SNc neurons are one of the most vulnerable and energy consuming neuronal clusters, due to their structural and functional properties. Here, we have listed down some of the plausible factors which make SNc cells to be most susceptible.

- *Complex axonal arbors*: Large axonal arborisation which requires large amounts of energy to drive currents along these axons (Bolam and Pissadaki, 2012; Pissadaki and Bolam, 2013).
- *Reactive neurotransmitter*: When a reactive neurotransmitter like dopamine is present in excess, it would readily oxidizes with proteins, nucleic acids and lipids (Sulzer, 2007) eventually leading to neurodegeneration. One of the mechanisms for sequestration of excess cytosolic dopamine is packing of dopamine into synaptic vesicles through vesicular monoamine transporter-2 (VMAT-2) using H+ concentration gradient which is maintained by H+-ATPase. In addition to that, in the case of substantia nigra, the expression of VMAT2 is lower than in the ventral tegmental area (Liang et al., 2004; Mosharov et al., 2009) which likely causes dopamine-mediated oxidative stress in SNc cells.
- *Auto-rhythmicity*: Uses L-type calcium channels for maintaining the pace-making type of firing which in turn requires higher amounts of energy to maintain calcium homeostasis (Surmeier et al., 2017) and lower expression of calcium-binding proteins (lower capacity of calcium buffering mechanism) adds additional burden on the cell's metabolic activity (German et al., 1992).
- *NMDA synaptic activation*: Due to pacemaker type of firing, magnesium blockage of NMDA receptors is ineffective, resulting in substantial NMDA receptor currents even with weak glutamatergic inputs resulting in additional burden to maintain calcium homeostasis; the resulting energy deficiency leads to excitotoxicity (Rodriguez et al., 1998; Surmeier et al., 2010).
- *Prone to neuroinflammation*: Astrocytes play a modulatory role in microglial activation (McGeer and McGeer, 2008; Glass et al., 2010; Rocha et al., 2012) and any miscommunication between them results in neuroinflammation which eventually leads to neurodegeneration (Waak et al., 2009; Booth et al., 2017). The risk of inflammation in SNc neurons is high due to the small proportion of astrocytes regulating the huge population of microglia in this region (Lawson et al., 1990; Whitton, 2007; Mena and García de Yébenes, 2008). It has been reported that neuromelanin can induce microglial activation (Zecca et al., 2008; Zhang et al., 2011). SNc neurons are more susceptible to neuro-melanin induced inflammation compared to VTA neurons due to their high neuro-melanin

biosynthesis as a result of underexpression of VMAT2 (Peter et al., 1995; Liang et al., 2004).
- *Weak microvasculature*: SNc neurons are more prone to environmental toxins due to weak surrounding cerebral microvasculature (Rite et al., 2007).

Since the metabolic demands of SNc neurons are particularly high when compared to any other neuronal types (Sulzer, 2007) including neurons of other dopaminergic systems (Bolam and Pissadaki, 2012; Pacelli et al., 2015; Giguère et al., 2018), any sustained insufficiency in the supply of energy can result in cellular degeneration, characteristic of PD (Mergenthaler et al., 2013).

According to the second approach, the overactivity of SubThlamic Nucleus (STN) in PD causes excessive release of glutamate to the SNc, which in turns causes degeneration of SNc neurons by glutamate excitotoxicity (Rodriguez et al., 1998). The above two approaches are interrelated and not entirely independent as one form of excitotoxicity - the 'weak excitotoxicity' - is thought to have its roots in impaired cellular metabolism (Albin and Greenamyre, 1992). Therefore, the insight behind these new lines of investigation is the mismatch in energy supply and demand which could be a primary factor underlying neurodegeneration in PD. Such a mismatch is more likely to take place in special nuclei like SNc due to their peculiar metabolic vulnerability (Bolam and Pissadaki, 2012; Pissadaki and Bolam, 2013; Sulzer and Surmeier, 2013; Pacelli et al., 2015; Surmeier et al., 2017; Giguère et al., 2018). Similar ideas have been proffered to account for other forms of neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Beal et al., 1993; Johri and Beal, 2012; Gao et al., 2017).

If metabolic factors are indeed the underlying reason behind PD pathogenesis, it is a hypothesis that deserves closer attention and merits a substantial investment of time and effort for an in-depth study. This is because any positive proof regarding the role of metabolic factors puts an entirely new spin on PD research. Several researchers proposed that systems-level energy imbalance probably a principal cause of PD (Wellstead and Cloutier, 2011; Bolam and Pissadaki, 2012; Pacelli et al., 2015). Unlike current therapeutic approaches that manage the symptoms rather than provide a cure, the new approach can in principle point to a more lasting solution. If inefficient energy delivery or energy transformation mechanisms are the reason behind degenerative cell death, relieving the metabolic load on the vulnerable neuronal clusters, by intervening through current clinically approved therapeutics (such as brain stimulation and pharmacology) could prove to be effective treatments (Adhihetty and Beal, 2008; Spieles-Engemann et al., 2010; Seidl and Potashkin, 2011; Musacchio et al., 2017).

In this paper, with the help of computational models, we investigate the hypothesis that the cellular energy deficiency in SNc could be the primary cause of SNc cell loss in PD. *The higher metabolic demand of SNc cells due to their unique molecular characteristics, complex morphologies, and other energy-demanding features perhaps make them more vulnerable to energy deficit.* Therefore, prolonged energy deprivation or

insufficiency in such cells creates metabolic stress, eventually leading to neurodegeneration. If we can aim to reduce the metabolic stress on SNc cells, we can delay the progression of cell loss in PD.

In the proposed modeling study, we focus on excitotoxicity in SNc caused by STN which is precipitated by energy deficiency (Greene and Greenamyre, 1996) and exploring simulated therapeutic strategies for slowing down SNc cell loss. With the help of computational models of neurovascular coupling, our group had earlier explored the effect of rhythms of energy delivery from the cerebrovascular system on neural function (Gandrakota et al., 2010; Chander and Chakravarthy, 2012; Chhabria and Chakravarthy, 2016; Philips et al., 2016). Recently, we proposed a preliminary computational spiking network model of STN-mediated excitotoxicity in SNc with a slightly abstract treatment of apoptosis (Muddapu and Chakravarthy, 2017). Building on the previous version of the model, we have improved the excitotoxicity model by incorporating more biologically plausible dopamine plasticity and also explored the therapeutic strategies to slow down or halt the SNc cell loss.

2. MATERIALS AND METHODS

All the nuclei were modeled as Izhikevich 2D neurons (**Figure 1**). All the simulations were performed by numerical integration using MATLAB (RRID:SCR_001622) with a time step (dt) of 0.1 s. The average time for 50-s simulation was around 10 h, and it reduced to 5 h when ran on GPU card (Nvidia Quadro K620).

2.1. Izhikevich Neuron Model

Computational neuroscientists are often required to select the level at which a given model of interest must cast, i.e., biophysical-level, conductance-based modeling level, spiking neuron-level or rate-coded level. Biophysical models capture a more biologically detailed dynamics but are computationally expensive whereas rate-coded, point-neuron models are computationally inexpensive but possess less biologically detailed dynamics. To overcome this predicament, Izhikevich

FIGURE 1 | Proposed model architecture. The model architecture of the proposed model of STN-mediated excitotoxicity in SNc. STN, SubThalamic Nucleus; SNc, Substantia Nigra pars compacta; GPe, Globus Pallidus externa.

(2003) developed spiking neuron models that are comparatively computationally inexpensive yet capture various neuronal dynamics. The proposed model of excitotoxicity consists of SNc, STN, and Globus Pallidus externa (GPe) modeled using Izhikevich neuron models arranged in a 2D lattice (**Figure 1**). The population sizes of these nuclei in the model were selected based on the neuronal numbers of these nuclei in rat basal ganglia (Oorschot, 1996). The Izhikevich parameters for STN and GPe were adapted from Michmizos and Nikita (2011) and Mandali et al. (2015) and the parameters for SNc were adapted from Cullen and Wong-Lin (2015). The firing rates of these neuronal types were tuned to match the published data (Modolo et al., 2007; Tripathy et al., 2014) by varying the external bias current (I_{ij}^x). All parameters values are given in the **Table 1**. The Izhikevich model consists of two variables, one for membrane potential (v^x) and the other one for membrane recovery variable (u^x).

$$\frac{dv_{ij}^x}{dt} = 0.04(v_{ij}^x)^2 + 5v_{ij}^x + 140 - u_{ij}^x + I_{ij}^x + I_{ij}^{syn} \quad (1)$$

$$\frac{du_{ij}^x}{dt} = a(bv_{ij}^x - u_{ij}^x) \quad (2)$$

$$if \quad v_{ij}^x \geq v_{peak} \left\{ \begin{array}{c} v_{ij}^x \leftarrow c \\ u_{ij}^x \leftarrow u_{ij}^x + d \end{array} \right\} \quad (3)$$

where, v_{ij}^x, u_{ij}^x, I_{ij}^{syn}, and I_{ij}^x are the membrane potential, the membrane recovery variable, the total amount synaptic current received and the external current applied to neuron x at location (i, j), respectively, v_{peak} is the maximum membrane voltage set to neuron (+30 mV) with x being GPe or SNc or STN neuron.

2.2. Synaptic Connections

The presence of excitatory synaptic connectivity from STN to SNc was observed from anatomical and electrophysiology studies (Kita and Kitai, 1987; Smith and Grace, 1992; Hamani et al., 2004, 2017) and these connections might take part in controlling the bursting activity of SNc (Smith and Grace 1992). The sizes (number of neurons) of SNc (8 × 8), STN (32 × 32) and GPe (32 × 32) nuclei in the model were selected such that they match the proportions as observed in the rat basal ganglia (Oorschot, 1996). We also modeled convergent projections from STN to SNc as per anatomical observations (Oorschot, 1996). Similarly, the synaptic connectivity between GPe and STN was considered one-to-one as in Dovzhenok and Rubchinsky (2012) and Mandali et al. (2015). The equations used to model synaptic connectivity are

$$\tau_{Recep} * \frac{dh_{ij}^{x \to y}}{dt} = -h_{ij}^{x \to y} + S_{ij}^x(t) \quad (4)$$

$$I_{ij}^{x \to y}(t) = W_{x \to y} * h_{ij}^{x \to y}(t) * (E_{Recep} - V_{ij}^y(t)) \quad (5)$$

The NMDA currents are regulated by voltage-dependent magnesium channel (Jahr and Stevens, 1990) which was modeled as,

$$B_{ij}(v_{ij}) = \frac{1}{1 + (\frac{Mg^{2+}}{3.57} * e^{-0.062 * V_{ij}^y(t)})} \quad (6)$$
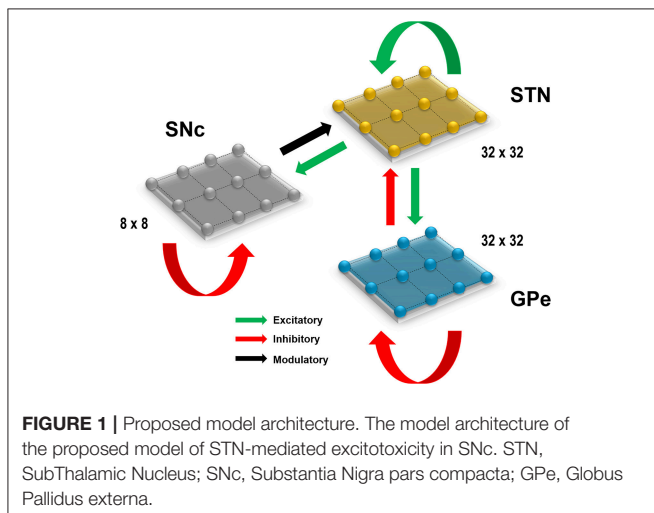
TABLE 1 | Parameter values used in the proposed model.

Parameter(s)	STN	SNc	GPe
Izhikevich parameters (a, b, c, d)	a = 0.005, b = 0.265, c = −65, d = 1.5	a = 0.0025, b = 0.2, c = −55, d = 2	a = 0.1, b = 0.2, c = −65, d = 2
External current (I^x)	I^{STN} = 3	I^{SNc} = 9	I^{GPe} = 4.25
Number of laterals ($nlat_x$)	$nlat_{STN}$ = 11	$nlat_{SNc}$ = 5	$nlat_{GPe}$ = 15
Radius of Gaussian laterals (R_x)	R_{STN} = 1.4	R_{SNc} = 1.6	R_{GPe} = 1.6
Synaptic strength within laterals (A_x)	A_{STN} = 1.3	A_{SNc} = 0.1	A_{GPe} = 0.1
Time decay constant for AMPA (τ_{AMPA})	6 ms	6 ms	6 ms
Time decay constant for NMDA (τ_{NMDA})	160 ms	160 ms	160 ms
Time decay constant for GABA (τ_{GABA})	4 ms	4 ms	4 ms
Synaptic potential of AMPA receptor (E_{AMPA})	0 mV	0 mV	0 mV
Synaptic potential of NMDA receptor (E_{NMDA})	0 mV	0 mV	0 mV
Synaptic potential of GABA receptor (E_{GABA})	−60 mV	−60 mV	−60 mV
Effect on the post-synaptic current ($cd2$)	0.1	0.1	0.1
Concentration of Magnesium (Mg^{2+})	1 nM	1 nM	1 nM

ms, milliseconds; mV, millivolts; nM, nanomolar.

where, S^x_{ij} is the spiking activity of neuron x at time t, τ_{Recep} is the decay constant for synaptic receptor, $h^{x \to y}_{ij}$ is the gating variable for the synaptic current from x to y, $W_{x \to y}$ is the synaptic weight from neuron x to y, Mg^{2+} is the magnesium ion concentration, V^y_{ij} is the membrane potential of the neuron y for the neuron at the location (i, j) and E_{Recep} is the receptor associated synaptic potential (Recep = NMDA/AMPA/GABA). The time constants of NMDA, AMPA, and GABA in GPe, SNc, and STN were chosen from Götz et al. (1997) are given in the **Table 1**.

2.3. Lateral Connections
Lateral connections are similar to collaterals of a neuron, and here it is defined as connections within each neuronal population. Earlier studies show the presence of lateral connections in STN (Kita et al., 1983) and GPe (Kita and Kita, 1994). In the case of SNc, the GABAergic interneurons were observed and their control of SNc activity revealed by immunohistochemistry studies (Hebb and Robertson, 1999; Tepper and Lee, 2007). To simplify the model, the GABAergic interneurons were replaced by GABAergic lateral connections in SNc population. Experimental studies show that synaptic current from lateral connections follows Gaussian distribution (Lukasiewicz and Werblin, 1990) that is, nearby neurons will have more influence than distant neurons. The lateral connections in various modules in the current network (STN, GPe, and SNc) were modeled as Gaussian neighborhoods (Mandali et al., 2015) and the parameters used are given in the **Table 1**. Each neuron receives synaptic input from a set number of neighboring neurons located in a 2D grid of size nxn.

$$w^{m \to m}_{ij,pq} = A_m * e^{\frac{-d^2_{ij,pq}}{R^2_m}} \qquad (7)$$

$$d^2_{ij,pq} = (i - p)^2 + (j - q)^2 \qquad (8)$$

where, $w^{m \to m}_{ij,pq}$ is the lateral connection weight of neuron type m at location (i, j), $d^2_{ij,pq}$ is the distance from center neuron (p, q), R_m is the variance of Gaussian, A_m is the strength of lateral synapse, m = GPe or SNc or STN.

2.4. Effect of DA on Synaptic Plasticity
Several experimental studies demonstrate dopamine-dependent synaptic plasticity in STN (Hassani et al., 1997; Magill et al., 2001; Yang et al., 2016) and GPe (Magill et al., 2001; Mamad et al., 2015). Experimental observations show an increase in synchrony in STN (Bergman et al., 1994, 1998) and GPe populations (Bergman et al., 1998) at low DA levels. The effect of low DA was implemented in the model by increasing in lateral connections strength in STN population as in Hansel et al. (1995) and similarly decrease in lateral connections strength in GPe as in Wang and Rinzel (1993). Similarly, SNc populations also showed an increase in synchrony at low DA levels (Hebb and Robertson, 1999; Vandecasteele et al., 2005; Tepper and Lee, 2007; Ford, 2014) which was modeled similarly to the model of DA-modulated GPe.

We modeled DA effect on the network as follows: as DA level increases, the strength of the lateral connections in STN decreases whereas, in GPe and SNc, lateral connection weights become stronger. As the lateral connection weights directly controls the amount of synaptic current each neuron receives. All the neurons in STN population will tend to fire together as the lateral connection weights increases (due to excitatory synapses). However, in the case of SNc and GPe it is contrary, that is, all the neurons will not tend to fire together as the lateral connection weights increases (due to inhibitory synapses). Lateral strength was modulated

by DA as follows,

$$A_{STN} = s_{STN}^{max} * e^{(-cd_{stn}*DA_s(t))} \qquad (9)$$

$$A_{GPe} = s_{GPe}^{max} * e^{(cd_{gpe}*DA_s(t))} \qquad (10)$$

$$A_{SNc} = s_{SNc}^{max} * e^{(cd_{snc}*DA_s(t))} \qquad (11)$$

where, s_{STN}^{max}, s_{GPe}^{max}, and s_{SNc}^{max} are strength of the lateral connections at the basal spontaneous activity of the population without any external influence in STN, GPe, and SNc, respectively. cd_{stn}, cd_{gpe}, and cd_{snc} were the factors by which dopamine affects the lateral connections in STN, GPe, and SNc populations, respectively, $DA_s(t)$ is the instantaneous dopamine level which is the spatial average activity of all the neurons in SNc.

According to experimental studies, DA causes post-synaptic effects on afferent currents in GPe and STN (Shen and Johnson, 2000; Smith and Kieval, 2000; Magill et al., 2001; Cragg et al., 2004; Fan et al., 2012). DA causes post-synaptic effects on afferent currents in SNc through somatodendritic DA receptors (Jang et al., 2011; Courtney et al., 2012; Ford, 2014). Thus, we included a factor ($cd2$), which regulates the effect of DA on synaptic currents of GPe, SNc, and STN. As observed in Kreiss et al. (1997), as DA level increases, the regulated current decreases as follows:

$$W_{x \rightarrow y} = (1 - cd2 * DA_s(t)) * w_{x \rightarrow y} \qquad (12)$$

where, $W_{x \rightarrow y}$ is the synaptic weight ($STN \rightarrow GPe$, $GPe \rightarrow STN$, $STN \rightarrow STN$, $GPe \rightarrow GPe$, $STN \rightarrow SNc$, $SNc \rightarrow SNc$), ($cd2$) is the parameter that affects the post-synaptic current, $DA_s(t)$ is the instantaneous dopamine level which is the spatial average activity of all the neurons in SNc.

2.5. Total Synaptic Current Received by Each Neuron

STN:

The total synaptic current received by a STN neuron at lattice position (i, j) is the summation of lateral glutamatergic input from other STN neurons considering both NMDA and AMPA currents and the GABAergic input from the GPe neurons.

$$I_{ij}^{STNsyn} = I_{ij}^{NMDAlat} + I_{ij}^{AMPAlat} + I_{ij}^{GABA \rightarrow STN} \qquad (13)$$

where, $I_{ij}^{NMDAlat}$ and $I_{ij}^{AMPAlat}$ are the lateral glutamatergic current from other STN neurons considering both NMDA and AMPA receptors, respectively, $I_{ij}^{GABA \rightarrow STN}$ is the GABAergic current from GPe neuron.

GPe:

The total synaptic current received by a GPe neuron at lattice position (i, j) is the summation of the lateral GABAergic current from other GPe neurons and the glutamatergic input from the STN neurons considering both NMDA and AMPA currents.

$$I_{ij}^{GPesyn} = I_{ij}^{GABAlat} + I_{ij}^{NMDA \rightarrow GPe} + I_{ij}^{AMPA \rightarrow GPe} \qquad (14)$$

where, $I_{ij}^{GABAlat}$ is the lateral GABAergic current from other GPe neurons, $I_{ij}^{NMDA \rightarrow GPe}$ and $I_{ij}^{AMPA \rightarrow GPe}$ are the glutamatergic current from STN neuron considering both NMDA and AMPA receptors, respectively.

SNc:

The total synaptic current received by a SNc neuron at lattice position (i, j) is the summation of the lateral GABAergic current from other SNc neurons and the glutamatergic input from the STN neurons considering both NMDA and AMPA currents.

$$I_{ij}^{SNcsyn} = I_{ij}^{GABAlat} + I_{ij}^{NMDA \rightarrow SNc} + I_{ij}^{AMPA \rightarrow SNc} \qquad (15)$$

where, $I_{ij}^{GABAlat}$ is the lateral GABAergic current from other SNc neurons, $I_{ij}^{NMDA \rightarrow SNc}$ and $I_{ij}^{AMPA \rightarrow SNc}$ are the glutamatergic current from STN neuron considering both NMDA and AMPA receptors, respectively.

2.6. Neurodegeneration

According to Rodriguez et al. (1998), dopamine deficiency in SNc leads to disinhibition and overactivity of the STN, which in turn causes excitotoxic damage to its target structures, including SNc itself. In order to simulate the SNc excitotoxicity induced by STN, we incorporate a mechanism of programmed cell death, whereby an SNc cell under high stress kills itself. The stress on a given SNc cell was calculated based on the firing history of the cell - higher firing activity causes higher stress.

The stress of each SNc neuron at lattice position (i, j) at time t due to excess firing is calculated as,

$$\tau_{stress} * \frac{dQ_{ij}^x}{dt} = -Q_{ij}^x + r_{ij}^x(t) \qquad (16)$$

where, $r_{ij}^x(t)$ is instantaneous mean firing rate of a SNc neuron at lattice position (i, j) at time t, calculated with a fixed sliding window Δt (1 s) (Dayan and Abbott, 2005) as,

$$r_{ij}^x(t) = \frac{1}{\Delta t} \int_{t-\Delta t}^{t} d\tau \rho(\tau) \qquad (17)$$

and,

$$\rho(\tau) = \sum_{i=1}^{n} \delta(t - t_i) \qquad (18)$$

Sequence of spike timing: $t_i = 1, 2, 3 \ldots n$

If stress variable (Q_{ij}^x) of a SNc neuron at lattice position (i, j) crosses certain threshold (S_{thres}) then that particular SNc neuron will be eliminated (Iglesias and Villa, 2008).

$$if \quad Q_{ij}^x(t) > S_{thres}, \qquad then \quad v_{ij}^x(t) = 0 \qquad (19)$$

2.6.1. Estimating Rate of Degeneration

For a given course of SNc cell loss, the half-life is the time taken for half of the SNc cells to be lost ($t_{1/2}$). The following equation was used to estimate the number of

SNc cells ($N_{sc}(t)$) for a given course that survived after a given time t.

$$N_{sc}(t) = N_{sc}^0 * e^{-\lambda t} \quad (20)$$

where, λ is the rate of degeneration (sec^{-1}), N_{sc}^0 is the number of surviving SNc cells at $t = 0$.

To estimate the rate of degeneration λ from a given course of SNc cell loss, the following equation was used,

$$\lambda = \frac{\ln 2}{t_{1/2}} \quad (21)$$

The instantaneous rate of degeneration $\lambda(t)$ was calculated by the following equation,

$$\lambda(t) = \frac{\ln(N_{sc}(t)) - ln(N_{sc}(t-1))}{t - (t-1)} \quad (22)$$

2.7. Neuroprotective Strategies

Pharmacological or surgical therapies that abolish the pathological oscillations in STN or block the receptors on SNc can be neuroprotective and might slow down the progression of SNc cell loss (Rodriguez et al., 1998).

2.7.1. Glutamate Inhibition Therapy

Glutamate drug therapy can have neuroprotective effect on SNc in two ways (1) Inactivation of NMDA (N-methyl-D-aspartate), AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazole-4-yl) propanoic acid) or excitatory metabotropic glutamate (Group-I - mGluR1/5) receptors (mGluR) by glutamate antagonists, and (2) Activation of metabotropic glutamate (Group-II/III - mGluR2,3/4,6,7,8) receptors by glutamate agonists. NMDA antagonist MK-801 showed reduction of SNc cell loss in the neurotoxic rats (Turski et al., 1991; Zuddas et al., 1992b; Brouillet and Beal, 1993; Blandini, 2001; Armentero et al., 2006) and primates (Zuddas et al., 1992a,b). AMPA antagonists such as NBQX (Merino et al., 1999), LY-503430 (Murray et al., 2003) and LY-404187 (O'Neill et al., 2004) exhibited neuroprotection of SNc cells in the neurotoxic animal models. mGluR-5 antagonist MPEP and MTEP showed neuroprotection in 6-OHDA lesioned rats (Armentero et al., 2006; Hsieh et al., 2012; Ferrigno et al., 2015; Fuzzati-Armentero et al., 2015) and MPTP-treated primates (Masilamoni et al., 2011), respectively. Broad-spectrum group II (Murray et al., 2002; Battaglia et al., 2003; Vernon et al., 2005) and group III (Vernon et al., 2005; Austin et al., 2010) agonists showed neuroprotection in neurotoxic rats. Selective mGluR2/3 agonist 2R,4R APDC (Chan et al., 2010) and mGluR4 agonist VU0155041 (Betts et al., 2012) significantly attenuated SNc cell loss in 6-OHDA lesioned rats.

The glutamate drug therapy was implemented in the proposed excitotoxicity model by the following criterion,

$$W_{STN \to SNc}(N_{sc}, t) = \begin{cases} W_{STN \to SNc}^0, & N_{sc}(t) > N_i \\ W_{STN \to SNc}^0 * \delta_{GI}, & N_{sc}(t) \le N_i \end{cases} \quad (23)$$

where, $W_{STN \to SNc}(N_{sc}, t)$ is the instantaneous change in synaptic weight of STN to SNc based on the number of surviving SNc neurons at time t $N_{sc}(t)$ is the instantaneous number of surviving SNc neurons, $W_{STN \to SNc}^0$ is the basal connection strength of STN to SNc, δ_{GI} is the proportion of glutamate inhibition, N_i is the number representing SNc cell loss $\langle i = 25\% \mid 50\% \mid 75\% \rangle$ at which therapeutic intervention was employed. In the present study, we have considered 25% (cells lost = 16), 50% (cells lost = 32) and 75% (cells lost = 48) SNc cell loss as early, intermediate and late stages of disease progression, respectively.

2.7.2. Dopamine Restoration Therapy

The neuroprotective effects of DA agonists therapy are thought to be due to one or more of the following mechanisms: (1) L-DOPA sparing, (2) Autoreceptor effects, (3) Antioxidant effects, (4) Antiapoptotic effects, and (5) Amelioration of STN-mediated excitotoxicity (Olanow et al., 1998; Grandas, 2000; Schapira, 2003; Zhang and Tan, 2016). In the present study, we focus on the amelioration of STN-mediated excitotoxicity. DA agonists can restore the dopaminergic tone in the dopamine-denervated brain, which results in increased inhibition in STN, thereby diminishing STN-induced excitotoxicity on SNc neurons (Olanow et al., 1998; Schapira and Olanow, 2003; Piccini and Pavese, 2006; Vaarmann et al., 2013).

The dopamine agonist therapy was implemented in the proposed excitotoxicity model by the following criterion,

$$DA(N_{sc}, t) = \begin{cases} DA_s(t), & N_{sc}(t) > N_i \\ DA_s(t) + \delta_{DAA}, & N_{sc}(t) \le N_i \end{cases} \quad (24)$$

where, $DA(N_{sc}, t)$ is the instantaneous change in dopamine level based on the number of surviving SNc neurons at time t $N_{sc}(t)$ is the instantaneous number of surviving SNc neurons, $DA_s(t)$ is the instantaneous dopamine signal from the SNc neurons, δ_{DAA} is the proportion of dopamine content restoration, N_i is the number representing SNc cell loss at which therapeutic intervention was employed.

2.7.3. Subthalamotomy

Subthalamotomy is still quite a common treatment amongst patients in advanced stages of PD where patients stop responding to L-DOPA (wearing-off) or chronic L-DOPA therapy results in motor complications such as L-DOPA Induced Dyskinesias (LID) (Alvarez et al., 2009; Obeso et al., 2017). It was reported that STN lesioning exhibits neuroprotective effect which acts as an antiglutamatergic effect in neurotoxic animal models (Piallat et al., 1996; Chen et al., 2000; Carvalho and Nikkhah, 2001; Paul et al., 2004; Wallace et al., 2007; Jourdain et al., 2014).

STN ablation was implemented in the proposed excitotoxicity model by the following criterion,

$$if \quad N_{sc}(t) \le N_i, \quad then \quad v_{ij}^{STN}(P_{les}, t) = 0 \quad (25)$$

where, P_{les} is the lesion percentage of STN which is selected from the following range: [5, 10, 20, 40, 60, 80, 100], $N_{sc}(t)$ is the instantaneous number of surviving SNc neurons, N_i is the number representing SNc cell loss at which therapeutic intervention was employed.

2.7.4. Deep Brain Stimulation (DBS) in STN

DBS therapy is preferred over ablation therapy of STN due to the potentially irreversible damage to the stimulated brain region in ablation therapy. It has been reported that long-term stimulation (DBS) of STN results in the slowdown of the progression of SNc cell loss in animal models (Benazzouz et al., 2000; Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Spieles-Engemann et al., 2010; Musacchio et al., 2017).

The DBS electrical stimulation was given in the form of current or voltage pulses to the target neuronal tissue (Cogan, 2008). The effect of DBS therapy was modeled as external stimulation current given to the entire or part of the STN module in the form of Gaussian distribution (Rubin and Terman, 2004; Hauptmann and Tass, 2007; Foutz and McIntyre, 2010; Mandali and Chakravarthy, 2016). The DBS parameters such as amplitude (A_{DBS}), frequency ($f_{DBS} = \frac{1}{T_{DBS}}$) and pulse width ($\delta_{DBS}$) were adjusted by using clinical settings as a constraint (Moro et al., 2002; Garcia et al., 2005), in order to reduce the synchrony in STN population along with the minimal rise in the firing rate. In addition to exploring DBS parameters, a range of stimulus waveforms (such as rectangular monophasic (MP) and biphasic (BP) current pulses) and different types of stimulation configurations (such as single contact point (SCP), four contact points (FCP) and multiple contact points (MCP)) were also implemented (**Figure 2**) (Cogan, 2008; Lee et al., 2016).

In the present study, the current pulses which given to neuronal network are in the form of monophasic and biphasic waveforms. The monophasic current pulse (P_{MP}) was generated as the following,

$$P_{MP}(t) = \begin{cases} A_{DBS}, & t_k \leq t < t_k + \delta_{DBS} \\ 0, & else \end{cases} \quad (26)$$

where, t_k are the onset times of the current pulses, A_{DBS} is the amplitude of the current pulse, δ_{DBS} is the current pulse width.

The biphasic current pulse (P_{BP}) was generated as the following,

$$P_{MP}(t) = \begin{cases} A_{DBS}, & t_k \leq t < t_k + \frac{\delta_{DBS}}{2} \\ -A_{DBS}, & t_k + \frac{\delta_{DBS}}{2} \leq t < t_k + \delta_{DBS} \\ 0, & else \end{cases} \quad (27)$$

where, t_k are the onset times of the current pulses, A_{DBS} is the amplitude of the current pulse, δ_{DBS} is the current pulse width.

The influence of stimulation on a particular neuron will depend on the position of the stimulation electrode in the neuronal network (Cogan, 2008). The effect of stimulation will decay as the distance between electrode position (i_c, j_c) and neuronal position (i, j) increased which was modeled as a Gaussian neighborhood (Mandali and Chakravarthy, 2016). We have assumed that the center of the electrode to be the mean of the Gaussian which coincides with the lattice position (i_c, j_c) and the spread of stimulus current was controlled by the width

of the Gaussian (σ).

$$I_{ij}^{DBS-STN}(t) = \sum_{\beta=1}^{N_{cp}^x} \mathcal{M}_\beta(t) * P_y(t) * e^{-\left[\frac{(i-i_c)^2 + (j-j_c)^2}{\sigma_{DBS-STN}^2}\right]} \quad (28)$$

where, $I_{ij}^{DBS-STN}(t)$ is the DBS current received by STN neuron at position (i, j) considering lattice position (i_c, j_c) as the electrode contact point at time t, $\mathcal{M}_\beta(t)$ is the indicator function which controls the activation of stimulation site β, N_{cp}^x is the number of activated stimulation contact points for different stimulation configurations $x = [SCP, FCP, MCP]$ ($N_{cp}^{SCP} = 1$, $N_{cp}^{FCP} = 4$, $N_{cp}^{MCP} = $ Number of neurons in simulated network - 1024), $P_y(t)$ is the current pulse at time t for $y = [MP, BP]$, $\sigma_{DBS-STN}$ is used to control the spread of stimulus current in STN network.

DBS was implemented in the proposed excitotoxicity model by the following criterion,

$$I_{ij}^{DBS-STN}(N_{sc}, t) = \begin{cases} 0, & N_{sc}(t) > N_i \\ I_{ij}^{DBS-STN}(t), & N_{sc}(t) \leq N_i \end{cases} \quad (29)$$

where, $I_{ij}^{DBS-STN}(t)$ is the instantaneous change in the stimulation current to STN neuron at position (i, j) based on the number of surviving SNc neurons at time t, $N_{sc}(t)$ is the instantaneous number of surviving SNc neurons, N_i is the number representing SNc cell loss at which therapeutic intervention was employed.

2.7.5. Antidromic Activation

The mechanism of how DBS alleviates advanced PD symptoms is not precise. One of the theories behind the therapeutic effect of DBS is activation of afferent connections of STN which results in antidromic activation of cortical, GPi or GPe neurons (Lee et al., 2004; McIntyre et al., 2004; Hammond et al., 2008; Montgomery and Gale, 2008; Kang and Lowery, 2014; Chiken and Nambu, 2015). In our study, we implemented the antidromic activation of GPe during DBS therapy. Antidromic activation was implemented similarly to Mandali and Chakravarthy (2016), where a percentage of DBS current given to STN neurons were given directly to GPe neurons. Similar to DBS applied to STN, external stimulation current was given to GPe neuron in the form of Gaussian distribution. The specifications of antidromic activation were described by the following equation,

$$I_{ij}^{DBS-GPe}(t) = \sum_{\beta=1}^{N_{cp}^x} \mathcal{M}_\beta(t) * P_y(t) * e^{-\left[\frac{(i-i_c)^2 + (j-j_c)^2}{\sigma_{DBS-GPe}^2}\right]} \quad (30)$$

where, $I_{ij}^{DBS-GPe}(t)$ is the DBS current received by GPe neuron at position (i, j) considering lattice position (i_c, j_c) as the electrode contact point, $\mathcal{M}_\beta(t)$ is the indicator function which controls the activation of stimulation site β, N_{cp}^x is the number of activated stimulation contact points for different stimulation configurations $x = [SCP, FCP, MCP]$ ($N_{cp}^{SCP} = 1$, $N_{cp}^{FCP} = 4$, $N_{cp}^{MCP} = $ Number of neurons in simulated network - 1024), $P_y(t)$ is the current pulse at time t for $y = [MP, BP]$, $A_{DBS-GPe}$ is the portion of DBS current pulse amplitude given as antidromic activation to
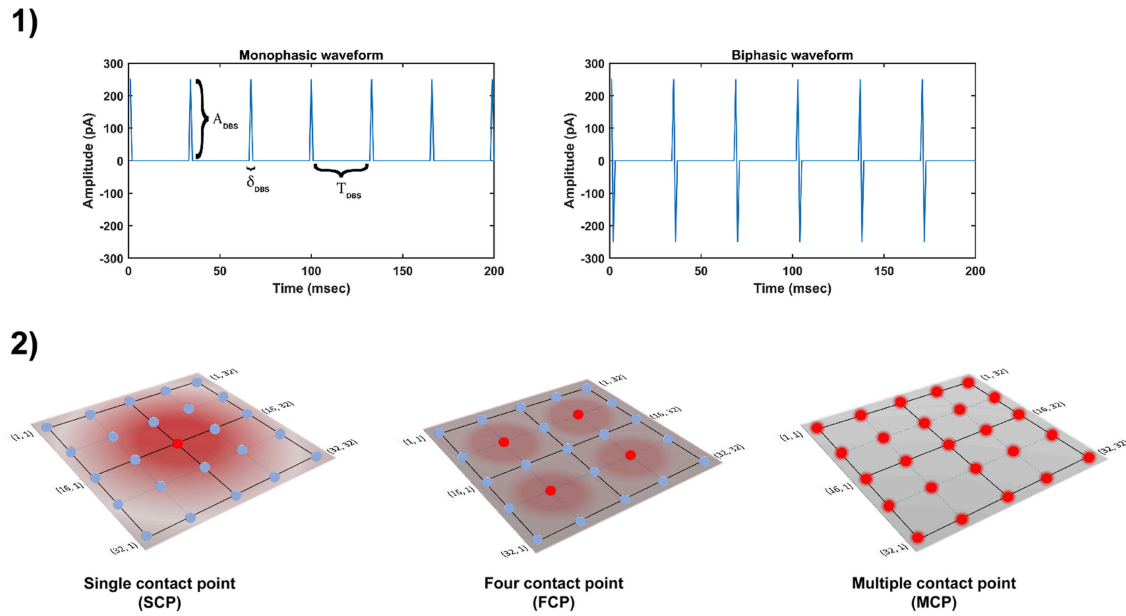
FIGURE 2 | Different DBS protocol used in the study. **(1)** DBS stimulation waveforms. **(2)** DBS stimulation configurations. DBS, Deep Brain Stimulation; A_{DBS}, Amplitude of DBS current pulse; δ_{DBS}, Pulse width of DBS current pulse; $T_{DBS}(1/f_{DBS})$, frequency of DBS current pulse; SCP, Single Contact Point; FCP, Four Contact Point; MCP, Multiple Contact Point.

GPe neurons, $\sigma_{DBS-GPe}$ is used to control the spread of stimulus current in GPe ensemble.

The DBS therapy with antidromic activation was implemented in the proposed excitotoxicity model by the following criterion,

$$
I_{ij}^{DBS-STN}(N_{sc}, t) = \begin{cases} 0, & N_{sc}(t) > N_i \\ I_{ij}^{DBS-STN-AA}(t), & N_{sc}(t) \leq N_i \end{cases}
$$
$$(31)$$

$$
I_{ij}^{DBS-GPe}(N_{sc}, t) = \begin{cases} 0, & N_{sc}(t) > N_i \\ I_{ij}^{DBS-GPe}(t), & N_{sc}(t) \leq N_i \end{cases} \quad (32)
$$

where, $I_{ij}^{DBS-STN-AA}(t)$ is the DBS current received by STN neuron at position (i, j) considering lattice position (i_c, j_c) as the electrode contact point with antidromic activation $(A_{DBS-GPe} = Per_{AA} * A_{DBS-STN}$; $A'_{DBS-STN} = (1 - Per_{AA}) * A_{DBS-STN})$, Per_{AA} is the proportion of $A_{DBS-STN}$ applied as $A_{DBS-GPe}$, $A'_{DBS-STN}$ is the portion of DBS current pulse amplitude given to STN neurons during antidromic activation, $N_{sc}(t)$ is the instantaneous number of surviving SNc neurons, N_i is the number representing SNc cell loss at which therapeutic intervention was employed.

2.7.6. STN Axonal & Synaptic Failures
In-vitro recordings observed depression in the synapse of STN neurons with SNc and is believed to be due to the delivery of continuous high-frequency stimulation pulses (Ledonne et al., 2012). This synaptic depression caused by increased STN activity during DBS arises due to an amalgamation of axonal and synaptic failures in the STN (Shen and Johnson, 2008; Ammari et al., 2011;

Moran et al., 2011, 2012; Zheng et al., 2011; Carron et al., 2013; Rosenbaum et al., 2014).

The effect of synaptic depression due to DBS of the STN was implemented by the following criterion,

$$
W_{STN \to SNc}(s_{DBS}, t) = \begin{cases} W_{STN \to SNc}, & s_{DBS} = OFF \\ W_{STN \to SNc} * W_{ASF}(Per_{ASF}), & s_{DBS} = ON \end{cases}
$$
$$(33)$$

where, $W_{STN \to SNc}(s_{DBS}, t)$ is the instantaneous change in synaptic weight of STN to SNc based $s_{DBS} = \{ON, OFF\}$, s_{DBS} is DBS stimulation, W_{ASF} is the weight matrix based on the percentage of axonal and synaptic failures (Per_{ASF}).

$$
W_{STN \to GPe}(s_{DBS}, t) = \begin{cases} W_{STN \to GPe}, & s_{DBS} = OFF \\ W_{STN \to GPe} * W_{ASF}(Per_{ASF}), & s_{DBS} = ON \end{cases}
$$
$$(34)$$

where, $W_{STN \to GPe}(s_{DBS}, t)$ is the instantaneous change in synaptic weight of STN to GPe based $s_{DBS} = \{ON, OFF\}$, s_{DBS} is DBS stimulation, W_{ASF} is the weight matrix based on the percentage of axonal and synaptic failures (Per_{ASF}).

2.8. Network Analysis
We analyzed the dynamics of the network (STN-GPe-SNc) by firing frequency (Dayan and Abbott, 2005), network synchrony (Pinsky and Rinzel, 1995) and bursting measures (van Elburg and van Ooyen, 2004). The equations used to compute these measures are described below.

2.8.1. Frequency of Firing

The spike-count firing rate is the measure of the number of action potentials for a given duration of time (Dayan and Abbott, 2005). The instantaneous mean firing rate ($r_{ij}^x(t)$) of a neuron at lattice position (i, j) at time t was calculated with a fixed sliding window Δt (0.1 s) which is similarly to Equations (17), (18). The mean firing rate of the population of neurons is simply the average of instantaneous mean firing rate across the number of neurons and the simulation time.

2.8.2. Synchronization

Neuronal synchronization is the measure of synchronicity (high synchrony - almost all neurons firing at once, low synchrony - least number of neurons firing at once) in the population of neurons within a network (Golomb, 2007). We had quantified the synchrony in the population of neurons at time t by following equation (Pinsky and Rinzel, 1995),

$$R_x(t) = \frac{1}{N * e^{i*\theta(t)}} \sum_{j=1}^{N} e^{i*\phi_j(t)} \qquad (35)$$

$$\phi_j(t) = 2 * \pi * \frac{(T_{j,k} - t_{j,k})}{t_{j,k+1} - t_{j,k}} \qquad (36)$$

where, $R_x(t)$ is the instantaenous synchronization measure ($0 \leq R_x(t) \leq 1$), x being GPe or SNc or STN neuron, N is the number of neurons in the network, $\theta(t)$ is the instantaneous average phase of neurons, $\phi_j(t)$ is the instantaneous phase of jth neuron, $t_{j,k}$ and $t_{j,k+1}$ are the spike times of kth and $(k + 1)$th spike of jth neuron, respectively, $T_{j,k} \in [t_{j,k}, t_{j,k+1}]$.

2.8.3. Bursting

If a neuron fires repeatedly with discrete groups of spikes, this dynamic state is termed as burst. Between two bursts, there is a period of quiescence where there will be no spikes. Burst can have two (doublet), three (triplet), four (quadruplet) or many spikes in it (Izhikevich, 2006). We had quantified the bursting of a neuron at lattice position (i, j) across time by following equation (van Elburg and van Ooyen, 2004),

$$B_{i,j} = \frac{2 * Var(t_{i,j,k+1} - t_{i,j,k}) - Var(t_{i,j,k+2} - t_{i,j,k})}{2 * E^2(t_{i,j,k+1} - t_{i,j,k})} \qquad (37)$$

where, $B_{i,j}$ is the measure of bursting of a neuron at lattice position (i, j), Var is the variance of the spike times, E is the expected value (mean) of the spike times, $t_{i,j,k}$, $t_{i,j,k+1}$ and $t_{i,j,k+2}$ are the spike times of kth, $(k + 1)$th and $(k + 2)$th spike of a neuron at lattice position (i, j), respectively.

3. RESULTS

We have investigated the Izhikevich parameters of STN, SNc and GPe which were chosen from the literature (Michmizos and Nikita, 2011; Cullen and Wong-Lin, 2015; Mandali et al., 2015) for their characteristic firing pattern and other biological properties (**Figure 3-1**). We then extensively studied the effect

of lateral connections in the network of neurons (**Figure 3-2**). Next, we have explored the effect of dopamine on the network of GPe, SNc, and STN neurons and compared with published data (**Figure 4**).

Then, we showed the results of the proposed excitotoxicity model which exhibits STN-mediated excitotoxicity in SNc (**Figures 5**, **6**) and studied their sensitivity to parameter uncertainty (**Figure 7**). Finally, we have explored current therapeutics such as glutamate inhibition (**Figure 8**), dopamine restoration (**Figure 9**), subthalamotomy (**Figure 10**) and deep brain stimulation (**Figures 11**, **12**) which might have a neuroprotective effect on the progression of SNc cell loss.

3.1. Characteristic Firing of Different Neuronal Types

The firing response of a single neuron to different external current input was characterized for the three different neuronal types involved in the excitotoxicity model (**Figure 3-1**). In the proposed model, we adjusted I_{ij}^x and other parameters of the Izhikevich model such that the basal firing frequencies of the different neuronal types match with experimental data (Modolo et al., 2007; Tripathy et al., 2014). The adjusted values can be seen in the **Table 1**.

The SNc neurons experimentally exhibit two distinct firing patterns: low-frequency irregular tonic or background firing (3–8 Hz) and high-frequency regular phasic or burst firing (~20 Hz) (Grace and Bunney, 1984a,b). The Izhikevich parameters which were chosen for SNc neurons configured the model to exhibit both types of firing patterns. Other properties such as doublet-spikes which were occasionally observed experimentally (Grace and Bunney, 1983) were also exhibited (**Figure 3-1A**). In the present model, SNc neuron basal firing rate were required to be ~4 Hz which is in the range of 3–8 Hz observed experimentally (Grace and Bunney, 1984a). Similar to SNc, STN neurons also exhibit tonic pacemaking firing and phasic high-frequency bursting (Beurrier et al., 1999; Allers et al., 2003). The basal firing rate of STN neurons was required to be ~13 Hz which is in the range of 6–30 Hz observed experimentally (Allers et al., 2003; Lindahl et al., 2013). The STN neurons also exhibit characteristic inhibitory rebound which was observed experimentally (**Figure 3-1B**) (Hamani et al., 2004; Johnson, 2008). Unlike SNc and STN, GPe neurons exhibit high-frequency tonic firing which was interpreted by bursts and pauses (Kita and Kita, 2011; Hegeman et al., 2016). The Izhikevich parameters which were chosen for GPe neurons were able to exhibit high-frequency firing without any bursts (**Figure 3-1C**). The basal firing rate of GPe neurons was required to be ~30 Hz which is in the range of 17–52 Hz observed experimentally (Lindahl et al., 2013).

3.2. Behavior Regimes With Varying Collateral Strength and Radius

We now study the network dynamics of each of the three neuronal types in a 2D array with lateral connections. The effect of network structural properties such as the strength and
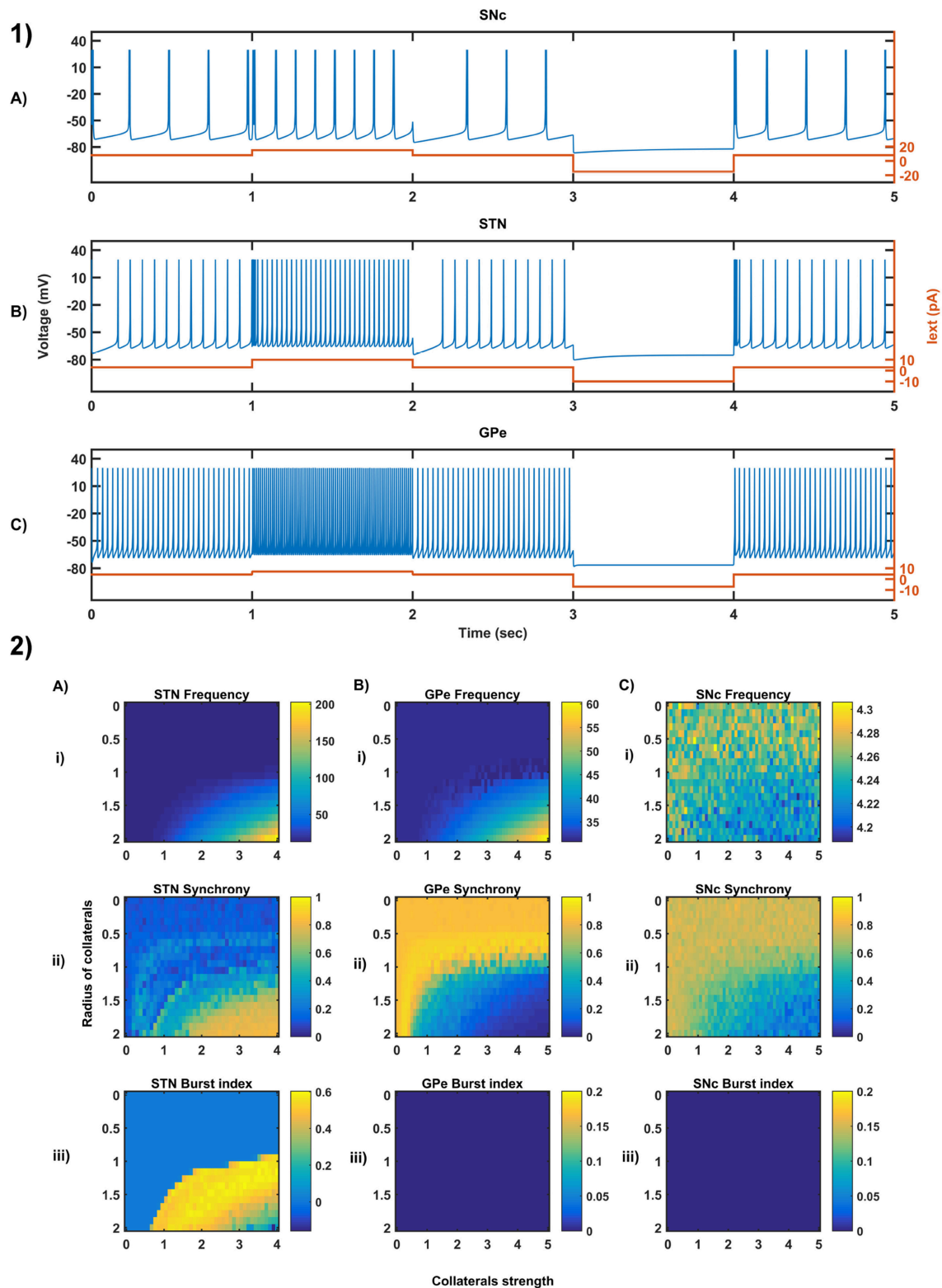
FIGURE 3 | Characteristic behavior in the single-neuron and the population of neurons of different neuronal types. **(1)** Characteristics firing patterns of SNc **(A)**, STN **(B)**, and GPe **(C)** for varying external currents (orange line - current in picoAmpere (pA)). **(2)** The response of STN **(A)**, GPe **(B)**, and SNc **(C)** populations for varying lateral connection strength (A_x) and radius (R_x) at the level of network properties [Frequency **(i)**, Synchrony **(ii)**, Burst Index **(iii)**]. I_{ext}, External current applied; STN, SubThalamic Nucleus; SNc, Substantia Nigra pars compacta; GPe, Globus Pallidus externa.
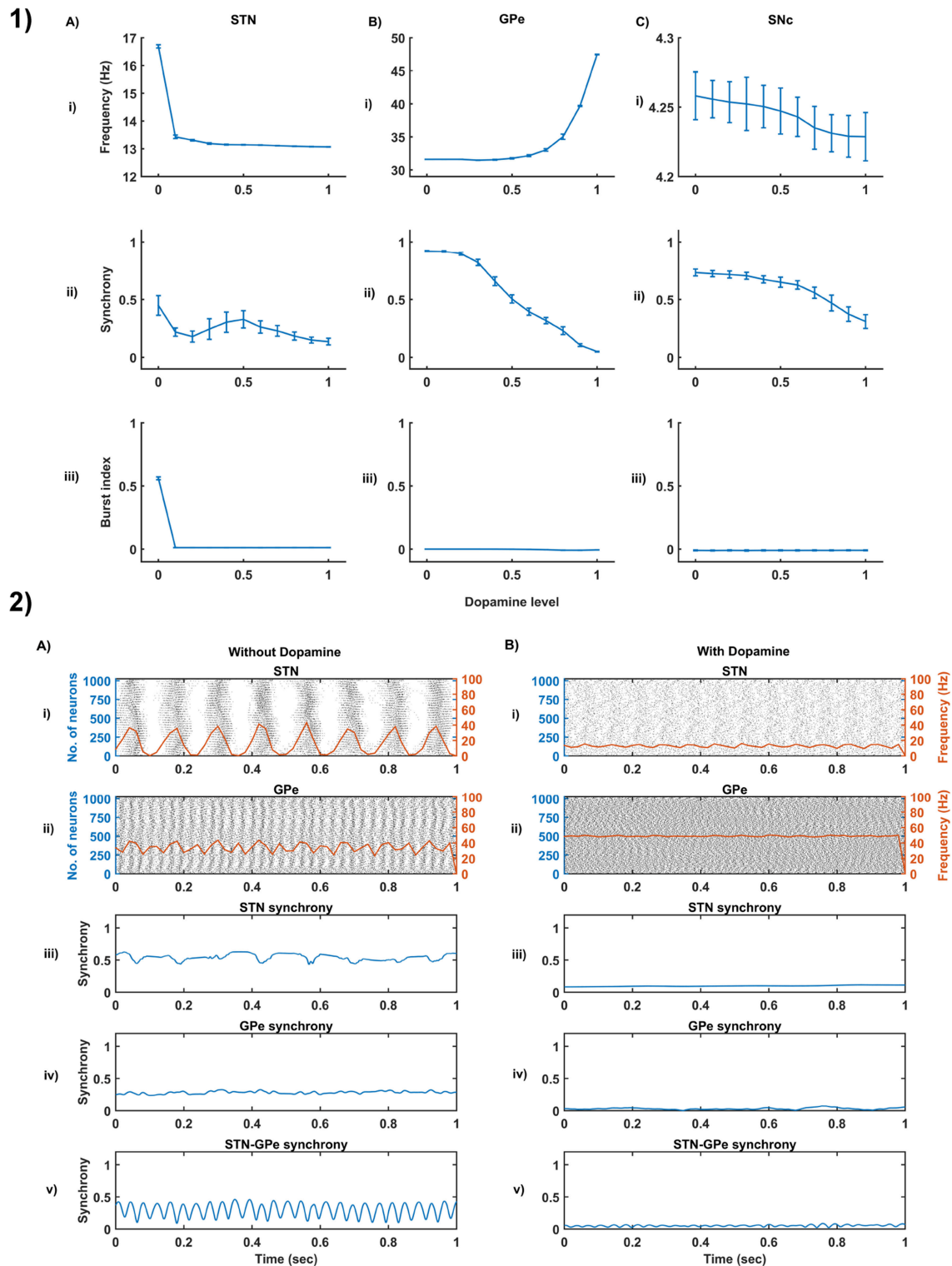
FIGURE 4 | Dopamine effect on the basal activity of different neuronal types. **(1)** The response of STN **(A)**, GPe **(B)**, and SNc **(C)** populations for varying dopamine levels at the level of network properties [Frequency **(i)**, Synchrony **(ii)**, and Burst Index **(iii)**]. **(2)** The response of STN-GPe network without **(A)** & with **(B)** dopamine - Raster plots of STN **(i)** & GPe **(ii)** populations overlaid with spike-count firing rate (orange line), Synchrony plots of STN **(iii)**, GPe **(iv)**, and combined STN-GPe **(v)**. STN, SubThalamic Nucleus; SNc, Substantia Nigra pars compacta; GPe, Globus Pallidus externa.
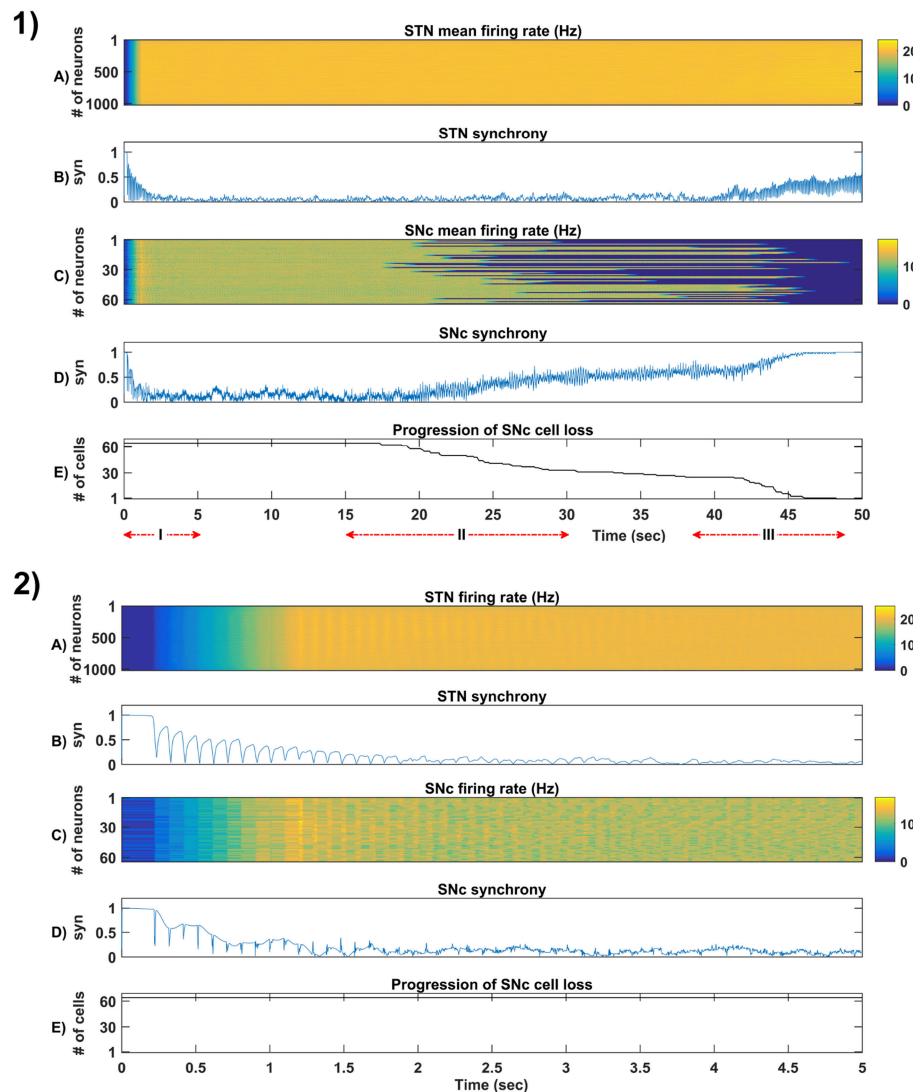
FIGURE 5 | Simulation plots of whole and step-wise mechanism (I) of the proposed excitotoxicity model. **(1)** Whole 50 sec simulation plots of the proposed excitotoxicity model. **(2)** Part-I of **(1)** Simulation plots of STN-SNc loop dynamics - Mean firing rate (1 s) of STN **(A)** & SNc **(C)**, Synchrony (syn) of STN **(B)** & SNc **(D)**, Progression of SNc cell loss **(E)**. STN, SubThalamic Nucleus; SNc, Substantia Nigra pars ompacta; GPe, Globus Pallidus externa.

neighborhood size of the lateral connections on the network functional properties such as average firing rate, network synchrony, and burst index was studied (**Figure 3-2**). The suitable values of lateral connection strength and radius for each neuronal type were chosen in correlation with experimental data (Humphries et al., 2006; Tepper and Lee, 2007). The selected values can be seen in the **Table 1**. As specified above, I_{ij}^{x}, A_x and R_x was adjusted such that the basal population activity correlated well with the experimental data (Humphries et al., 2006; Tepper and Lee, 2007).

The network dynamics of STN plays a vital role in the proposed model of excitotoxicity, in this scenario we have studied the role of lateral connections in regulating STN network properties. The basal STN population activity without lateral connections showed regular spiking without any bursting type

of behavior. Contrarily, the basal STN population activity with lateral connections showed the bursting type of activity (not shown here).

3.3. Dopamine Effect on the Basal Activity of Different Neuronal Populations

From the simulated results, it is clear that as DA level increases the mean firing rate decreases in STN, increases in GPe and decreases in SNc (**Figure 4-1**). The network synchrony decreases in all neuronal populations as DA levels increases. However, in the case of STN, the decrease is not monotonic (**Figure 4-1Aii**) where high synchrony was observed at moderate levels of DA, with synchrony falling on either side. This high synchronicity at moderate levels of DA is a result of the change in firing pattern from asynchronous bursting to synchronous spiking
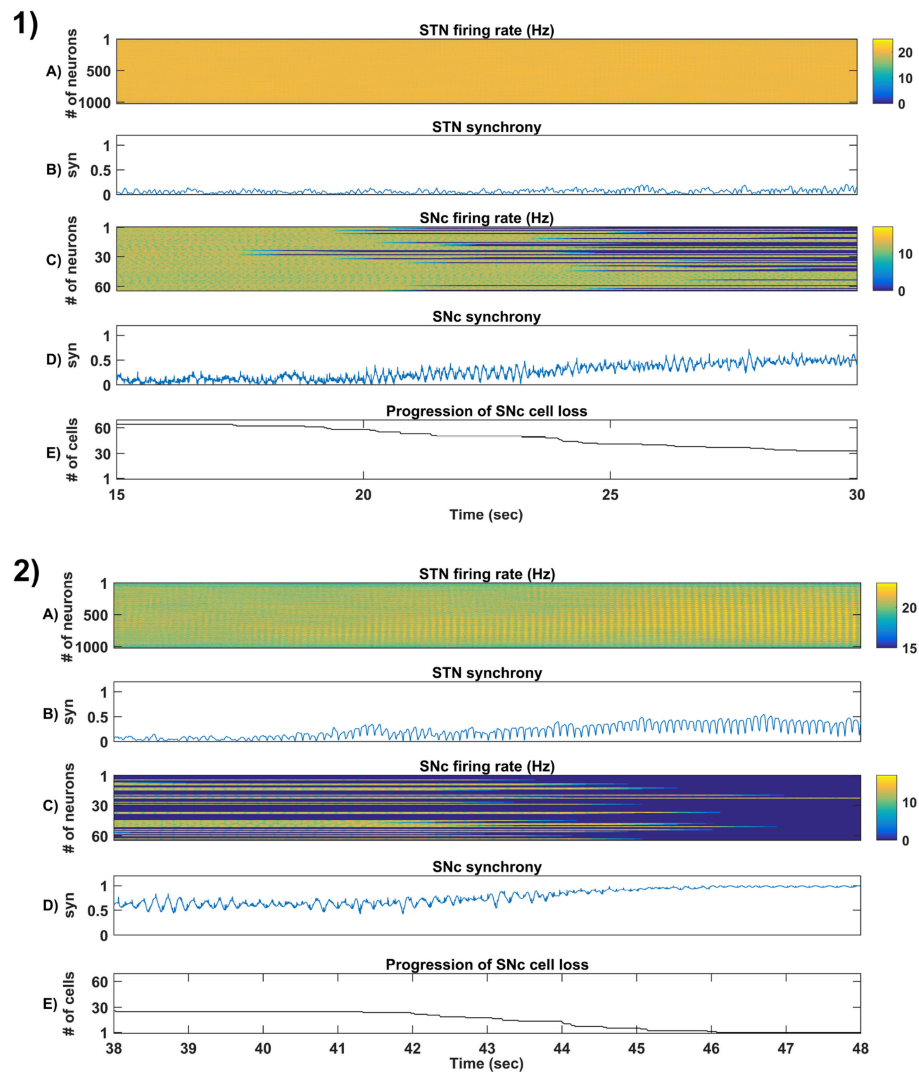
FIGURE 6 | Simulation plots of step-wise mechanisms (II, III) of the proposed excitotoxicity model. **(1)** Part-II of (**Figure 5-1**) Stress-induced neurodegeneration in SNc. **(2)** Part-III of (**Figure 5-1**) STN-mediated runaway effect of neurodegeneration in SNc - Mean firing rate (1 s) of STN **(A)** & SNc **(C)**, Synchrony (syn) of STN **(B)** & SNc **(D)**, Progression of SNc cell loss **(E)**. STN, SubThalamic Nucleus; SNc, Substantia Nigra pars compacta; GPe, Globus Pallidus externa.

which can be correlated with burst index (**Figure 4-1Aiii**) in STN population. In the dopamine-depleted condition, STN shows the bursting type of firing pattern which was exhibited by our model consistent with published studies (Vila et al., 2000; Ammari et al., 2011; Park et al., 2015). The following trend of STN activity was observed when DA level increases from 0 to 1: synchronous bursting, asynchronous bursting, synchronous spiking and asynchronous spiking. At very low DA levels (0–0.1), the STN exhibits regular bursting (**Figure 4-1Aiii**) with high synchrony (**Figure 4-1Aii**). At low DA levels (0.1–0.3), the STN exhibits an irregular mixed mode of bursting and singlet-spiking with low synchrony (**Figure 4-1Aii**). At moderate DA levels (0.3–0.7), the STN exhibits regular singlet-spiking (**Figure 4-1Aiii**) with high synchrony (**Figure 4-1Aii**). Moreover, at high DA levels (0.3–1), the STN exhibits irregular singlet-spiking with low synchrony (**Figure 4-1Aii**).

STN-GPe dynamics is known to play an important role in PD pathological oscillations that are thought to be strongly related to the cardinal symptoms of PD (Bergman et al., 1994; Brown, 2003; Litvak et al., 2011; Park et al., 2011). Numerous computational models were developed to explain the pathological oscillations in STN-GPe (Terman et al., 2002; Pavlides et al., 2015; Shouno et al., 2017). The connectivity pattern between STN and GPe was explored by using a conductance-based model (Terman et al., 2002) which exhibited different rhythmic behaviors. In our model, the connectivity pattern between STN and GPe was considered to be dopamine-dependent (Cragg et al., 2004; Mandali et al., 2015) and spontaneous activity of the STN-GPe network was studied with no external input current. Under normal DA conditions, low synchrony and minimal oscillations were exhibited by the STN-GPe network (**Figure 4-2B**) (Kang and Lowery, 2013).
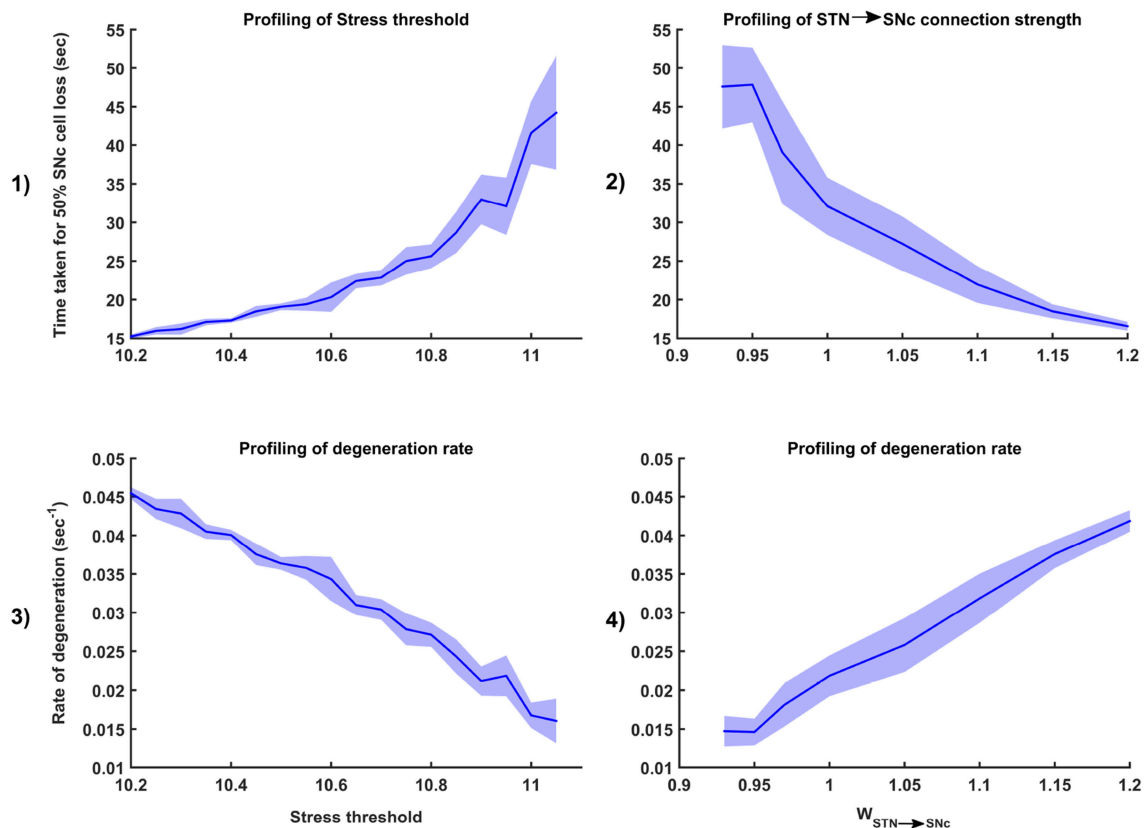
FIGURE 7 | Sensitivity of the proposed model toward parameter uncertainty. Time taken for 50% SNc cell loss for varying stress threshold (Q_{thres}) **(1)** and connection strength from STN→SNc ($W_{STN→SNc}$) **(2)**. Rate of degeneration (λ) for varying stress threshold (Q_{thres}) **(3)** and connection strength from STN→SNc ($W_{STN→SNc}$) **(4)**. STN, SubThalamic Nucleus; SNc, Substantia Nigra pars compacta.

It was reported that dopamine-depleted condition results in pathological oscillations in STN characterized by high synchrony and beta range oscillations (Brown et al., 2001; Weinberger et al., 2006; Park et al., 2010, 2011; Lintas et al., 2012; Kang and Lowery, 2013; Pavlides et al., 2015). In our model during dopamine-depleted conditions, high synchrony and the higher rate of oscillations were exhibited in the STN-GPe network, and beta range oscillations were also observed in STN population (**Figure 4-2A**).

3.4. STN-Induced Excitotoxicity in SNc

The proposed excitotoxicity model was able to exhibit STN-mediated excitotoxicity in SNc which was precipitated by energy deficiency (Albin and Greenamyre, 1992; Beal et al., 1993; Greene and Greenamyre, 1996; Rodriguez et al., 1998; Blandini, 2001, 2010; Ambrosi et al., 2014) (**Figures 5, 6**). For a more detailed explanation of the excitotoxicity results obtained, we have sub-divided 50 s simulation into three parts - (I) STN-SNc loop dynamics (normal condition), (II) Stress-induced neurodegeneration in SNc (pre-symptomatic PD condition), and (III) STN-mediated runaway effect of neurodegeneration in SNc (symptomatic PD condition).

3.4.1. (I) STN-SNc Loop Dynamics

In the first part of the simulation, connectivity between STN and SNc were introduced at $t = 0$, and the model exhibited decreased synchrony in STN and SNc over time (**Figure 5-2B**). The results showed the pivotal role of dopamine in modulating STN activity (Cragg et al., 2004; Lintas et al., 2012; Yang et al., 2016). The excitatory drive from STN to SNc results in decreased synchrony in SNc due to increased inhibitory drive from lateral connections (**Figure 5-2D**). During this whole process, the stress threshold ($Q_{thres} = 11.3$) was fixed, and there was no SNc cell loss due to stress (**Figure 5-2E**).

3.4.2. (II) Stress-Induced Neurodegeneration in SNc

In the second part of the simulation, stress threshold was slightly reduced from $Q_{thres} = 11.3$ to $Q_{thres} = 10.8$ at $t = 10s$ to replicate PD-like condition in the model where stress-induced neurodegeneration gets initiated. The model exhibited stress-induced neurodegeneration in SNc where SNc cells start dying when stress variable (Q_{ij}^x) exceeds the stress threshold (Q_{thres}) which acts like an apoptotic threshold (**Figure 6-1E**). It was observed that there was no increased synchrony in the STN population as a result of SNc cell loss (**Figure 6-1B**). However, there was increased synchrony
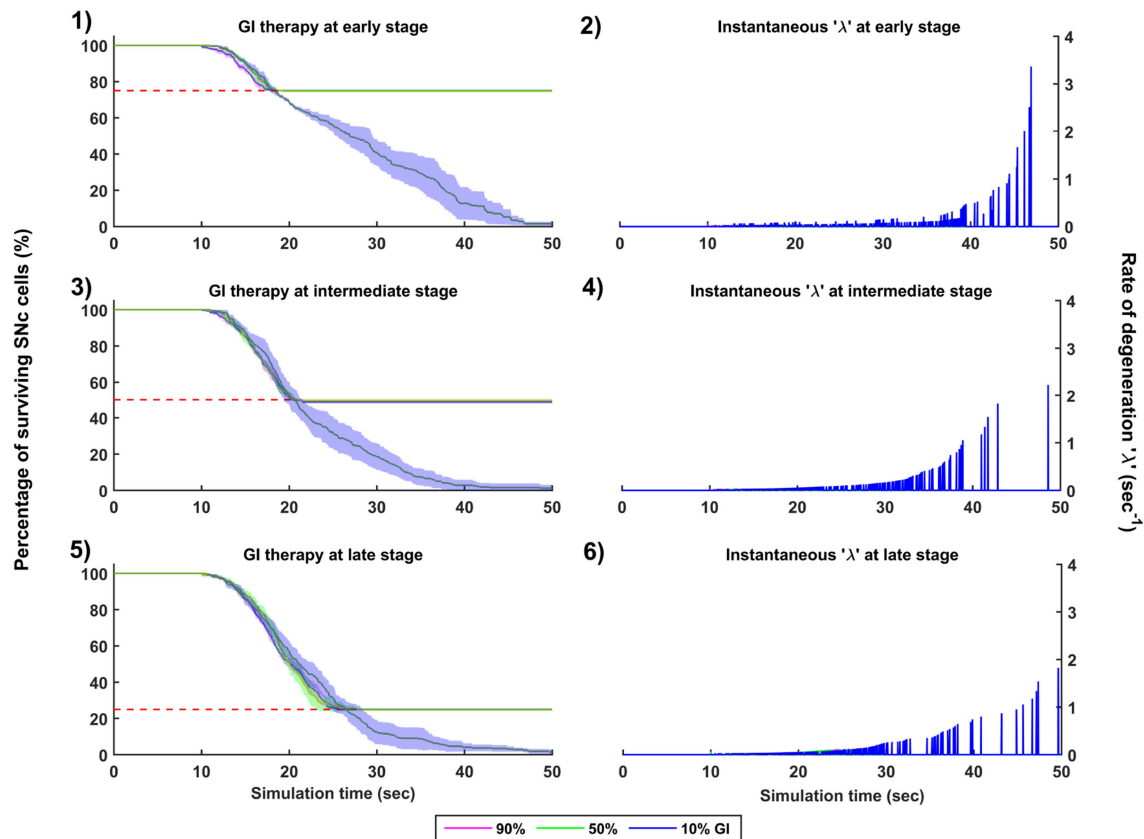
FIGURE 8 | Simulation plots for Glutamate Inhibition (GI) therapy. Progression of SNc cell loss for 90, 50, and 10% GI at early (25%) **(1)**, intermediate (50%) **(3)** and late (75%) **(5)** stages of SNc cell loss. Instantaneous rate of degeneration (λ) for 90, 50, and 10% GI at early (25%) **(2)**, intermediate (50%) **(4)** and late (75%) **(6)** stages of SNc cell loss. SNc, Substantia Nigra pars compacta.

in the SNc population (**Figure 6-1D**) which might be due to reduced inhibitory drive from lateral connections as a result of SNc cell loss.

3.4.3. (III) STN-Mediated Runaway Effect of Neurodegeneration in SNc

In the third part of the simulation, no parameters were changed, but after $t = 40s$, there was a rise in STN synchrony as a result of stress-induced SNc cell loss (**Figure 6-2**). A substantial amount of SNc cell loss (more than 50%) resulted in increased synchrony (**Figure 6-2B**) and firing rates (**Figure 6-2A**) of the STN population. As the STN synchrony increased, runaway effect kicks in where increased STN excitatory drive to SNc cells result in hastening the stress-induced neurodegeneration of remaining SNc cells (**Figure 6-2E**).

3.5. Sensitivity of Excitotoxicity Model Toward Parameter Uncertainty

To check the sensitivity of excitotoxicity model for different parametric values, we have considered two factors which can maximally influence the output results. Firstly, stress threshold (Q_{thres}) which is analogous to the apoptotic

threshold and is assumed to be dependent on the amount of available energy to the cell (Albin and Greenamyre, 1992; Greene and Greenamyre, 1996). Secondly, the synaptic weight between STN and SNc ($W_{STN \rightarrow SNc}$) which is analogous to synaptic modification and is assumed to be modulated by the excitatory drive from STN to SNc (Hasselmo, 1994, 1997).

3.5.1. Stress Threshold (Q_{thres})

Simulation results showed that the time taken for 50% SNc cell loss ($t_{1/2}$) increases as the stress threshold increases (**Figure 7-1**). The rate of degeneration or degeneration constant (λ) is the ratio of the number of SNc cells that degenerate in a given period of time compared with the total number of SNc cells present at the beginning of that period. The rate of degeneration (λ) decreases as the stress threshold increases (**Figure 7-3**). These results show the importance of stress threshold in regulating excitotoxic damage to SNc and also support the idea of "weak excitotoxicity hypothesis" where SNc cells showed increased susceptibility to glutamate due to impaired cellular energy metabolism (Albin and Greenamyre, 1992; Greene and Greenamyre, 1996).
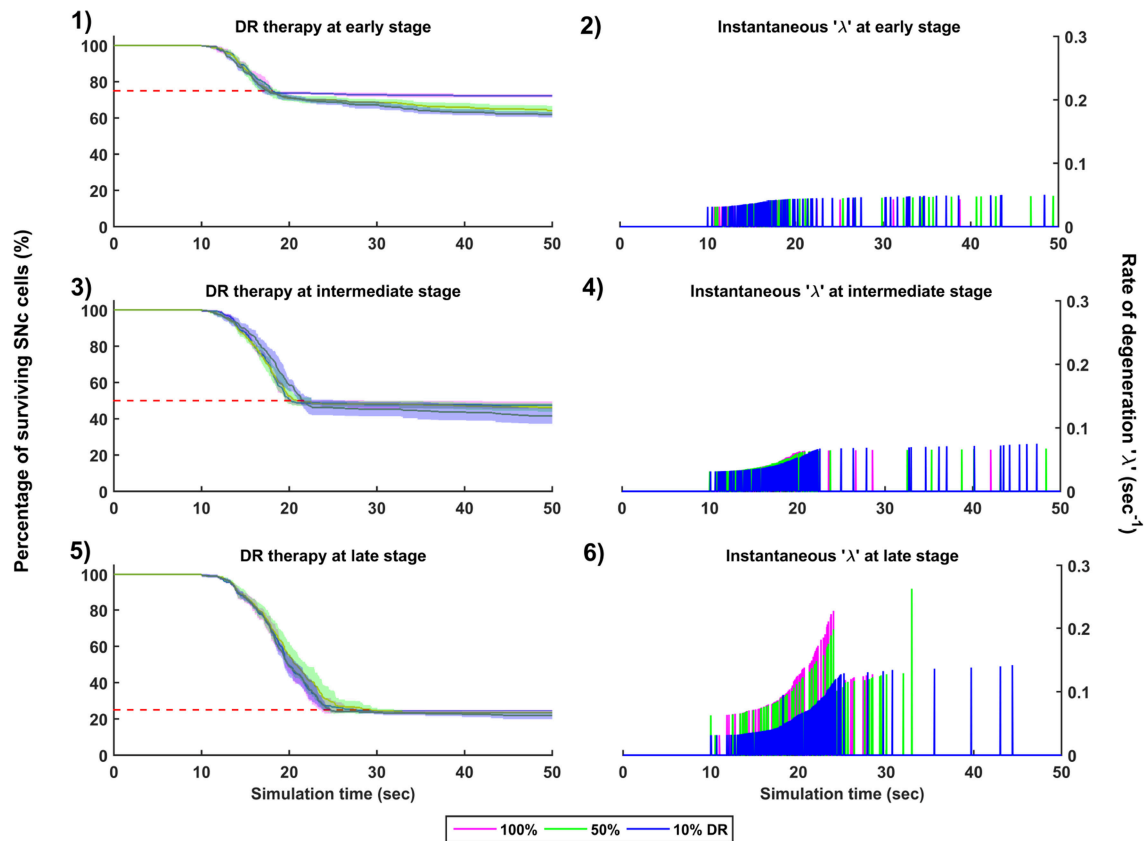
FIGURE 9 | Simulation plots for Dopamine Restoration (DR) therapy. Progression of SNc cell loss for 100, 50, and 10% DR at early (25%) **(1)**, intermediate (50%) **(3)** and late (75%) **(5)** stages of SNc cell loss. Instantaneous rate of degeneration (λ) for 100, 50, and 10% DR at early (25%) **(2)**, intermediate (50%) **(4)** and late (75%) **(6)** stages of SNc cell loss. SNc, Substantia Nigra pars compacta.

3.5.2. STN-SNc Synaptic Weight ($W_{STN \to SNc}$)

Simulation results showed that time taken for 50% SNc cell loss ($t_{1/2}$) decreases as the STN-SNc synaptic weight increases (**Figure 7-2**). The rate of degeneration (λ) increases as the STN-SNc synaptic weight increases (**Figure 7-4**). These results show the extent of STN influence in the causation of excitotoxicity in SNc. They also support the notion that STN-mediated excitotoxicity might play a major role in SNc cell loss in PD condition (Rodriguez et al., 1998; Blandini, 2001, 2010; Ambrosi et al., 2014).

3.6. Strategies for Neuroprotection of SNc

We now extend the proposed excitotoxic model to study the effect of various therapeutic interventions on the progression of SNc cell loss. The following three types of interventions which were simulated: (1) drugs, (2) surgical interventions, and (3) Deep Brain Stimulation (DBS).

3.6.1. Glutamate Inhibition Therapy

The effect of glutamate agonists and antagonists on the progression of SNc cell loss was implemented in the manner specified in the methods section. The onset of glutamate therapy at different stages of SNc cell loss showed that cell loss was

delayed or halted (**Figure 8**). For the glutamate therapy which is initiated at 25, 50, and 75% SNc cell loss, the progression of SNc cell loss was halted when the percentage of glutamate inhibition administrated was above 50%. As the glutamate dosage increases the progression of SNc cell loss delays and after a particular dosage of glutamate inhibitors the SNc cell loss halts. There was no change in the course of SNc cell loss for low levels of glutamate inhibition (**Figures 8-1, 8-3, 8-5**). The peak of the instantaneous rate of degeneration decreases as the therapeutic intervention is delayed in the case of 10% glutamate inhibition (**Figures 8-2, 8-4, 8-6**).

3.6.2. Dopamine Restoration Therapy

The effect of dopamine agonists on the progression of SNc cell loss was also implemented in the manner specified in the methods section. The onset of dopamine agonist therapy at different stages of SNc cell loss showed that the progression of cell loss was only delayed (**Figure 9**). For the dopamine agonists therapy which is initiated at 25, 50, and 75% SNc cell loss, the progression of SNc cell loss was delayed when the percentage of dopamine restoration was a mere 10%. The neuroprotective effect of dopamine agonist therapy is dependent on the level of restoration of dopamine tone on the STN.
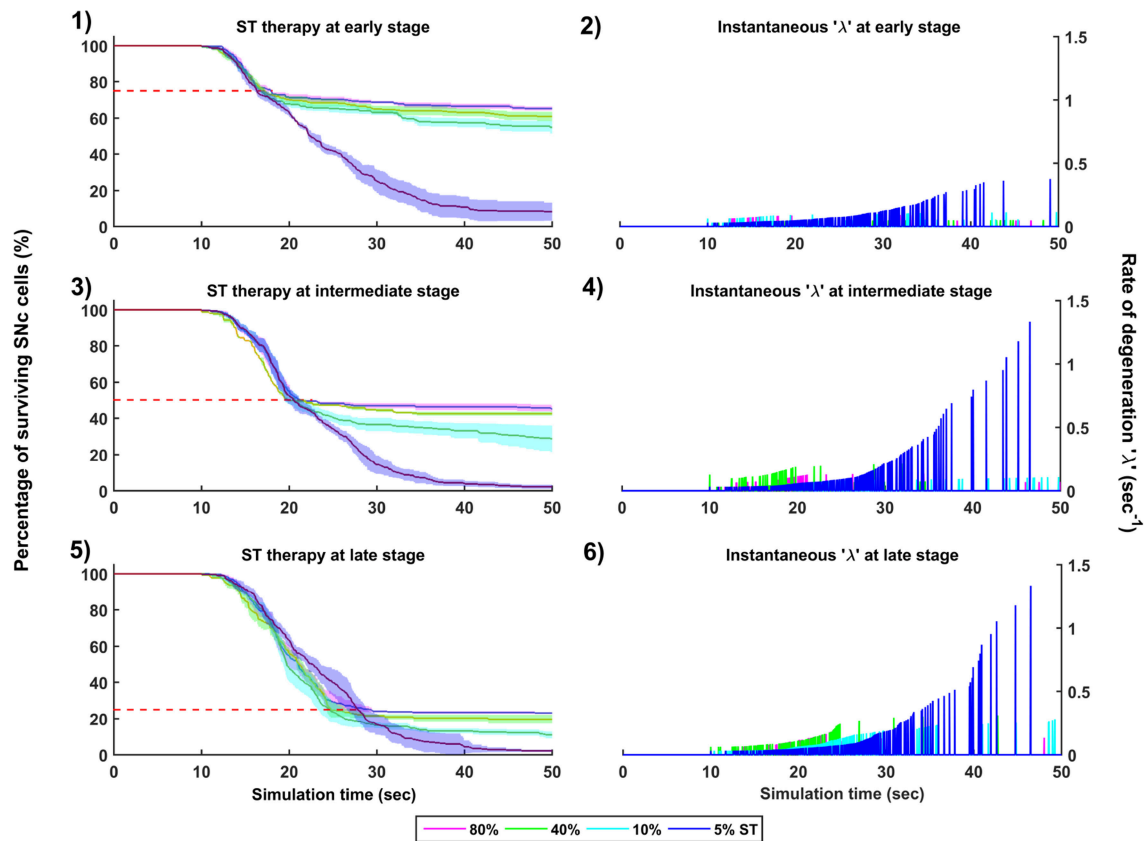
FIGURE 10 | Simulation plots for SuthalamoTomy (ST) therapy. Progression of SNc cell loss for 80, 40, 10, and 5% ST at early (25%) **(1)**, intermediate (50%) **(3)** and late (75%) **(5)** stages of SNc cell loss. Instantaneous rate of degeneration (λ) for 80, 40, 10, and 5% GI at early (25%) **(2)**, intermediate (50%) **(4)** and late (75%) **(6)** stages of SNc cell loss. SNc, Substantia Nigra pars compacta.

In other words, as the dopamine content in STN increases, the progression of SNc cell loss delays. Unlike glutamate inhibition, the progression of SNc cell loss was not halted even at 100% dopamine restored in all the case of intervention (**Figures 9-1**, **9-3**, **9-5**). The dopamine restoration therapy did not have much effect on the instantaneous rate of degeneration (**Figures 9-2**, **9-4**, **9-6**).

3.6.3. Subthalamotomy

The effect of subthalamotomy on the progression of SNc cell loss was implemented in a way described in the methods section. The onset of STN ablation therapy at different stages of SNc cell loss showed that progression of cell loss was delayed or halted (**Figure 10**). The neuroprotective effect of subthalamotomy is dependent on the proportion of lesioning of STN population. In other words, as the proportion of STN lesioning increases the progression of SNc cell loss delays and halts only when almost all of the STN population is lesioned (**Figures 10-1**, **10-3**, **10-5**). The progression of SNc cell loss is halted only at 100% STN lesioning in all cases of intervention (not shown here). However, as the proportion of STN lesioning decreases, the rate of degeneration increases. Similarly to dopamine restoration therapy, subthalamotomy also did not

have much effect on the instantaneous rate of degeneration (**Figures 10-2**, **10-4**, **10-6**).

3.6.4. Deep Brain Stimulation of STN

The effect of deep brain stimulation on the progression of SNc cell loss was implemented in the way described in the methods section. Along with the stimulation of STN, the inhibitory drive to STN through the afferent connections as result of antidromic activation of the GPe population and the synaptic depression in STN as result of increased axonal and synaptic failures in STN were incorporated in the model.

As specified earlier, different stimulation configurations and stimulus waveforms were implemented while exploring the optimal DBS parameters for therapeutic benefits. The STN population response for different types of DBS protocol was simulated. To study the neuroprotective effect, stimulation parameters which reduce the STN overactivity (Meissner et al., 2005) during dopamine depletion condition were chosen (**Table 2**). The biphasic stimulus pulse shows more therapeutic benefits than monophasic stimulus pulse; biphasic current alleviates the STN pathological activity without increasing the firing rate of STN population as a whole. The four-contact point type of stimulation configuration required lesser stimulus

FIGURE 11 | Simulation plots for Monophasic DBS therapy. Profiling of monophasic stimulus waveform for different stimulation configuration in order to achieve the maximal neuroprotective effect of DBS. Confusion matrices for SCP **(1)**, FCP **(2)**, and MCP **(3)** configurations showing survival ratios of SNc cells for different percentage activation of antidromic activation and STN axonal & synaptic failures at early (25%) **(A)**, intermediate (50%) **(B)** and late (75%) **(C)** stages of SNc cell loss. Ratios around 0 is indicated as forthwith (indigo), ratios around 0.5 is indicated as delayed (light green), and ratios around 1 is indicated as halted (yellow). DBS, Deep Brain Stimulation; SNc, Substantia Nigra pars compacta; STN, SubThalamic Nucleus; SCP, Single Contact Point; FCP, Four Contact Point; MCP, Multiple Contact Point.

amplitude for producing the same effect when compared with the other two configurations. From these studies, we can say that four-contact point configuration with biphasic stimulus pulse gives maximum therapeutic benefits from the neuroprotective point of view.

To understand the neuroprotective therapeutic mechanism of DBS in PD (Benazzouz et al., 2000; Maesawa et al., 2004; Wallace et al., 2007; Spieles-Engemann et al., 2010; Musacchio et al., 2017), we have investigated some of the prominent hypotheses regarding the therapeutic effect of DBS viz., (1) excitation hypothesis, (2) inhibition hypothesis and most recent one (3) disruptive hypothesis (McIntyre et al., 2004; Chiken and Nambu, 2015).

The excitation hypothesis was implemented by direct stimulation of the STN population in the proposed excitotoxicity model. The simulation results show that DBS to STN diminishes the pathological synchronized activity but in turn increases the firing rate of the STN population which was not apt for neuroprotection. Next, we have implemented the inhibition hypothesis where antidromic activation of GPe neurons during STN-DBS is highlighted, thereby increasing the inhibitory drive to STN (Mandali and Chakravarthy, 2016). In this scenario also, the inhibitory drive from GPe was not sufficient

to produce comprehensive neuroprotection (**Figures 11**, **12**). On average FCP stimulus configuration produced better neuroprotective effect compared to other two configurations in both monophasic and biphasic current (**Figures 11-2**, **12-2**). Moreover, MCP stimulus configuration results in worsening the disease progression by hastening the SNc cell loss in monophasic stimulus (**Figure 11-3**), but in biphasic stimulus, neuroprotection increased with higher levels of antidromic activation in all stages of therapeutic intervention (**Figure 12-3**).

Finally, the disruptive hypothesis was implemented by increasing the proportion of axonal and synaptic failures in STN population (Rosenbaum et al., 2014). From simulation results, it was observed that the progression of SNc cell loss was delayed or halted as the percentage of STN axonal and synaptic failures increased (**Figures 11**, **12**). On average FCP stimulus configuration produced better neuroprotective effect compared to other two configurations in both monophasic and biphasic currents (**Figures 11-2**, **12-2**). For the higher percentage of STN axonal and synaptic failures also, the neuroprotective effect was not pronounced in monophasic MCP DBS setting (**Figure 11-3**), but in biphasic MCP DBS setting neuroprotection increased with the higher percentage of STN axonal and synaptic failures (**Figure 12-3**).
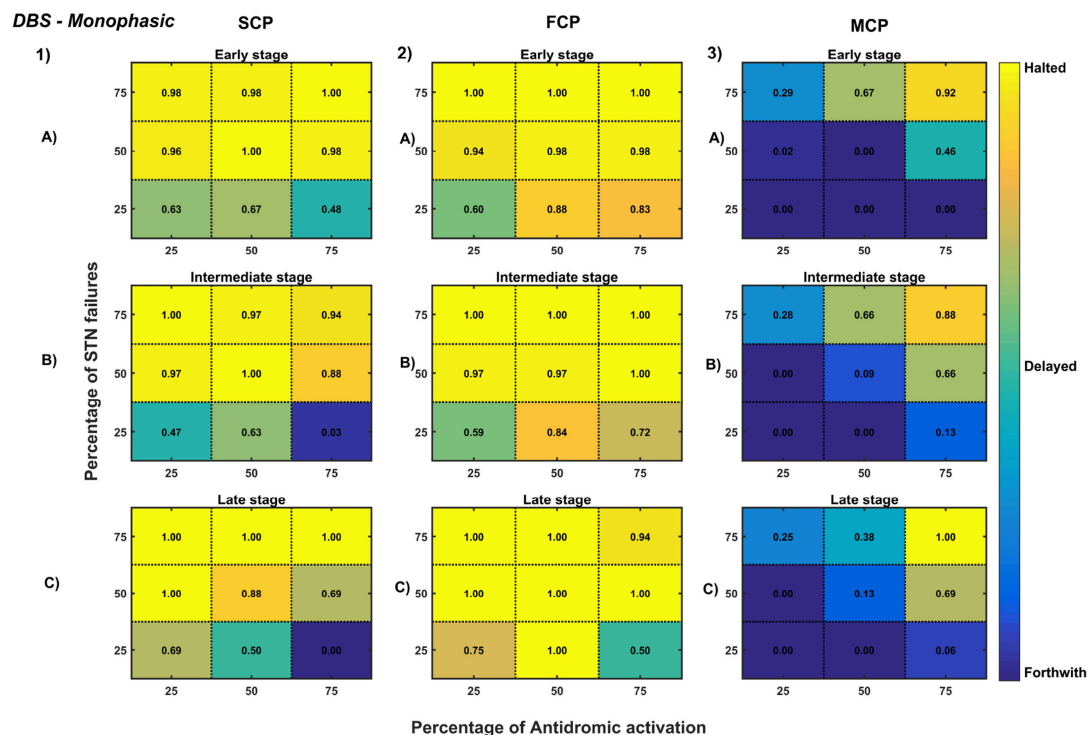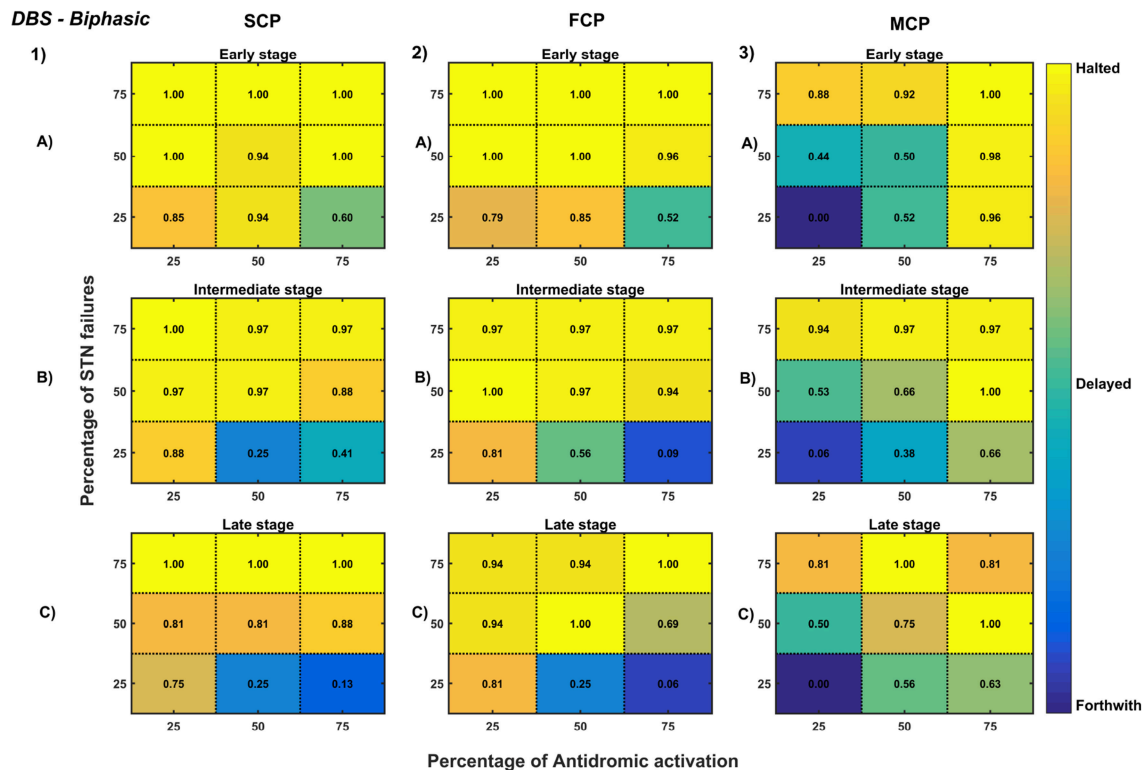
FIGURE 12 | Simulation plots for Biphasic DBS therapy. Profiling of biphasic stimulus waveform for different stimulation configuration in order to achieve the maximal neuroprotective effect of DBS. Confusion matrices for SCP **(1)**, FCP **(2)**, and MCP **(3)** configurations showing survival ratios of SNc cells for different percentage activation of antidromic activation and STN axonal & synaptic failures at early (25%) **(A)**, intermediate (50%) **(B)** and late (75%) **(C)** stages of SNc cell loss. Ratios around 0 is indicated as forthwith (indigo), ratios around 0.5 is indicated as delayed (light green), and ratios around 1 is indicated as halted (yellow). DBS, Deep Brain Stimulation; SNc, Substantia Nigra pars compacta; STN, SubThalamic Nucleus; SCP, Single Contact Point; FCP, Four Contact Point; MCP, Multiple Contact Point.

4. DISCUSSION

4.1. Excitotoxicity Model

The goal of this work was to develop a model which investigates the role of excitotoxicity in SNc cell loss, where excitotoxicity was caused by STN and precipitated by energy deficiency. The study suggests that excitotoxicity in SNc is initially driven by an energy deficit which leads to an initial dopamine reduction as a result of SNc cell loss. This initial dopamine reduction causes disinhibition of STN which in turns leads to excitotoxic damage due to excessive release of glutamate to its target nuclei including SNc (Rodriguez et al., 1998). The excitotoxicity which was driven by energy deficit, termed as "weak excitotoxicity," results in increased vulnerability of SNc neurons to even physiological concentration of glutamate. The excitotoxicity which was driven by overactive excitatory STN neurons termed as "strong excitotoxicity" results in overactivation of glutamatergic receptors on SNc neurons (Albin and Greenamyre, 1992). In summary, it appears that the excitotoxic cause of SNc cell loss in PD might be initiated by weak excitotoxicity mediated by energy deficit, and followed by strong excitotoxicity, mediated by disinhibited STN.

The results from the proposed model reinforce the role of STN in regulating SNc cell loss (Hamani et al., 2004, 2017). The model results show that although cell loss was observed, there was no increased synchrony in the STN population which is a pathological marker of the PD condition (Lintas et al., 2012). Thus, the SNc cell loss and STN synchrony have a threshold-like relation where there is an increased STN synchrony only after substantial SNc cell loss. The initial SNc cell loss leads to further activation of STN by disinhibition, which in turn further activates SNc compensating for the dopamine loss, acting as a pre-symptomatic compensatory mechanism (Bezard et al., 2003). It was reported that the onset of PD symptoms occurs only after there is more than 50% SNc cell loss (Bezard et al., 2001). This was observed in our simulation results also where only at around 50–70% SNc cell loss there is in an increased STN synchrony. As a result of substantial SNc cell loss, decreased dopamine causes disinhibition of STN which in turn overactivates STN, eventually producing a runaway effect that causes an unstoppable SNc cell loss due to excitotoxic damage (Rodriguez et al., 1998). The threshold-like behavior of SNc cell loss and STN synchrony might also be facilitated by the inhibitory drive from GPe to STN: the proliferation of GPe-STN synapses (Fan et al., 2012) also acts as a presymptomatic compensatory mechanism. It was also reported that lesioning of GPe caused progressive SNc cell loss by increasing STN activity (Wright et al., 2002) and lesioning of STN proved to be neuroprotective (Wright and Arbuthnott, 2007).

To summarize, up to a point of stress threshold, SNc cells can survive indefinitely; but if, for any reason, there is loss of cells in SNc, and the SNc cell count falls below a threshold, from that point onwards, the aforementioned runaway effect kicks in leading to a progressive and irrevocable cell loss. Such cell loss is strongly reminiscent of cell loss due to neurodegeneration.

4.2. Neuroprotective Strategies

A variety of conventional therapies are simulated in the model to test their efficacy in slowing down or arresting SNc cell loss. Among the current therapeutics, glutamate inhibition, dopamine restoration, subthalamotomy and deep brain stimulation showed superior neuroprotective effects in the proposed model. Glutamate inhibition and subthalamotomy were successful in delaying or arresting the SNc cell loss by inhibiting the excitatory drive from STN to SNc (Lee et al., 2003; Wallace et al., 2007; Austin et al., 2010), and in case of dopamine restoration it is by replenishing the dopamine tone to the STN which in turn restores inhibition on itself (Olanow et al., 1998; Vaarmann et al., 2013), thereby diminishing STN-mediated excitotoxicity in SNc. The neuroprotective effect of glutamate inhibition, dopamine restoration and subthalamotomy was dependent on the dosage of glutamate inhibitors, the extent of dopamine restored and proportion of STN lesioned, respectively. As the disease progresses, the effect of glutamate inhibition on the rate of degeneration increased but in the case of dopamine restoration and subthalamotomy, it was decreased. In the late stages of disease progression, our computational study indicates that the neuroprotective effect of glutamate inhibition is more prominent than dopamine restoration and subthalamotomy.

From our study, it can be said that subthalamotomy mostly delays the SNc cell loss but very rarely halts it. This phenomenon was not much evident in the late stages of disease progression in the model which is consistent with the standard clinical understanding that the neuroprotective effect of subthalamotomy in advanced PD is not quite successful (Guridi and Obeso, 2015). Early treatment with subthalamotomy in PD can have a neuroprotective effect (Guridi et al., 2016) a trend that was reflected in our computational study. Another factor underlying the neuroprotective effect of subthalamotomy during the early stage

of PD is the involvement of presymptomatic compensation mechanisms (Bezard et al., 2003). One of the compensatory mechanisms is the increased activity of STN before any significant striatal dopamine loss which leads to excess excitatory drive from STN to the remaining SNc cells to restore the dopamine loss due to initial cell loss (Bezard et al., 1999). This excess excitatory drive from STN eventually leads to excitotoxicity in SNc neurons. To overcome this excitotoxicity, subthalamotomy had to be applied very early after diagnosis of PD to have any neuroprotective effect (Guridi et al., 2016).

In our modeling study, we have explored various aspects of DBS protocol from stimulus waveforms to stimulus configurations and other DBS parameters. From the simulation results, it can be suggested that biphasic stimulus waveform with four-contact point stimulation configuration showed maximal neuroprotective effect since biphasic stimulus guarantees charge-balance in the stimulated neuronal tissue (Hofmann et al., 2011) and DBS parameters were given in the **Table 2**.

It has been reported that long-term stimulation (DBS) of STN results in the slowdown of the SNc cell loss in animal models (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Spieles-Engemann et al., 2010; Musacchio et al., 2017), but the mechanism behind the neuroprotective benefits of DBS is not elucidated. To understand the neuroprotective effect of DBS in PD, we have investigated three prominent hypotheses viz., excitation, inhibition and disruptive actions of DBS (Chiken and Nambu, 2015). In the excitation hypothesis, only DBS was applied which results in increased firing rate in STN and leads to more excitatory drive to SNc which eventually kills the SNc cells due to stress. Therefore, considering only the excitation hypothesis cannot explain the neuroprotective effect of DBS. Next, inhibition hypothesis was implemented where antidromic activation of GPe result in the increased inhibitory drive to STN (Mandali and Chakravarthy, 2016). In this scenario also, the neuroprotective effect of DBS could not be comprehensively explained. Finally, the disruptive hypothesis was implemented by increasing the axonal and synaptic failures in STN population during DBS therapy (Rosenbaum et al., 2014). From simulation results, it was observed that the progression of SNc cell loss kept on delaying as the percentage of STN axonal and synaptic failure increased. Therefore, it can be inferred that DBS blocks the propagation of

TABLE 2 | DBS parameter values obtained from the preliminary studies.

Parameter(s)	SCP	FCP	MCP
DBS frequency (f_{DBS}) in Hz	130	130	130
Monophasic pulse width (δ_{DBS}) in ms	100	100	100
Biphasic pulse width (δ_{DBS}) in ms	200	200	200
Monophasic DBS amplitude (A_{DBS}) in pA	650	650	650
Biphasic DBS amplitude (A_{DBS}) in pA	1,000	1,000	1,000
Spread of the current (σ_{DBS})	5	2	0
Electrode contact point(s)	(16, 16)	(8, 8) (8, 24) (24, 8) (24, 24)	Many

Hz, Hertz; ms, milliseconds; pA, picoamperes.

pathological oscillations occurring in STN to other nuclei; in other words DBS disrupts the information transfer through the stimulation site, producing neuroprotection effect in SNc (Ledonne et al., 2012).

4.3. Limitations and Future Directions

Though the model captures the exciting results of excitotoxicity, it is not without limitations. The timescales which are represented in the results of the proposed model are not realistic, as the neurodegeneration which occurs over the years in PD was exhibited in a few tens of seconds in the model. This limitation is inevitable due to the practical challenges faced by computer simulations since it is impractical to simulate the model for months and years. The difficulty arises due to the fact that the simulation must span widely separated time scales - sub-millisecond time scales to describe spiking activity and years to describe neurodegenerative processes.

The major inputs to the SNc neurons come from the striatum which was not included in the model. As our objective was to investigate the extent of STN-mediated excitotoxicity in SNc, we avoided any other structures which can influence this phenomenon at present.

In the proposed model, the variability of stress threshold, which is analogous to an apoptotic threshold (that can be broadly associated with the available energy represented as (ATP/ADP) ratio), is sensitive enough to alter the model results is a constant parameter. In order to achieve variability in this parameter, astrocytic and vascular network-level models can be introduced. With the astrocyte layer introduced, the effect of astrocytes on the therapeutic effect of DBS can be explored (Fenoy et al., 2014).

In the future, we plan to simulate the SNc activity using a detailed biophysical model to study the dynamics at the molecular level and also to investigate the cellular pathways related to PD pathology. We would like to include Spike-timing-dependent plasticity (STDP) learning in STN population for the long-term effect of DBS (Ebert et al., 2014).

Our hypothesis behind this whole study is to understand the pathogenesis of PD as cellular energy deficiency in SNc as a cause. As Wellstead and Cloutier pointed out (Wellstead and Cloutier, 2011), PD should be understood by placing the failure of brain energy delivery mechanisms in the center as a core pathological process, with other manifestations of pathology as deriving from that core process (see the Figure 12 in Wellstead, 2010).

CODE ACCESSIBILITY

The code of the proposed excitotoxicity model is available on ModelDB server (McDougal et al., 2017), and access code will be provided on request. (https://senselab.med.yale.edu/modeldb/enterCode.cshtml?model=244384).

AUTHOR CONTRIBUTIONS

VM and VC: conceived, developed the model and prepared the manuscript. AM: conceived and developed the model. SR: prepared the manuscript.

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Cellular, Synaptic and Network Effects of Acetylcholine in the Neocortex

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The neocortex is densely innervated by basal forebrain (BF) cholinergic neurons. Long-range axons of cholinergic neurons regulate higher-order cognitive function and dysfunction in the neocortex by releasing acetylcholine (ACh). ACh release dynamically reconfigures neocortical microcircuitry through differential spatiotemporal actions on cell-types and their synaptic connections. At the cellular level, ACh release controls neuronal excitability and firing rate, by hyperpolarizing or depolarizing target neurons. At the synaptic level, ACh impacts transmission dynamics not only by altering the presynaptic probability of release, but also the magnitude of the postsynaptic response. Despite the crucial role of ACh release in physiology and pathophysiology, a comprehensive understanding of the way it regulates the activity of diverse neocortical cell-types and synaptic connections has remained elusive. This review aims to summarize the state-of-the-art anatomical and physiological data to develop a functional map of the cellular, synaptic and microcircuit effects of ACh in the neocortex of rodents and non-human primates, and to serve as a quantitative reference for those intending to build data-driven computational models on the role of ACh in governing brain states.

Keywords: neuromodulation, acetylcholine, Ach receptors, neocortex, cellular excitability, synaptic transmission, network activity

INTRODUCTION

The cholinergic system is one of the most well-studied neuromodulatory systems, and perhaps phylogenetically the oldest. Acetylcholine (ACh) is found in both vertebrates and invertebrates and together with adrenaline and noradrenaline (NA), it acts as one of the main effectors of the autonomic nervous system. In the central nervous system (CNS), ACh impacts cellular and synaptic physiology and may switch network dynamics resulting in behavioral transitions such as from sleep to wakefulness, distraction to attention, and learning and recall (Hasselmo and Sarter, 2011; Lee and Dan, 2012).

Cholinergic effects have been studied for more than a century. In 1869, Schmiedeberg and Koppe (1869) demonstrated how extracts of a common mushroom, *Amanita muscaria*, could slow, and at a higher concentration arrest the beat of the frog heart. They purified the extract and named it muscarine. This substance, when applied to the brain and spinal cord was able to produce flaccidity and weaken the peripheral reflexes. However, the pharmacology of the nitrite ester of choline was different in that it had considerable nicotinic activity (nicotine is the major alkaloid of tobacco, first isolated by Posselt and Reiman from *Nicotiniana tabacum* leaves in 1828; Koukouli et al., 2017).

In 1921 experimental proof was obtained for ACh's role as a chemical transmitter at the cardiac vagal endings. The active substance was initially named "vagusstoff" by Otto Loewi in 1921 (Loewi, 1924). Sir Henry Dale further described that muscarinic responses were antagonized by atropine, whereas the nicotine actions were antagonized by curare (Dale, 1914).

It has long been known that ACh is also present at the level of the CNS, however, it was not until 1953 that evidence of the release of ACh in the brain was provided (Eccles et al., 1953). Prior to this discovery, it was known that anti-cholinergic drugs could influence learning and memory—pharmacological activation of muscarinic ACh receptors (mAChRs) was known to produce delirium symptoms, while receptor blockade generates severe anterograde amnesia. Moreover, the dementia of Alzheimer's and Parkinson's diseases has been associated with the loss of cortical cholinergic innervation (Little et al., 1998; Giacobini, 2003; Sabri et al., 2008; Hasselmo and Sarter, 2011), and chronic administration of nicotine reverses hypofrontality in animal models of addiction and schizophrenia (Koukouli et al., 2017).

Classical notions sustain the view that the central cholinergic system works by a diffuse release of ACh across the cortex, activating its receptors globally and producing slow responses. While this view might be applicable to long-lasting behavioral phenomena, such as cortical arousal, it does not explain the modulation of processes that happen on a much faster scale, such as sensory gating, or plasticity (Muñoz and Rudy, 2014). ACh release in the neocortex originates from neurons distributed within the basal forebrain (BF) nuclei, including the medial septum, the vertical and horizontal diagonal band of Broca, the substantia innominata, and the nucleus basalis of Meynert (NBM). Release occurs through topographical projections, and all the projections arise from six groups of choline acetyl-transferase (ChAT)-positive neurons in the BF (Ch1–Ch4) and brainstem (Ch5–Ch6; Wevers, 2011). The innervation sparsely reaches all cortical layers, but layer 5 is more heavily innervated, particularly in the motor and sensory areas; cholinergic pathways often provide *en passant* innervation (Dani and Bertrand, 2007) to the neocortex. Additionally, ACh-releasing cells are found in cortical layer 2/3. These cells exhibit a bipolar morphology, stain positive for calretinin (CR) and vasoactive intestinal peptide (VIP), and are GABAergic (von Engelhardt et al., 2007; Granger et al., 2018).

The function of a neuromodulatory system is largely defined by the anatomy of its projections. Projections from the BF selectively control cortical activity and target neocortical regions more specifically than previously assumed (Hasselmo and Sarter, 2011; Muñoz and Rudy, 2014; Obermayer et al., 2017). Recent evidence suggests that a roughly topographical organizational scheme exists in the rostro-caudal sequence of neurons of the BF (Zaborszky et al., 2015) and that specific BF nuclei innervate specific cortical areas, as opposed to what happens with noradrenergic fibers originating from the locus coeruleus (Chaves-Coira et al., 2016; Kim et al., 2016). Cholinergic fibers can take one of four different routes to cortical structures: the septal pathway (which projects mainly to the hippocampal cortex) the medial pathway, the lateral

pathway, or the internal capsule projection (which preferentially project to the neocortex; Poorthuis et al., 2014). Cholinergic terminals that reach the neocortex, mainly *via* layer 1 or layer 6 (Obermayer et al., 2017), can either exert a spread out control of cortical activity and regulate processes such as the transition from sleep to wakefulness and arousal, or contact a restricted number of cortical elements and have cell-type specific effects; here contextual cholinergic signals act in concert with local processing of sensory inputs in order to guide behavior.

The aim of this review is to bring together current knowledge of cholinergic modulation in the neocortex and to identify the gaps to propose future directions to advance the field of neuromodulation. Here, we summarize existing literature on ACh release in the neocortex of rodents and non-human primates, specifically focusing on how ACh-induced effects on the diversity of cell-types and synapses shape the emergence of network states and review theories that bridge the modulation of local circuit properties and the consequent reconfiguration of cortical states. Data-driven computational models allow predictions on the potential role of ACh in reconfiguring neocortical states (Ramaswamy et al., 2018). Therefore, this review reconciles the minimal, although sparse, datasets required to build a multi-scale computational model of the neocortical cholinergic system.

VOLUME vs. SYNAPTIC TRANSMISSION

A major factor that determines the spatiotemporal precision of ACh action is the transmission mode at cholinergic terminals. Cholinergic cortical signaling has historically been considered a slow and diffuse process, which was established upon examination of the functional organization of cholinergic projections and was mainly based on reports indicating a nearly complete absence of classical synapses at the level of cholinergic terminals (Muñoz and Rudy, 2014). Before optogenetic techniques were available, cholinergic pathways could not be activated in a selective manner, and thus evidence of the existence of fast cholinergic synaptic transmission was lacking, although some proof of fast nicotinic responses was already available from hippocampal recordings (Kalmbach et al., 2012; Obermayer et al., 2017).

In the cerebral cortex, cholinergic fibers are distributed in an intricate network with a characteristic laminar pattern. They have a higher density in the deeper layers. Cholinergic innervation reflects the classic organizational scheme of information processing systems (Kennedy and Bullier, 1985), with a higher number of projections being present in higher-order areas. Presumed cholinergic release sites have been ultra-structurally inspected and the subtle presence of synapse-like contacts has indeed been revealed; however, a relatively large number of these small varicosities, which are often associated with accumulated synaptic vesicles, do not seem to effectively establish synaptic contact with neighboring neurons, or exhibit only a few morphologically identifiable synapses Furthermore, the scarceness of astrocytic processes in the immediate vicinity of ChAT-immuno-reactive axons (when compared to

glutamatergic terminals) may also allow greater diffusion of ACh within the extracellular space (Aoki and Kabak, 1992). Thus, relatively low concentrations of ACh will reach locations that are distant from the release site. This produces volume transmission or bulk release: neuromodulators slowly diffuse in a wide cortical area and bind to a large pool of extra-synaptic receptors (Dani and Bertrand, 2007).

Many studies (Umbriaco et al., 1994; Descarries and Mechawar, 2000; Sarter et al., 2009; Yamasaki et al., 2010) conducted in the neocortex have suggested that ACh acts preferentially non-synaptically; however, central cholinergic synapses had already been observed in the early '90s. Actual synapses were found on cholinergic varicosities in the cingulate cortex of the rat (Umbriaco et al., 1994), and in macaque more than 40 percent of cholinergic varicosities contained synaptic specializations (Mrzijak et al., 1995). Later, Turrini et al. (2001) provide definitive evidence that suggests that synaptic mechanisms of cholinergic transmission not only exist but prevail in the rat neocortex. Ultrastructural observations that most (66%) cholinergic boutons—as revealed by IR assays for the specific cholinergic marker, vesicular ACh transporter (vAChT)—establish classical synapses in layer 5 of the rat parietal cortex. By applying an improved fixation protocol and by using an antibody for vAChT, Turrini et al. (2001) demonstrated that cholinergic boutons predominantly established symmetric synapses on layer 5 dendritic shafts. The authors also found that immuno-stained varicosities occasionally established asymmetric contacts, but always on dendritic spines.

Another study probed the molecular-anatomical relationship between detectable cholinergic varicosities and the most abundant receptor subtype in the cortex—the muscarinic receptor subtype M1 (Yamasaki et al., 2010). This study established that in the mouse neocortex M1 can be found almost exclusively on the extra-synaptic membrane of pyramidal cells (PCs). Here, they observed that M1 distribution is far denser than the putative cholinergic release sites and that it does not show any apposition pattern to the varicosities, nor to the cytomatrix active zone proteins that are normally found at glutamatergic terminals. Hence, M1's function in cortical PCs may be to sense ambient ACh released from cholinergic terminals at variable distances, and the main modality through which it is recruited is likely to be volume transmission. These approaches not only contribute to building a more refined knowledge of the subcellular localization of receptor subtypes but also provide a method to qualitatively discriminate between two major modes of transmission. Because of a substantial difference in the distribution pattern of cholinergic receptors across species, it is very likely that experiments performed in different species will yield conflicting results. For instance, even though a low incidence of classical synapses was reported for the rodent brain, a much higher proportion of cholinergic synapses was found in primates (Smiley et al., 1997). In the human cerebral cortex, the same authors found that up to 67% of all cholinergic varicosities established synaptic contacts, suggesting that ACh signaling in humans is mostly mediated by point-to-point synaptic transmission; this mechanism appears to prevail

in the primate brain, but whether the same can be said for rodents is still a matter of open debate.

Cholinergic innervation from the BF is more specific than previously considered; ACh can control cortical activity on a fine spatial scale as well. Indeed, these findings agree with the evidence of ACh signaling occurring through direct fast point-to-point synaptic transmission brought about by the application of optogenetic tools (Kalmbach et al., 2012). Overall, it is not completely clear yet whether one mode of cholinergic transmission prevails over the other. Instead, a growing body of evidence suggests that volume and synaptic transmission may be complementary mechanisms by which ACh modulates cortical function (Sarter et al., 2009). While bulk release is thought to cause a more tonic change in extracellular ACh concentration, in the scale of seconds and minutes, and is mainly mediated by activation of extra-synaptic receptors, ACh release occurring at junctional sites would have a more circumscribed influence, with the modulation of circuit activity being restricted to the contacted cortical elements and to a much more delimited spatiotemporal scale (Muñoz and Rudy, 2014). Taken together, evidence shows that ACh modulates microcircuit activity with different modalities, ranging from synaptic release to volume transmission, and exerts its effects by modifying membrane excitability or synaptic activity.

Instead of trying to proclaim one modality over the other, future research should address the issue of whether they can occur simultaneously and have a differential impact on the temporal aspects of the response. Traditional bath application of agonists results in broad spatial and temporal activation that might not reflect the accuracy of endogenous ACh release (Urban-Ciecko et al., 2018). It is thus of crucial importance to determine whether the different ways in which cholinergic agonists are experimentally applied reflect different transmission modalities, and how faithfully stimulation protocols replicate physiological conditions. In the future, ACh application should be standardized according to precisely obtained dose-response and sensitization kinetics curves, and ascending concentrations should be used in order to detect eventual dose-dependent responses. Furthermore, it would be of outstanding interest to better understand how ACh release obtained by optogenetic stimulation of cholinergic afferents compares against bath application of cholinergic agonists. In a recent study, optogenetic recruitment of cholinergic fibers was performed in parallel with 1 mM ACh bath-application to detect changes in Martinotti cells (MCs) activity: the two techniques yielded very similar results (Obermayer et al., 2018). Perhaps the high concentration of ACh used in this case is comparable with a more physiological activation of the cholinergic system. Further clarification is required on the matter, and future studies should, therefore, consider this issue and design their experiments accordingly.

Cholinergic projections are likely to be arranged according to a modular pattern, with isolated bands of neighboring ChAT^{+} cells in the BF having defined cortical targets that are, in turn, functionally associated. When retrograde dyes are injected in distant cortical areas, labeled regions of cholinergic cells in the BF still largely overlap, even though the innervated

cortical space is quite restricted (Muñoz and Rudy, 2014). Furthermore, Zaborszky et al. (2015) assert that the degree of overlap of labeled neuronal locations within the BF is positively correlated to the connection strength between the different injected cortical regions. Such an organization could induce widespread modulation even when the system is only focally activated (Muñoz and Rudy, 2014). Nevertheless, the response to neuromodulatory inputs is determined by the interplay of multiple factors, such as post-synaptic target, receptor type and subunit composition, subcellular localization of the receptors and their sensitivity. This way, a diffusely-organized projection system can fine-tune microcircuit activity. The cholinergic projection system should be viewed as a highly dynamic structure, able to propagate inputs either selectively or diffusely, switching from one modality to another, depending on the needs.

The next section aims to unravel the contribution of each subtype of cholinergic receptor to microcircuit modulation and attempts to determine the physiological relevance of their compartmentalized distribution and differential activation.

CHOLINERGIC RECEPTORS

Even though the differential pharmacological effects had already been characterized, it was not until the early 1950s that the idea of "receptors" as the binding site for ACh was firmly established by Eccles et al. (1953). Cholinergic receptors are composed of two classes of transmembrane macromolecular complexes, the muscarinic and the nicotinic receptor families, each of which is further divided into subclasses. The occurrence of many ACh receptor subtypes and their differential dendritic, somatic, axonal, and synaptic localization contribute to the varied roles that these receptors play in the CNS. Cholinergic receptors have been found on axons originating from thalamic, cortical or basalo-cortical fibers as well as on cortical pyramidal excitatory neurons and inhibitory GABAergic interneurons (Groleau et al., 2015). The precise layer-wise distribution of cholinergic terminals, the identification of cell-types that actually express cholinergic receptors, and the subcellular localization of these receptors are described in the following sections.

MUSCARINIC RECEPTORS

Cholinergic synapses throughout the CNS are composed of muscarinic receptors (mAChRs), which can be further differentiated into subtypes that are encoded by a single gene (Venter et al., 1988; Van der Zee and Luiten, 1999). Five genetically defined and pharmacologically characterized (M1 to M5) mAChR subtypes have been identified in the CNS with high levels of expression in subcortical structures and the cerebral cortex (Wevers, 2011). Immunocytochemical approaches have identified different levels of expression of mAChRs throughout the cerebral cortex. These studies have detected moderate levels of mAChRs in the frontal cortex, parietal cortex, temporal cortex, entorhinal cortex, occipital cortex, insular and cingulate cortex, with the highest values for

the temporal and occipital cortex. M1 receptors are the most abundantly expressed among all subtypes of mAChRs (Wevers, 2011). The density of cholinergic terminals in the rat neocortex differs between the six layers and depends on the cortical region studied (Eckenstein et al., 1988; Lysakowski et al., 1989). The pattern of cellular staining for mAChRs in the neocortex is characterized by a clear laminar distribution: in most of the cortical mantle, especially in neocortical areas, predominantly layer 5 PCs (L5PCs) show strong immunoreactivity across mammals such as the mouse, golden hamster, rat, cat, and human (Van der Zee and Luiten, 1999).

The density of each mAChR subtype differs throughout the brain with M1 being the most abundantly expressed and M5 the least (Alger et al., 2014). In the hippocampus and neocortex, M1 is present at high levels, M3 is moderately represented (though generally low elsewhere) and M4 is present in high density, as almost anywhere else in the brain, even though its concentration is considerably lower than M1. M2 instead, is found at very low densities, and this class of receptors seems to be distributed according to a precise pattern. M2 receptors frequently reside on presynaptic axonal terminals, whereas M1 receptors are often located on somato-dendritic regions of neurons. The M5 subtype is believed to play an important role in cortical perfusion, and it is mainly expressed on endothelial cells of the cerebral vascular system (Elhusseiny and Hamel, 2000; Gericke et al., 2011) even though recent evidence suggests that the M3 subtype is also involved in this kind of process (Zuccolo et al., 2017). In the rodent visual cortex, the subtypes M1 and M2 predominate, while in primates the subtypes M1, M2 and M4 prevail. Besides a few regional variations, highest labeling densities have been observed in the superficial layers of most cortical areas for both M1 and M2 (Wevers, 2011).

Most cholinergic receptors are metabotropic and mediate slow responses, which are typically associated with volume transmission. In the neonatal and adult cortices of rodents and primates, M1–M5 subtypes of mAChRs occur in both pre-synaptic and post-synaptic positions (Mrzljak et al., 1993; Groleau et al., 2015). All mAChRs are transmembrane macromolecular complexes that are coupled to membrane-embedded G-proteins of different kinds; g-proteins act as intracellular effectors and initiate signaling cascades that ultimately have an effect on intracellular processes, leading to the opening or closing of some ion channel, or to the production of long-term modifications of genetic activity and protein expression. Different mAChRs are coupled to specific G-proteins. The pre-synaptic mAChRs M2 and M4 preferentially couple to G_i and G_o proteins that generally have inhibitory effects on voltage-activated calcium channels or extend the opening of potassium channels. The resulting decrease in c-AMP signaling suppresses neurotransmitter release (Groleau et al., 2015). M1, M3 and M5 subtypes are preferentially coupled to G_q and G_{11} proteins and are mainly located post-synaptically. Their activation seems to trigger membrane depolarization and increases the input-resistance of the cell membrane. M1-like (M1-M3-M5) receptors are known to potentiate NMDA currents and also influence and modulate voltage-dependent calcium currents, mostly by upregulating phospholipase C

(PLC) signaling and inositol triphosphate (IP$_3$) turnover. One major effect that can be attributed to M1-type receptors is the inhibition of potassium currents, including the I$_m$ and the I$_{AHP}$ (both medium and slow rate). However, M1-type receptors can also potentiate cationic currents like the I$_h$ and the TRP currents, and the I$_{cat}$ (Teles-Grilo Ruivo and Mellor, 2013). For a more detailed description of the effects of ACh on various currents and their associated intracellular signaling pathways, we direct the reader to the section "Subcellular Nicotinic and Muscarinic Pathways" of this review.

PRE-SYNAPTIC LOCALIZATION

What anatomical and functional evidence exists on the distribution of mAChRs in the neocortex? Muscarinic cholinergic activity influences sensory processing by facilitating or depressing neuronal responses to specific stimuli, and by modulating connections strength and neural synchronization: this results in the fine-tuning of cellular and network properties during developmental processes, the execution of attention tasks and perceptual learning (Groleau et al., 2015). These effects can largely be attributed to M1 and M2 subtypes, which appear to be highly prevalent in the neocortex. The presence of M1 and M2 mAChRs on PC somata and apical dendrites in non-human primates is well established, but M2 receptors are also found on excitatory and inhibitory axons in the primate neocortex (Mrzljak et al., 1993). Disney et al. (2006) report that M1 and M2 receptor labeling can be observed, but is quite weak in axons and terminals in the macaque visual cortex, whereas mAChRs are mostly expressed at the level of the soma of GABAergic neurons and in the dendritic compartments of glutamatergic cells.

Among the presynaptic receptors in the rodent and human visual cortex, M2 is very abundant while M4 is less prevalent (Groleau et al., 2015). M2 and M4 are mostly found at the presynaptic terminals; activation of these receptor subtypes causes membrane hyperpolarization and conveys a self-inhibitory signal. Thus, extracellular levels of ACh are regulated by means of negative feedback. In the rat's primary visual cortex (V1) M2 is mainly found at the level of cholinergic terminals in layer 4 and layer 5. Being the main inhibitory auto-receptor, it contributes to the suppression of presynaptic ACh release (Mrzljak et al., 1993).

It is not yet clear whether the presence of M2-like subtypes at the level of the presynaptic terminal is a distinctive feature of cholinergic axons innervating the neocortex. Conflicting results emerge when looking at rodent studies, while experiments done on non-human primates and cats corroborate M2 receptors as the main auto-receptors localized on BF cholinergic axons. Subsequent research should, therefore, address this issue and determine the extent to which presynaptic M2-like receptors account for negative feedback *via* auto-inhibition, since this type of self-regulatory process is crucial for the fine-tuning of the response. Moreover, given that BF fibers originating from distinct neuron clusters differentially innervate separate cortical areas (Zaborszky et al., 2015; Chaves-Coira et al., 2016; Kim et al., 2016), discrepancies should be expected

when assessing receptor subtype distributions across neocortical regions. Estimation of the physiological presynaptic distribution profile of inhibitory auto-receptors in the rodent sensory cortex is of key importance to understanding the system's self-calibrating features. A systematic anatomical profiling of receptor expression should be performed in the rodent models, and quantitative comparisons should be made across sensory areas.

POST-SYNAPTIC LOCALIZATION

Neocortical PCs and inhibitory interneurons are strongly innervated by cholinergic axons, with L5PCs being the most densely innervated cells; however, numerous immuno-reactive interneurons can be found in all layers, but most frequently in layer 2/3 and layer 5. Here, the mAChR positive interneurons are intermingled with labeled PCs, but in general, the immunostaining of interneurons is less dense than that of the PCs (Van der Zee and Luiten, 1999). While mAChRs are more easily found in the dendritic compartments of PCs, their expression profile throughout the diversity of inhibitory interneurons is quite homogeneous, as these receptors are detected in proximity of the somatic compartment (Disney et al., 2006). mAChRs are expressed by different types of interneurons. In macaque, M2 receptors are found in 31% of PV neurons, 23% of CB neurons, and 25% of CR neurons. 87% of PV$^+$ neurons, 60% of CB$^+$ neurons and 40% of CR$^+$ neurons however, express M1-type mAChRs. The M1 subtype is found across the cortical mantle on the cell bodies and dendrites of post-synaptic PCs, and it appears to be present mainly in layers 2/3 and 6, but it can be found across all cortical layers. In macaque V1, M1 is mostly expressed on GABAergic interneurons, but it is also found on cortico-cortical fibers (Mrzljak et al., 1993; Groleau et al., 2015). M1 immuno-reactivity is also observable in interneurons of the rat neocortex (Levey et al., 1991), although other studies have pointed to a low expression of M1 in primary sensory cortices of rats, such as S1 and V1. Some found M1 expression on PV$^+$ neurons to be low or even undetectable in mice neocortex (Yamasaki et al., 2010). The significant difference in expression between rodents and primates could be explained by the fact that M1 receptors are much more associated to the extra-synaptic membrane compartments and are usually activated by volume transmission. Given that the BF cholinergic projection system is scaled-up in primates relative to rodents, there could be a more widespread distribution of M1 receptors throughout cortical interneurons. M1 immuno-reactivity is also detected at the synaptic level, in both inhibitory and excitatory synapses across cortical layers, but more frequently on asymmetric synapses, and here, preferentially on dendritic spines, as opposed to symmetric synapses where M1 is found mostly on dendritic shafts (Mrzljak et al., 1993). This preferential distribution perspective is challenged though, by experimental evidence that cholinergic boutons form synapses mainly with dendritic shafts, much fewer with dendritic spines and only occasionally on neuronal somata (Beaulieu and Somogyi, 1991; Mrzljak et al., 1993; Umbriaco et al., 1994). However, in mice, the highest density of M1 immuno-particles is observed in small-caliber

oblique dendrites (smaller than 0.66 μm in diameter) of PCs (Yamasaki et al., 2010).

In L5PCs, M2 mAChRs are mainly localized postsynaptically, where they bring about a decrease in excitatory conductances, but M2 and M4 receptors are also present on the cell bodies of GABAergic interneurons in layers 2/3 and 4; here, M2 activation inhibits GABA release. The M3 subtype is localized postsynaptically in rodent inhibitory neurons and dendrites, where it enhances inhibitory transmission (Mrzljak et al., 1993; Groleau et al., 2015). Finally, M4 mAChRs are expressed in cortical excitatory neurons, in particular, in layer 4 spiny stellate neurons (L4SS) across different neocortical regions—S1, V1, and prefrontal cortex (PFC)—where they generate a persistent

hyperpolarizing response (Radnikow and Feldmeyer, 2018). Perhaps the presence of M4 mAChRs is a marker to tell apart layer 4 from other layers.

Cholinergic inputs to the cortex generate different responses depending on which receptor is recruited: while M1-like (M1-M3-M5) receptors activation generally leads to an increase in postsynaptic conductance, M2-like receptors (M2-M4) have the opposite tendency to decrease synaptic transmission, by regulating presynaptic ACh release or by directly hyperpolarizing the post-synaptic membrane. mAChRs thus seem to be distributed both at the presynaptic and the postsynaptic level, and the resulting effect depends mostly on which subtype is activated. A detailed understanding of the cellular localization
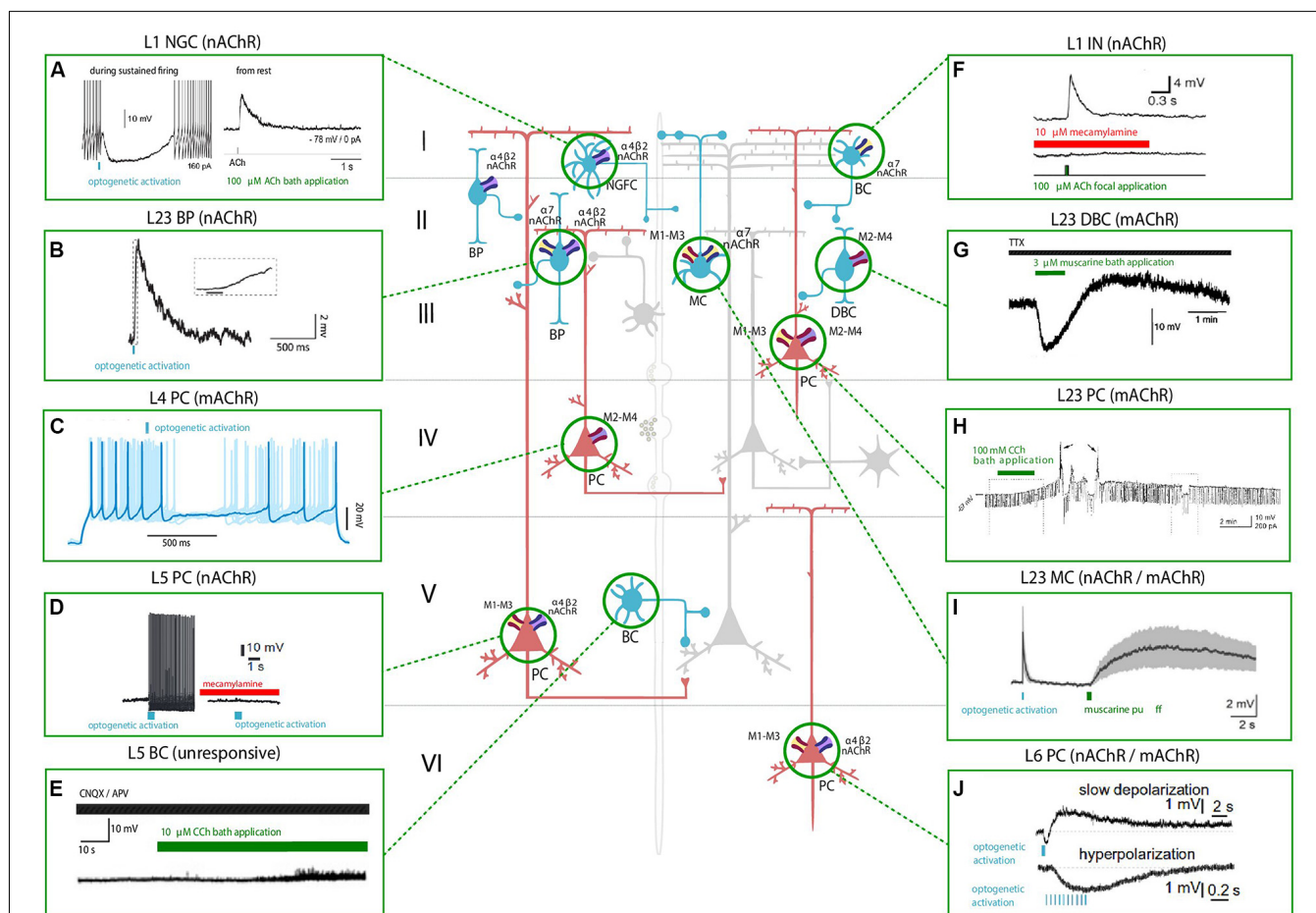
FIGURE 1 | Effect of nicotinic acetylcholine receptors (nAChRs) and muscarinic ACh receptors (mAChRs) activation on the membrane potential of various neocortical cell types. The central schema represents the main cell types in the neocortex. Excitatory neurons are shown in red and inhibitory GABAergic neurons are shown in blue. The electrophysiological responses to the optogenetic activation of cholinergic fibers (in light blue) or the application of a cholinergic agonist (shown in green) or antagonist (shown in red) of each cell type are depicted in the inserts. Timing of cholinergic manipulation is shown as a vertical or horizontal bar. Muscarinic and nicotinic cholinergic receptors associated with the observed response, when known, are shown as four main subtypes: M1-M3-M5 like receptors (yellow and red), M2-M4 like receptors (violet and red), α4β2 heteromeric nAChRs (violet and blue) and α7 homomeric nAChRs (yellow and blue). All shown experimental traces reflect studies listed in **Tables 1**, **2**. Selected traces were recorded in sensory areas of the rodent neocortex. Inclusion criteria for the experimental traces comprise knowledge of the cell-types and the receptor subtype (nicotinic or muscarinic) involved in the electrophysiological response. Abbreviations: PC, pyramidal cell; SS, spiny-stellate cell; IN, interneuron; MC, Martinotti cell; BC, basket cell; DBC, double-bouquet cell; NGFC, neurogliaform cell; BPC, bipolar cell. Reproduced and adapted from: (left, top to bottom): **(A)**. Brombas et al., 2014; **(B)** Arroyo et al., 2012; **(C)** Dasgupta et al., 2018; **(D)** Hedrick and Waters, 2015; **(E)** Kawaguchi, 1997 (Right, top to bottom): **(F)** Gulledge et al., 2007; **(G)** Kawaguchi, 1997; **(H)** Shalinsky et al., 2002; **(I)** Dasgupta et al., 2018; **(J)** Hedrick and Waters, 2015. For more exhaustive information on agonist concentration, species and cortical area examined, see **Tables 1**, **2**.

of each receptor subtype for every cell-type is still lacking; some generalizations can be made (as can be seen in **Figure 3**), but in order to precisely understand how neuromodulatory signals affect neural computation, a detailed knowledge of the amount and distribution of receptor subtypes at the level of each compartment is essential. Furthermore, it is of vital importance to gather this information for each neocortical cell-type. Neuromodulatory inputs very likely affect each cell-type differently, unlocking the possibility of fine-tuning the response and allowing delicate recalibration based on contextual information processing. This is most likely achieved by differentially distributing receptors along cellular compartments, thus creating modulatory micro-domains.

REGULATION OF NEURONAL AND SYNAPTIC PHYSIOLOGY

ACh can either increase or decrease neurotransmitter release probability, consistent with its role as a neuromodulator rather than a transmitter, and the effect on synaptic release probability depends on the identity of the pre and postsynaptic partners. Cell-types in the neocortex are differentially regulated by ACh, and the effects of cholinergic release include modulation of membrane properties (**Figure 1**) and synaptic dynamics (**Figure 2**).

The effects of ACh on neocortical PCs have been thoroughly investigated, and many studies (Gil et al., 1997;

FIGURE 2 | Effect of nAChRs and mAChRs activation on neocortical synaptic dynamics. The central schema represents the main neocortical cell types and their synaptic connections. A fiber of subcortical provenance associated with cholinergic boutons is also shown. Excitatory neurons are shown in red and inhibitory GABAergic neurons are shown in blue. The electrophysiological responses to the application of a cholinergic agonist or antagonist or to basal forebrain (BF) optical stimulation are depicted in the inserts. Panels show the modulation of synaptic dynamics in terms of increase or decrease in PSP/PSC size. Muscarinic and nicotinic cholinergic receptors associated with the observed response, when known, are shown as four main subtypes: M1-M3-M5 like receptors (yellow and red), M2-M4 like receptors (violet and red), α4β2 heteromeric nAChRs (violet and blue) and α7 homomeric nAChRs (yellow and blue). All shown experimental traces reflect studies listed in **Table 3**. Selected traces were recorded in sensory areas of the rodent neocortex. Inclusion criteria for the experimental traces comprise knowledge of the pre and postsynaptic cell-types and the receptor subtype (nicotinic or muscarinic) involved in the response. Abbreviations: PC, pyramidal cell; TTPC, thick tufted pyramidal cell; STPC, slender tufted pyramidal cell; SS, spiny-stellate cell; MC, Martinotti cell; BC, basket cell; NGFC, neurogliaform cell; BPC, bipolar pyramidal cell; IPC, inverted pyramidal cell. Reproduced and adapted from: (left, top to bottom): **(A)** Brombas et al., 2014; **(B)** Urban-Ciecko et al., 2018; **(C)** Kruglikov and Rudy, 2008; **(D)** Dasgupta et al., 2018; **(E)** Yamamoto et al., 2010; **(F)** Salgado et al., 2007; **(G,H)** Eggermann and Feldmeyer, 2009; **(I)** Kruglikov and Rudy, 2008; **(J)** Markram et al., 1997. For more exhaustive information on technique, species and cortical area examined, see **Table 3**.

FIGURE 3 | Differential expression of cholinergic receptors in various neuronal compartments across cell-types. Heatmap matrices show the occurrence of cholinergic receptor subtypes at the level of different cell-types. The presence of a given subtype in a cellular compartment is classified as consistently expressed (consistent findings across experimental studies), sometimes expressed (evidence of its presence is only partial) and never expressed (presence of a given subtype is undetectable). Abbreviations: PC, pyramidal cell; M1, M2, M3, M4, muscarinic cholinergic receptors 1–4; nAChR, nicotinic acetylcholine (ACh) receptor.

(SNR) of incoming thalamic inputs. ACh seems to plays a role in enhancing circuit responses to relevant stimuli, providing a mechanism to regulate sensory processing during learning and attention.

The involvement of mAChRs in the depolarizing response of PCs to BF cholinergic inputs has been established by numerous studies (McCormick and Prince, 1985; Delmas and Brown, 2005; Gulledge and Stuart, 2005; Carr and Surmeier, 2007; Zhang and Séguéla, 2010), which report that muscarinic activation in PCs leads to an initial SK-mediated hyperpolarization, followed by a more sustained and slow depolarization (**Table 1**, **Figure 1**). Interestingly, the same biphasic response can be induced by bath perfusion of muscarinic agonists in hippocampal interneurons (Heys and Hasselmo, 2012; Heys et al., 2012). The mechanism by which this depolarization emerges has not been fully clarified yet, but some authors suggest the suppression of muscarinic-sensitive and voltage-dependent K^+ conductance termed the M current (I_m) or the activation of a non-specific cationic current both support the observed depolarization (McCormick and Prince, 1985; Krnjević, 2004).

In L5PCs, transient activation of M1-type mAChRs induces calcium release from IP_3-sensitive intracellular calcium stores and subsequent activation of an apamin-sensitive, SK-type calcium-activated potassium conductance (Gulledge et al., 2007). Conversely, M4-mediated activation of a potassium conductance (K_{ir3}) in L4SS generates a persistent membrane

Disney et al., 2007) have come to the conclusion that besides generating direct PC depolarization, cholinergic modulation has an overall effect of increasing the signal to noise ratio

TABLE 1 | Effect of muscarinic acetylcholine receptors (mAChRs) activation on membrane potential in various neocortical cell types.

Cell type	Receptor	Effect	Area	Technique—Reference
L5 PC	M1 (soma)	Transient hyperpolarization	Rat PMC/V1/PFC	Optogenetics (Hedrick and Waters, 2015)
	M1 (soma)	Slow depolarization	Rat PMC/V1/PFC	1. Optogenetics (Hedrick and Waters, 2015)
				2. Somatic puff 100 μM ACh/CCh (Gulledge and Stuart, 2005)
				3. 100 μM ACh focally applied (Gulledge et al., 2007)
	M1 (soma)	Hyperpolarization	Rat SSC	100 μM ACh focally applied (Gulledge et al., 2007)
	M1 (soma)	Depolarization	Rat mPFC	30 μm muscarine or oxotremorine bath application (Haj-Dahmane and Andrade, 1996)
L23 PC	Muscarinic	Depolarization	Mouse V1	*In vivo* 2-photon imaging (Alitto and Dan, 2013)
	Muscarinic	Prolonged depolarization	Rat EC layer II	100 mM CCh bath application (Shalinsky et al., 2002)
	M2–M4	Hyperpolarization	Mouse SSC (p12–p16)	Optogenetics (Dasgupta et al., 2018)
L4 PC	M2–M4	Persistent hyperpolarization	Rat SSC	100 μM ACh, puff (Eggermann and Feldmeyer, 2009)
L4 SS	M4 (soma)	Persistent hyperpolarization	Rat SSC	100 μM ACh, puff (Eggermann and Feldmeyer, 2009)
L1 BC	Muscarinic	Depolarization	Mouse V1	*In vivo* 2-photon imaging (Alitto and Dan, 2013)
L1 DBC	Muscarinic	Depolarization	1. Mouse V1	1. *In vivo* 2-photon imaging (Alitto and Dan, 2013)
			2. Rat PFC	2. 10 μM CCh or 3 μM muscarine bath application (Kawaguchi, 1997)
L23 DBC	M2	1. Hyperpolarization	1. Rat SSC	1. 100 μM ACh focally applied (Gulledge et al., 2007)
		2. Hyperpolarization + slow depolarization	2. Rat PFC	2. 10 μM CCh or 3 μM muscarine bath application (Kawaguchi, 1997)
L23 MC	M1–M3	Depolarization	Mouse SSC	1. Muñoz et al. (2017; M1- M3 KO lines)
				2. Optogenetics (Dasgupta et al., 2018)
	Muscarinic	Depolarization	Mouse V1	1 μM/10 mM ACh application (Chen et al., 2015)
L23 BC		Not responsive (NR)	1. Rat SSC	1. 100 μM ACh focally applied (Gulledge et al., 2007)
			2. Rat PFC	2. 10 μM CCh or 3 μM muscarine bath application (Kawaguchi, 1997)
L5 BC		NR	Rat SSC	100 μM ACh focally applied (Gulledge et al., 2007)
L5 MC	Muscarinic	NR/slight depolarization	Rat SSC	100 μM ACh focally applied (Gulledge et al., 2007)

The table links the distribution and localization (when known, in brackets) of muscarinic receptors across neocortical cell types, with respect to cortical layers, with the effect of their activation. The effect of receptor activation is represented in terms of variation of membrane potential. Age of the specimen is given in brackets, when known. When biphasic effects occur, they are listed as multiple effects. Inclusion criteria for the listed studies comprise: (1) recordings performed in the rodent neocortex; (2) knowledge of the morphological type involved; and (3) knowledge of the receptor subtype involved in the response. Abbreviations: PC, pyramidal cell; SS, spiny-stellate cell; IN, interneuron; MC, Martinotti cell; BC, basket cell; DBC, double-bouquet cell; NGFC, neurogliaform cell; BPC, bipolar cell; NBC, nest basket cell; RS, regular spiking. PMC, primary motor cortex; V1, primary visual area; PFC, prefrontal cortex; mPFC, medial prefrontal cortex; EC, entorhinal cortex; SSC, somatosensory cortex, ACh, acetylcholine; CCh, carbachol.

hyperpolarization and induces suppression of neurotransmitter release (**Table 1**, **Figure 1**). The observed hyperpolarizing response is supported by a decrease in presynaptic calcium conductance, at synapses between L4PCs and also at synapses between L4PCs and L23PCs (see **Table 3**, **Figure 2**; Eggermann and Feldmeyer, 2009). Focal application of ACh onto the soma of L5PCs evokes a biphasic response in which a transient membrane hyperpolarization precedes a slower and longer-lasting depolarization. Pharmacological evidence suggests that this effect is mediated by M1 receptors. Compared with the pressure application of ACh, activation of cholinergic synapses with brief bursts provides relatively weak activation of mAChRs that often fails to affect the somatic membrane potential at rest (Hedrick and Waters, 2015). One possible interpretation of these results might be that synaptically released ACh activates first nAChRs and usually fails to activate mAChRs, whereas pressure ejection onto the soma recruits primarily mAChRs.

Muscarinic activation modulates K^+ conductances (McCormick, 1992), but the reversal potential for K^+ is approximately -90 mV: mAChR activation, therefore, exerts a little effect at resting membrane potential. However, when a neuron is depolarized, the observable mAChR-mediated hyperpolarization and subsequent depolarization are larger. The reported biphasic effect affects both cortico-pontine (CPn) and commissural (COM) pyramidal neurons; however, COM neurons show a more pronounced inhibitory phase, while CPn neurons have a larger and longer-lasting depolarizing phase (Baker et al., 2018). While these effects have been characterized thoroughly in deep-layers PCs, others report that ACh has limited ability to inhibit superficial PCs *via* changes in membrane potential (Gulledge et al., 2007).

Cortical inhibitory interneurons are, as well as PCs, a prominent target of cholinergic neuromodulation. The ways in which ACh modulates the dynamics of local interneurons have not been completely clarified yet, because the effects of BF cholinergic stimulation and bath application of cholinergic agonists (**Table 1**) strongly depend on the inhibitory cell-type.

Exogenous application is unlikely to mimic accurately the spatiotemporal profile of ACh release from cholinergic axons, and furthermore, there seems to be no agreement within

TABLE 2 | Effect of nAChRs activation on membrane potential in various neocortical cell types.

Cell type	Receptor	Effect	Area	Technique—Reference
L5 PC	$\alpha_4\beta_2$ (soma and main dendrite)	Medium depolarization	Mouse PMC/V1/PFC	Optogenetics (Hedrick and Waters, 2015)
	α4 α5	Depolarization Persistent spiking (starting from subthreshold)	Mouse PMC/V1/PFC	Optogenetics (Hedrick and Waters, 2015)
L6 PC	α4 α5 (soma and main dendrite)	Depolarization	Mouse PMC/V1/PFC	Optogenetics (Hedrick and Waters, 2015)
	$\alpha_4\beta_2$	Depolarization	Rat PFC (p7–p27)	Kassam et al. (2008; bath application of 10 μM ACh to 1 mM)
L1 NGFC	Nicotinic (non-α7)	Depolarization (from RP) Suppression of activity (from subthreshold)	Rat SSC	Iontophoretic application or bath application of 100 μM ACh (Brombas et al., 2014)
L1 BC	Nicotinic	Suppression of activity (at low levels of cortical desynchronization)	Mouse V1	*In vivo* 2-photon imaging (Alitto and Dan, 2013)
L1 INs	Nicotinic	Fast depolarization (from RP)	Rat SSC	100 μM ACh focally applied (Christophe et al., 2002) and (Gulledge et al., 2007)
NBC	Nicotinic	Depolarization	Rat SSC	100 μM ACh focally applied (Gulledge et al., 2007)
BPC	Nicotinic	Depolarization	Rat SSC	100 μM ACh focally applied (Gulledge et al., 2007)
DBC	Nicotinic	Depolarization	Rat PFC	10 μM CCh or 3 μM muscarine bath application (Kawaguchi, 1997)
L23 MC	Nicotinic	Depolarization	Mouse V1	1 μM/10 mM ACh application (Chen et al., 2015)
	Nicotinic	Depolarization	Rat SSC	Optogenetics (Dasgupta et al., 2018)
	α4β2	Depolarization	Mouse S1 and mPFC	Optogenetics or 1 mM ACh bath-application (Obermayer et al., 2018)
L23 BC	Nicotinic	Some are depolarized Some are hyperpolarized	Mouse V1	*In vivo* 2-photon imaging (Alitto and Dan, 2013)
L23 CHAT+ BPC	α4β2	Depolarization	Mouse SSC (P20–P40)	Optogenetics (Arroyo et al., 2012)
L23 BPC	α4β2 and α7	Depolarization	Mouse and rat SSC	Optogenetics (Arroyo et al., 2012) and (Dasgupta et al., 2018); 100 μM ACh focally applied (Gulledge et al., 2007)
L5 MC	α4β2	Depolarization	Mouse S1 and mPFC	Optogenetics or 1 mM ACh bath-application (Obermayer et al., 2018)

The table links the distribution and localization (when known, in brackets) of nicotinic receptors across neocortical cell types, with respect to cortical layers, with the effect of their activation. The effect of receptor activation is represented in terms of variation of membrane potential. Age of the specimen is given in brackets, when known. When biphasic effects occur, they are listed as multiple effects. Inclusion criteria for the listed studies comprise: (1) recordings performed in the rodent neocortex, (2) knowledge of the morphological type involved and (3) knowledge of the receptor subtype involved in the response. Abbreviations: PC, pyramidal cell; SS, spiny-stellate cell; IN, interneuron; MC, Martinotti cell; BC, basket cell; DBC, double-bouquet cell; NGFC, neurogliaform cell; BPC, bipolar cell, NBC, nest basket cell; RS, regular spiking. PMC, primary motor cortex; V1, primary visual area; PFC, prefrontal cortex; SSC, somatosensory cortex, ACh, acetylcholine; CCh, carbachol; RP, resting potential; NR, not responsive.

TABLE 3 | Cholinergic mediated modulation of neocortical synaptic dynamics.

Connection type	Receptor	Effect	Area	Technique—Reference
L5 PC-L5 PC	Muscarinic	Reduction in depression rate of consecutive EPSPs	Rat SSC	Bath application of 50 μM ACh (Tsodyks and Markram, 1997)
	M1 (perisomatic)	Enhancement of EPSCs	Rat SSC (p14–p16)	1–10 μM ACh local puff (Nuñez et al., 2012)
	M2 (basal dendrites)	Reduction of IPSCs	Rat SSC (p14–p16)	50–100 μM ACh local puff (Nuñez et al., 2012)
	Nicotinic	Increase in EPSPs	Rat SSC (p14–p16)	1–10 μM ACh local puff (Nuñez et al., 2012)
L5 PC-L5 MC	Nicotinic heteromeric	Decrease in onset delay, increase in time course; no change in EPSP size	Mouse S1 and mPFC	Optogenetics or 1 mM ACh bath-application (Obermayer et al., 2018)
L4 PC-L4 PC	M4	Reduction in first EPSP amplitude	Rat SSC	Bath application of 100 μM ACh (Eggermann and Feldmeyer, 2009)
L4 SS-L4 SS				
L4 PC-L23 PC	M4	Reduction in first EPSP amplitude	Rat SSC	Bath application of 100 μM ACh (Eggermann and Feldmeyer, 2009)
L23 PC-L23 PC	M1/M3	Reduction in EPSC amplitude	Rat A1 (p21–p28)	Bath application of 10 μM oxotremorine or muscarine (Atzori et al., 2005)
	Muscarinic (apical dendrite)	Reduction in EPSP amplitude	Rat PFC	Iontophoretic application of 0.05 M muscarine (Vidal and Changeux, 1993)
	Nicotinic (apical dendrite)	Increase in EPSPs	Rat PFC	Iontophoretic application of 0.05 M muscarine (Vidal and Changeux, 1993)
TC fibers-L4 PC	Muscarinic	Increase in EPSP depression rate	Rats and mice TC slice (p21–p28)	Bath application of 5–10 μM muscarine (Gil et al., 1997)
CHAT⁺ fibers—L4 PC	Muscarinic	IPSC	Mouse TC slice (p12–p16)	Optogenetic activation (Dasgupta et al., 2018)
L23 PC-L23 MC	Nicotinic	Increase in EPSPs	Mouse SSC	Bath application of 20 μM CCh (Urban-Ciecko et al., 2018) and optogenetics
L1 NGF-L23 PC	Nicotinic heteromeric	No change in EPSP size	Mouse S1 and mPFC	Optogenetics or 1 mM ACh bath-application (Obermayer et al., 2018)
	M1 (perisomatic)	Connection is silenced (L23 PC is disinhibited)	Rat SSC (p24–p31)	Iontophoretic application or bath application of 100 μM ACh (Brombas et al., 2014)
	Nicotinic (non α7)	Connection is silenced	Rat SSC (p24–p31)	Iontophoretic application or bath application of 100 μM ACh (Brombas et al., 2014)
L23 BC-L5 PC	Muscarinic	Reduction in IPSPs amplitudes (connection is silenced)	Mouse SSC	Bath application of 10 μM muscarine (Kruglikov and Rudy, 2008)
L5 BC-L5 PC	M2/M4	Reduction in u-IPSC amplitude	Mouse SSC	Bath application of 10 μM muscarine (Kruglikov and Rudy, 2008)
	Muscarinic	Reduction in u-IPSC amplitude	Rat insular cortex	Bath application of 10 μM CCh (Yamamoto et al., 2010)
L5 MC-L5 PC	Nicotinic heteromeric	Decrease in onset delay, no change in IPSP size	Mouse S1 and mPFC	Optogenetics or 1 mM ACh bath-application (Obermayer et al., 2018)
L5 BC-L5 BC	Nicotinic	Increase in IPSP size	Mouse A1	Bath application of 10 μM CCh (Hilscher et al., 2017)
L5 RS IN-L5 PC	Muscarinic	Decrease in IPSCs amplitudes	Rat insular cortex	Bath application of 10 μM CCh (Yamamoto et al., 2010)
L5 BC-L5 RS IN	Muscarinic	Increase in first IPSCs amplitudes	Rat insular cortex	Bath application of 10 μM CCh (Yamamoto et al., 2010)
L5 RS IN-L5 RS IN	Muscarinic	Increase in first IPSCs amplitudes	Rat insular cortex	Bath application of 10 μM CCh (Yamamoto et al., 2010)

The table links the distribution and localization (when known, in brackets) of nicotinic and muscarinic receptors across neocortical cell types, with the effect of their activation on synaptic dynamics. Effect is represented in terms of increase or decrease in PSP/PSC size. Age of the specimen is given in brackets, when known. Inclusion criteria for the listed studies comprise: (1) recordings performed in the rodent neocortex; (2) knowledge of the pre and post synaptic morphological types involved; and (3) knowledge of the receptor subtype involved. Abbreviations: PC, pyramidal cell; SS, spiny-stellate cell; IN, interneuron; MC, Martinotti cell; BC, basket cell; DBC, double-bouquet cell; NGFC, neurogliaform cell; BPC, bipolar cell; NBC, nest basket cell; RS, regular spiking. A1, primary auditory area; PFC, prefrontal cortex; SSC, somatosensory cortex; TC thalamo-cortical; ACh, acetylcholine; CCh, carbachol; EPSP, excitatory post-synaptic potential; IPSP, inhibitory post-synaptic potential; EPSC, excitatory post-synaptic current; IPSC, inhibitory post-synaptic current.

the neuroscientific community on which concentration of cholinergic agonists should be used to promote activation of the cholinergic receptors. The applied dose ranges from 10 to 100 micromolar across different experimental groups, and in other cases, it even spans the millimolar range. These discrepancies arise from the fact that to measure the physiological extracellular concentration of ACh is experimentally challenging, because of the prompt intervention of hydrolases in the synaptic cleft. Application of acetylcholinesterase inhibitors cannot be avoided, making it extremely difficult to detect physiological levels of ACh in the extracellular space. Moreover, while mAChR agonists have been extensively used and are known to generate a multitude of responses in cortical neurons, much fewer studies (Hedrick and Waters, 2015; Dasgupta et al., 2018) have discerned muscarinic responses evoked by endogenous ACh release (see **Figures 1, 2**).

Cholecystokinin-immunoreactive (CCK) cells are affected heterogeneously by cholinergic agonists depending on their sizes. For example, small CCK cells are promptly depolarized by cholinergic inputs, while bigger CCK cells show a biphasic response comprising an initial hyperpolarization and a subsequent depolarization similarly to PCs (Kawaguchi, 1997). There is a general consensus (Gulledge et al., 2007; Kruglikov and Rudy, 2008; Poorthuis et al., 2013) that cholinergic modulation of fast-spiking PV positive (PV$^+$) interneurons does not produce any effect on membrane excitability (**Table 1**). However, evidence also shows the opposite. For example, Alitto and Dan (2013) report in their review that PV$^+$ interneurons are depolarized *via* muscarinic activation, but when mAChRs are blocked by antagonist application, the excitation is converted to inhibition; in turn inhibition of PV$^+$ cells is converted to excitation when nAChRs are blocked, suggesting that excitation and inhibition compete in the same population of PV$^+$ interneurons through the activity of the different receptors.

The subpopulation of dendrite-targeting interneurons, that is identified as somatostatin (Sst) expressing (Sst$^+$) interneurons (MCs), can be depolarized by activation of mAChRs (Fanselow et al., 2008). However, some studies report that only very few Sst$^+$ interneurons display excitation or inhibition in response to BF stimulation and that the inhibitory cells displaying the strongest excitation by ACh are L1 and VIP$^+$ interneurons). Recent findings outlined by Muñoz et al. (2017) challenge these results. In their study, they claim that cholinergic modulation of Sst$^+$ interneurons *via* M1 and/or M3 mAChRs provides a major excitatory drive to these cells during whisking activity.

VIP expressing interneurons are highly responsive to cholinergic inputs and show a mixed activation profile that is partially blocked by both nicotinic and muscarinic receptor antagonists (Kawaguchi and Kubota, 1997).

In summary, muscarinic activation has differential effects on membrane potential, based on which subtypes are expressed in a specific cell-type and in cellular compartments. These heterogeneous responses might play different roles in neocortical information processing: the initial hyperpolarizing phase observed in PCs and some CCK$^+$ cells could be used to push the cell away from threshold, while the subsequent depolarization selectively augments inputs that are strong enough to reach

threshold, therefore increasing the SNR, and at the same time promoting synchronization of neural activity. At the same time, the presynaptic inhibition of excitatory feedback could serve as a mechanism to prevent interference during the encoding of new stimuli and reduce top-down influences on perceptive processes. In addition, muscarinic receptors contribute to the generation of the gamma rhythm by inducing synchronized oscillations in both excitatory and inhibitory neurons (Heys et al., 2012).

Another class of receptors contributes to cholinergic signaling in the neocortex. Nicotinic receptors exert fast cortical actions, playing a key role in many cognitive processes (Dani and Bertrand, 2007), as described in the following section (Dani and Bertrand, 2007).

NICOTINIC RECEPTORS

ACh is primarily regarded as a neuromodulator rather than a neurotransmitter in the CNS because its physiological effects have a latency of onset of tens of milliseconds to minutes (Van der Zee and Luiten, 1999). This great variability in the response of cortical neurons to ACh stimulation originates from the fact that there are two main types of ACh receptor proteins. Neuronal nicotinic receptors (nAChRs) are ionotropic receptors which are composed of combinations of twelve different nAChR subunits: α2 to α10 and β2, β3, β4. Each receptor is made of five subunits. It is generally assumed that nicotinic actions are fast and precise; however, the depolarization rate produced by the opening of the nicotinic channel can vary depending on the specific subunit composition. Because mAChR signaling acts through G-proteins, mAChR signaling might be expected to be slower than ionic nAChR signaling. However, homomeric (α7) nAChRs can also mediate slow responses, and the time course of muscarinic action may also vary widely, depending on the signal pathways involved (Muñoz and Rudy, 2014).

The nicotinic branch of the AChR family can be further divided into at least two classes, based on the affinity that their binding sites have for nicotine itself or the snake toxin α-bungarotoxin. At their simplest neuronal nAChRs are homomeric (constituted from five identical subunits) while the more complex forms are heteromeric, composed of at least one α and one β subtype. Binding studies using [^{3}H]-nicotine have shown that high-affinity nAChR binding sites are very common for the human cerebral cortex, while autoradiographic labeling of nAChRs shows an inhomogeneous distribution over architectonically identified cortical areas of the rat brain, with highest concentrations in the medial PFC (mPFC) and generally frontal areas.

As for mAChRs, the expression of different subunit combinations varies across layers and across cortical areas. Given the involvement of the nicotinergic system in the treatment of tobacco addiction, many studies have been performed in the human brain. Most data on the distribution of nAChRs has been obtained from human autopsy tissue homogenates using techniques such as ligand binding, RT-PCR, immunoprecipitation, and Western blot.

Currently-available nAChR agonists and antagonists used for receptor auto-radiography are not subtype specific, although

they act on nAChR subtypes with a distinct profile: labeling experiments carried out with different probes revealed that nAChRs are widely expressed in the cortex, both at the level of gray and white matter; many fibers show immunoreactivity at the neuropil level (Schröder, 1992). Five α subunits (3–7) and three β subunits (2–4) are expressed in the human brain. The expression of α4 and β2 subunits in the frontal cortex, parietal cortex, and temporal cortex shows a characteristic laminar distribution. Higher receptor binding is observed in layers 1, 3 and 5. These results are in agreement with the observed distribution of α3 and α4 mRNAs that are mostly found in PCs of layer 2/3 and layer 5 of the frontal cortex (Wevers, 2011). However, other studies report that the α3 mRNA is exclusively expressed in layer 4, while α4 subunit is moderately expressed in all layers (Radnikow and Feldmeyer, 2018). The α7 subunit is found mostly in layer 1–3 and 5 and is virtually absent in layer 4, while α4 and β2 immunoreactive fibers were observed in layer 4 of the PFC (Sparks et al., 2018). The α2 subunit is a characteristic feature of L5MCs that project to layer 1 and specifically target L5TTPCs (Hilscher et al., 2017). The detection of nicotinic subunits is possible because of the existence of specific antisubunit-antibodies and the introduction of nAChR subunit-Cre mouse lines. Nevertheless, nicotinic receptors are made up of multiple subunits and are either homomeric or heteromeric. The most abundant receptor subtypes in the neocortex are the homomeric receptor α7 and the heteromeric α4β2 channel (which is often associated with the regulatory subunit α5; Radnikow and Feldmeyer, 2018). Nicotinic receptors can be activated both *via* volume transmission and fast synaptic activity (Dani and Bertrand, 2007; Hedrick and Waters, 2015; Hay et al., 2016).

PRE-SYNAPTIC LOCALIZATION

None of the studies mentioned above investigates the precise cellular localization of cholinergic receptors, which is crucial in determining the outcome of the response. This is especially true for nAChRs, because their activation directly leads to a cation influx into the cell, and immediately results in a voltage change in the underlying compartment.

nAChRs are expressed on glutamatergic inputs to layer 5, mostly contacting layer 5 interneurons and L5/L6 PCs. L5PCs and L6PCs are modulated by α7 and β2 nAChRs, respectively, while L23PCs and glutamatergic inputs to these cells do not contain nAChRs. Interneurons across layers contain mixed combinations of nAChRs (Poorthuis et al., 2013). Some subtypes, such as α7 homomeric receptors, are preponderantly expressed in presynaptic areas, whereas heteromeric receptors are more expressed on cell bodies and main dendrites (Bertrand, 2010). Cholinergic axons that diffusely innervate the cortex are thought to make *en passant* connections in the area of the main dendrite of the PCs from layer 5 and VI, therefore causing a volume release of ACh. Pre-synaptically, nAChRs generally increase the release of GABA and glutamate (Dani and Bertrand, 2007). However, both nAChR and mAChRs can reduce EPSPs by acting pre-synaptically (Levy et al., 2006).

POST-SYNAPTIC LOCALIZATION

The distribution of nAChRs at the light and electron microscopic level was studied in the human cerebral cortex using anti-nAChR monoclonal antibody (mAb) WF-6, which is not subunit selective (Schröder et al., 1990): nAChR immunoreactivity revealed a pattern for the frontal and temporal cortex that was very similar to that obtained with the auto-radiography. In the frontal cortex, *in situ* hybridization techniques display numerous labeled neurons, mostly PCs bearing the α7 mRNA in the cell body and in the apical dendrite. In the motor cortex, many PCs showed signals in the proximal part of their apical dendrite.

As reported by Schröder et al. (1989) and Schröder (1992) nAChR localization is predominant in L23 and L5 PCs; a few nAChR-expressing fusiform cells can be detected in layer 4 and VI. Many PCs show nAChRs on basal dendrites that originate in layer 5, cross the superficial layers of the cortex perpendicular to the pial surface, and branch between layers 1 and 2. Immuno-precipitate is detectable both in cell bodies and in their apical dendrites, in branches of various diameters, and in the PSD of synaptic junctions. In a double-labeling approach conducted in the temporal cortex, it was further demonstrated that PV[+] interneurons express α4 and α7 subunit protein (Wevers, 2011). Double-labeling studies have shown that at least 30% of cortical neurons contain both nAChR and mAChR proteins, the majority of these being PCs. In the human cortex, nicotinic immuno-staining in individual neurons appears generally comparable to that seen in the rodent model (Schröder et al., 1989; Schröder, 1992): as in the rat occipital cortex, nAChRs can be detected on the cell bodies and dendrites of L23 and L5 PCs.

Most studies agree that nAChRs are preferentially found in infragranular layers, mostly at the level of L5 and L6PCs, but also at the level of inhibitory interneurons; CB-immunoreactive neurons, as well as PV^{+} neurons all express nAChRs, while that is not true for CR-ir neurons (Coppola and Disney, 2018); furthermore, nAChRs are expressed at the level of layer 2/3 as well, both in PC bodies and in the apical dendrites of deeper-layer placed cells. However, only a small subset of layer 2/3 excitatory neurons and no layer 4 neurons express nAChRs; layer 6 expression profile can be set apart from the rest, given that these neurons predominantly express the slowly desensitizing heteromeric α4β2 channel (Radnikow and Feldmeyer, 2018).

The distribution of nAChRs and the subunits combination, therefore, depends on cell-types, laminar position and on the cortical area studied, similarly to mAChRs; nowadays the possibility of systematically studying the distribution profile of cholinergic receptors has greatly increased, due to the advancement in the production of anti-subunit-specific-antisera and to the development of better immunoprecipitation and ligand binding techniques. Such studies exist and are quite informative as regards, for instance, the striatum (Zoli et al., 2002), but a comprehensive and detailed investigation of the expression of subunits in the neocortex is still lacking. Nicotinic activation prevalently modulates the excitability of deep cortical layers: in the next section, we move on and

explore the contribution of nicotinic stimulation to local circuit properties and examine studies that investigated the involvement of the nicotinergic system in the modulation of neocortical activity.

REGULATION OF NEURONAL AND SYNAPTIC PHYSIOLOGY

Even though nAChRs are predominantly expressed pre-synaptically, where their activation modulates neurotransmitter release through calcium influx or terminal depolarization (Nashmi and Lester, 2006), there is evidence that nAChRs may also influence post-synaptic signaling and that these effects vary based on the subcellular localization of the receptor (**Tables 2**, **3**). nAChRs expressed on distal dendrites are thought to cause the generation of fast excitatory post-synaptic potentials since activation of nAChRs on distal apical dendrites promotes PC depolarization and leads to an increase in action potential firing. On the contrary, activation of nAChRs on the proximal apical dendrites (closer to the cell body) reduces membrane impedance and shunts signal incoming from the apical tuft: when the nAChRs opens, the membrane resistance of the PC decreases and signals incoming from the apical dendrites get attenuated (Dani and Bertrand, 2007).

Optogenetic activation of cortical cholinergic input generates an increase in membrane excitability (**Table 2**) mediated by nAChRs and promotes spiking in L5PCs (Hedrick and Waters, 2015). When the stimulation is paired with additional depolarization, spiking activity becomes persistent and can be blocked by BAPTA application, suggesting that the observed depolarization is mediated by intracellular Ca^{++} transients. As suggested by kinetic analysis it is likely that non-α7 nAChRs determine this response. The depolarizing response spans all layers, but occurs with laminar and regional differences; additionally, the effect of the depolarization can be moderate and transitory or pronounced and persistent depending on the cell membrane potential. Although the modulatory effect was found to be stronger in deeper layers, the authors report that it was similar in M1, V1 and prefrontal (PF) cortices. The preferential modulation of deep neocortical layers is likely to influence the flow of excitation occurring throughout the neocortex that originates in layer 4 and then propagates to the superficial layers, whose role is to modify the output of layer 5. Altogether this study showed that nAChR activation increases the excitability of neocortical PCs; in the light of previous evidence that α4 and α5 subunits are highly expressed in layer 6 (Tribollet et al., 2004), and nAChR-mediated responses in layer 6 of the PFC have already been reported by many studies (Kassam et al., 2008; Bailey et al., 2010; Poorthuis et al., 2013), the authors suggest that the presence of α4 and α5-mediated PSPs could be a characteristic feature of L6PCs across neocortical regions (see **Table 2**, **Figure 1**).

Pyramidal-to-PCs connections in layer 5 can be potentiated by using an spike-timing-dependent-plasticity (STDP) protocol. Bath-application of 10 μM (or 300 nM) nicotine impairs L5PC to L5PC potentiation and favors the induction of LTD. When monitoring spontaneous synaptic events, application of nicotine increases the frequency and amplitude of sEPSCs. Evoked excitatory post-synaptic currents (EPSCs) behave differently and are reduced in amplitude by nicotine. However, puffing nicotine directly on PCs fails to elicit an inward current, and application of gabazine prevents the de-potentiation. Therefore, the effects of nicotine on L5PC to L5PC synapses should be attributed to an enhancement of GABAergic transmission, rather than the direct activation of a PCs (Couey et al., 2007).

nAChRs are known to be distributed throughout the dendritic trees of cortical PCs (van der Zee et al., 1992), but a comprehensive mapping of cholinergic synapses apposition remains elusive. To provide concomitant information on receptor localization while recording electrical responses more researchers should apply the strategy used by Hedrick and Waters (2015), who measured nicotinic PSPs during restricted illumination of the slice: illumination of the tuft dendrites failed to evoke a nicotinic PSP at the soma and therefore the authors concluded that nAChRs that contribute to the somatic depolarization are likely to be within 300 μm of the soma and many are probably located in the proximal 50 μm of the apical and basal arbor. This technique sheds light on the compartmental origin of the observed response and it is immensely useful to causally link the distribution of cholinergic receptors and their physiological role. A subsequent investigation should combine this strategy with pharmacological inactivation of specific receptor subunits and provide further proof that PCs responses to cholinergic inputs in different layers are mediated by specific receptor subunits and that their distribution profile is greatly involved in determining the outcome of neural computations.

Although nAChRs are mainly found on PCs, there is extensive evidence that nAChRs are expressed on the membrane of cortical interneurons (**Table 2**), such as MC, chandelier cells (ChCs) and basket cells (BCs), where they contribute to the modulation of GABAergic signaling (Couey et al., 2007; Wevers, 2011). The subpopulation of serotonin receptor 5-HT3aR expressing GABAergic interneurons is depolarized by ACh *via* nAChRs (Gulledge et al., 2007; Poorthuis et al., 2013); this embryologically distinguished subpopulation, that accounts for about 30% of the total number of cortical inhibitory interneurons, is heterogeneous and includes all the VIP$^+$ interneurons, as well as the VIP$^-$ neurogliaform cells (NGCs; Rudy et al., 2011). VIP$^+$ interneurons show a mixed activation profile in which both nicotinic and muscarinic receptors are involved (**Figure 1**; Kawaguchi, 1997).

Prominent nAChRs expression is a hallmark of layer 1 inhibitory interneurons both in rodents and humans (Letzkus et al., 2011; Alitto and Dan, 2013) and endogenous cholinergic release is known to rapidly recruit this receptor subpopulation during locomotion and attentive processes. These fast, nicotinic responses are mediated by α7 and β2 containing receptors (Poorthuis et al., 2018). When at rest, all layer 1 interneurons are depolarized *via* nicotinic activation (**Figure 1**, **Table 2**); however, when these interneurons are engaged in repetitive firing, ACh inhibits the activity of L1 NGCs (Brombas et al., 2014). Conversely, single bouquet cells (SBCs) are activated by ACh in the regime of repetitive firing (Jiang et al., 2013). Layer

1 interneurons responses are abolished by application of nAChR antagonists (**Figure 1**; Christophe et al., 2002).

ACh enhances the activation of neocortical deep-layers PCs by ascending thalamic inputs *via* mAChR-mediated depolarization and subsequent enhanced glutamate release from thalamocortical terminals in layer 4 (Gil et al., 1997; Metherate and Hsieh, 2004; Disney et al., 2007), but it also releases inhibition on superficial layers PCs. There is extensive evidence that ACh mediates activation of layer 1 and layer 2/3 non-fast spiking PV⁻ cortical interneurons *via* non-α7 nAChRs. These interneurons, in turn, inhibit MCs and BCs that directly target PCs: nAChR-mediated inhibition of superficial interneurons reduces inhibition of superficial PCs (Gulledge et al., 2007; Arroyo et al., 2012; Brombas et al., 2014). Photostimulation of ChAT⁺ neurons in the BF evokes a prolonged disynaptic inhibition in PCs; pharmacological manipulation of the response suggests that it is supported by non-α7 mediated excitation of specific interneurons subtypes. This finding indicates that nicotinic cholinergic input originating from BF fibers is also comprised of a slow component. The observed delayed barrage of inhibitory post-synaptic current (IPSC) in L23PCs exhibits a long latency (of about 26 ms) characteristic of dysynaptic inhibition. Layer 1 and layer 2/3 inhibitory interneurons, and in particular in late-spiking cells and L23 ChAT⁺ bipolar cells are responsible for this phenomenon (Arroyo et al., 2012). In agreement with previous reports (Poorthuis et al., 2014) fast-spiking cells such as BCs and ChCs do not exhibit EPSPs in response to optogenetic stimulation of ChAT⁺ BF neurons, but rather respond similarly to PCs and are swamped by an IPSC barrage as well. While layer 1 and layer 2/3 late spiking cells (LS) exhibit both a fast and a slow response, L23 ChAT bipolar cells display only a slow response. This study demonstrates that the fast and slow components are mediated by α7 receptors and non-α7 receptors, respectively, and that non-α7 receptor-mediated excitation elicits action potentials in cortical interneurons that in turn produce a delayed and prolonged wave of inhibition in L23PCs and FS cells. One proposed explanation for the slow response is that it may arise from a cholinergic bulk transmission and that it may sustain the high metabolic demand of processes such as attention and memory (Cauli et al., 2004). Cortical ChAT⁺/VIP⁺ interneurons have been shown to dilate local microvasculature to increase blood supply during periods of elevated neuronal activity (Kocharyan et al., 2008) during the execution of memory and attention tasks, following electrical BF stimulation. The fast component of the cholinergic response may also be implicated in the emergence of a broader phenomenon like synchronized neuronal activity; it has been shown that LS cells are connected *via* gap junctions, and this fast response may thus play a fundamental role in the emergence of network oscillations that sustain plasticity and attention mechanisms.

Couey et al. (2007) realized that the effect of nicotine on L5PC to L5PC connections is mostly due to an enhancement of GABAergic transmission, and they decided to dissect the effects of nicotine on three different interneurons types. First, they looked at the activity of FS cells in layer 5, and observed no effect when adding nicotine to the bath; later they stained the cells for certain neuropeptides and several nAChR subunits and found an extremely low amount of mRNA coding for nicotinic subunits in FS cells, which might explain their unresponsiveness. Once again, another piece of evidence emerges confirming that (putative) BCs have a tendency not to respond to the application of cholinergic agonists. The authors identified another type of interneuron as a regular-spiking-non-PC (RSNPC), and observed a fast inward current after application of nicotine. LTS cells (putative MC) showed an even bigger inward current response; in both cell-types the most abundantly stained nicotinic subunit was α4, but β2 and α7 were also present. In this study, nicotine application increases the frequency and amplitude of spontaneous EPSCs in putative BCs and MCs; as for putative ChC (RSNP) a decrease in the frequency, but not the amplitude of sEPSCs can be observed (Couey et al., 2007).

Pyramidal to SST⁺ interneurons neocortical connections are relatively weak, but local excitatory input to SST neurons is selectively enhanced during cholinergic modulation of network activity. In a recent 2018 study, it was shown that endogenous ACh release activates presynaptic nAChRs and boosts glutamatergic input in a target-cell specific manner (Urban-Ciecko et al., 2018). Thus, there is evidence that local excitatory input to SST neurons is selectively enhanced during nicotinic modulation of network activity (**Table 2**, **Figure 2**). In a recent study by Obermayer et al. (2018) examined PC-MC-PC disynaptic connections in both layer 2/3 and layer 5 and found that the typical delayed disynaptic inhibitory response in the post-synaptic PC is faster and stronger when cholinergic inputs are activated optogenetically, or by means of 1 mM ACh bath application. When looking at the activity of a single MC, they observed that ACh inputs lead to a significant decrease of the onset delay of AP firing and increases the number of APs fired in MCs, which can account for the earlier onset and prolonged duration of disynaptic inhibition. This effect was abolished by application of 10 μM DHβE demonstrating that it is mediated by heteromeric nicotinic receptors (**Table 2**, **Figure 2**). However, when they recorded from synaptically connected PC-MC pairs during concurrent activation of cholinergic fibers, they could only observe an increase in the membrane depolarization level, but not in EPSP sizes. The same effect was found in MC-PC connections, and this as well was confirmed to be nicotinic in nature, contradicting the result obtained by Urban-Ciecko and others and others. The setups of the two experiments are comparable: both studies were performed in the adult mouse somatosensory cortex. However, the first remarkable difference lies in the nature of the cholinergic input used in the two experiments: while Obermayer et al. (2018) used bath-application of 1 mM ACh and optogenetic activation, Urban-Ciecko et al. (2018) decided to record activity in the presence of 20 μM CCh, a non-hydrolyzable analogue of ACh. Not only the two concentrations differ by two orders of magnitude, but the two cholinergic agonists work in fundamentally different ways. While ACh is almost immediately hydrolyzed by the cholinesterase in the synaptic cleft (within a few milliseconds), carbachol has a much more prolonged effect (Katz and Miledi, 1973). Nevertheless, the results obtained by bath-application of ACh are in agreement with the results achieved by optogenetic activation of the cholinergic system,

which is supposed to be a more physiological way of stimulating cholinergic release (Obermayer et al., 2018).

Interestingly, optogenetic activation of cholinergic inputs did not affect the typical fast disynaptic post-PC response mediated by BCs, which provides yet another example of how BCs tend to be unresponsive to cholinergic release in both layer 2/3 and layer 5, or more generally show a more heterogeneous response profile to ACh inputs (Obermayer et al., 2018). This could be explained by the lack of a precise morphological identification of various subtypes of BCs, which could express cholinergic receptors in different subcellular locations or in a different amount, and therefore show differential responses to ACh inputs. These findings indicate that subcortical neuromodulatory projections recruit nicotinic receptors to alter network function through increased inhibition and provide a potential mechanism by which attention controls the gain of local circuits.

NICOTINIC AND MUSCARINIC KINETICS

What are the receptor affinities to various agonists and can this be related to the actual amount of nicotinergic modulation? The relative activation of receptors vs. the concentration of agonist has been measured (**Table 4**).

Muscarine reversibly reduces Ca^{2+} currents in a dose-dependent manner. The modulation is rapid, with an onset time constant of 1.2 s. A slowly developing component of the modulation also is observed, with a time constant of 17 s. Under elevated Ca^{2+} conditions, the fast component is due to a reduction in both N- and P-type calcium currents, whereas the slow component involves L-type current (Stewart et al., 1999). Receptor properties such as conductance, open time, and sensitivity to ACh depend on the nicotinic subunit composition (**Table 4**). (α4)2(β2)3 nAChRs are sensitive to micromolar scale changes, while (α7)5 receptors have a half-maximal sensitivity of more than a hundred micromolar. Extracellular choline is normally 3–5 μM but can attain 20 μM in some pathological cases. However, ACh reaches the millimolar range at the site of release (Alkondon and Albuquerque, 2004). Responses mediated by α_7 nAChRs are short-lasting, whereas those mediated by $\alpha_4\beta_2$ nAChRs are long-lasting. This is because the mean open time of α_7 nAChRs is shorter than that of $\alpha_4\beta_2$ nAChRs. Also, α_7 nAChRs desensitize much faster than $\alpha_4\beta_2$ nAChRs (Alkondon et al., 1999).

An interesting hypothesis was put forward by Albuquerque et al. (2000). α_7 but not $\alpha_4\beta_2$ nAChRs can be fully activated by choline (Nguyen et al., 1995; Alkondon et al., 1999). Choline and acetate are the products of hydrolyzation of synaptically released ACh by ACh-esterase in the synaptic cleft. This process occurs quickly, but reuptake of choline into presynaptic terminals is slow. Therefore, the ACh concentration in the synaptic cleft should decay rapidly, with only low levels of diffusing ACh reaching peri-synaptic sites. But choline levels should rapidly rise in the synaptic cleft with high levels of diffusing choline reaching peri-synaptic sites. This implies that extrasynaptically located α4β2 nAChRs (i.e., the high affinity nAChRs) could be activated by diffusing, low levels of ACh, extrasynaptically located while low-affinity α7 nAChRs may be activated by diffusing choline.

TABLE 4 | Nicotinic homomeric and heteromeric receptors kinetics.

Receptor type	Single channel conductance	Open time	PO$_{max}$	EC50 for ACh or nicotine	Kinetics
Nicotinic heteromeric (α3)2(β4)3	29 pS	0.71 ± 0.14 and 3.5 ± 0.4 ms			
Nicotinic heteromeric (α3)2(β4)3 (α3β2)2α5	29 pS (Stetzer et al., 1996), 18.2 ± 0.46 (Rovira et al., 1998)	147 ms (Stetzer et al., 1996)		EC50 ACh 1.70–1.83 μM for ACh EC50 Nicotine 2.91 μM IC50 Nicotine 2.92 μM (Kuryatov et al., 2011) EC50 Nicotine 4.64 (Kuryatov et al., 2011) IC50 Ach 115–122 μM EC50 Nicotine 16.7 μM (Kuryatov et al., 2011)	Fast: 40–121 ms; slow: 274–1039 ms (Figl and Cohen, 2000)
(α3β4)2 α5					Fast 4–6 ms; slow 30–53 ms (Figl and Cohen, 2000)
Nicotinic heteromeric (α4)2(β2)3	31.3 pS, 40.5 pS (high state) and 21.9 pS (low; Hales et al., 2006)	207 ± 38 ms (Hsiao et al., 2008)	0.8 (Li and Steinbach, 2010)	High affinity is 1.6 μM, low affinity is 62 μM (Buisson and Bertrand, 2001) EC50 for ACh in activating α7 is 3 μM (Albuquerque et al., 2000)	
Nicotinic heteromeric (α4β2)2 α5				EC50 ACh 1.44–1.64 μM for variants tested EC50 Nicotine 0.62 μM IC50 Nicotine 0.0872 μM (Kuryatov et al., 2011)	
Nicotinic homomeric (α7)5	82.9 pS (Albuquerque et al., 2000)	108 μs and 92.7 μs for channels activated by 11 and 10 mM Ach, respectively (Albuquerque et al., 2000)		Choline: EC50 1.6 mM; IC50 37 μM (Alkondon and Albuquerque, 2004). 200 μM ACh (Buisson and Bertrand, 2001). EC50 for ACh in activating α7 130 μM (Albuquerque et al., 2000)	

The table lists properties of nicotinic homomeric and heteromeric receptors (single-channel conductance, open time and open probability and EC50 and kinetics).
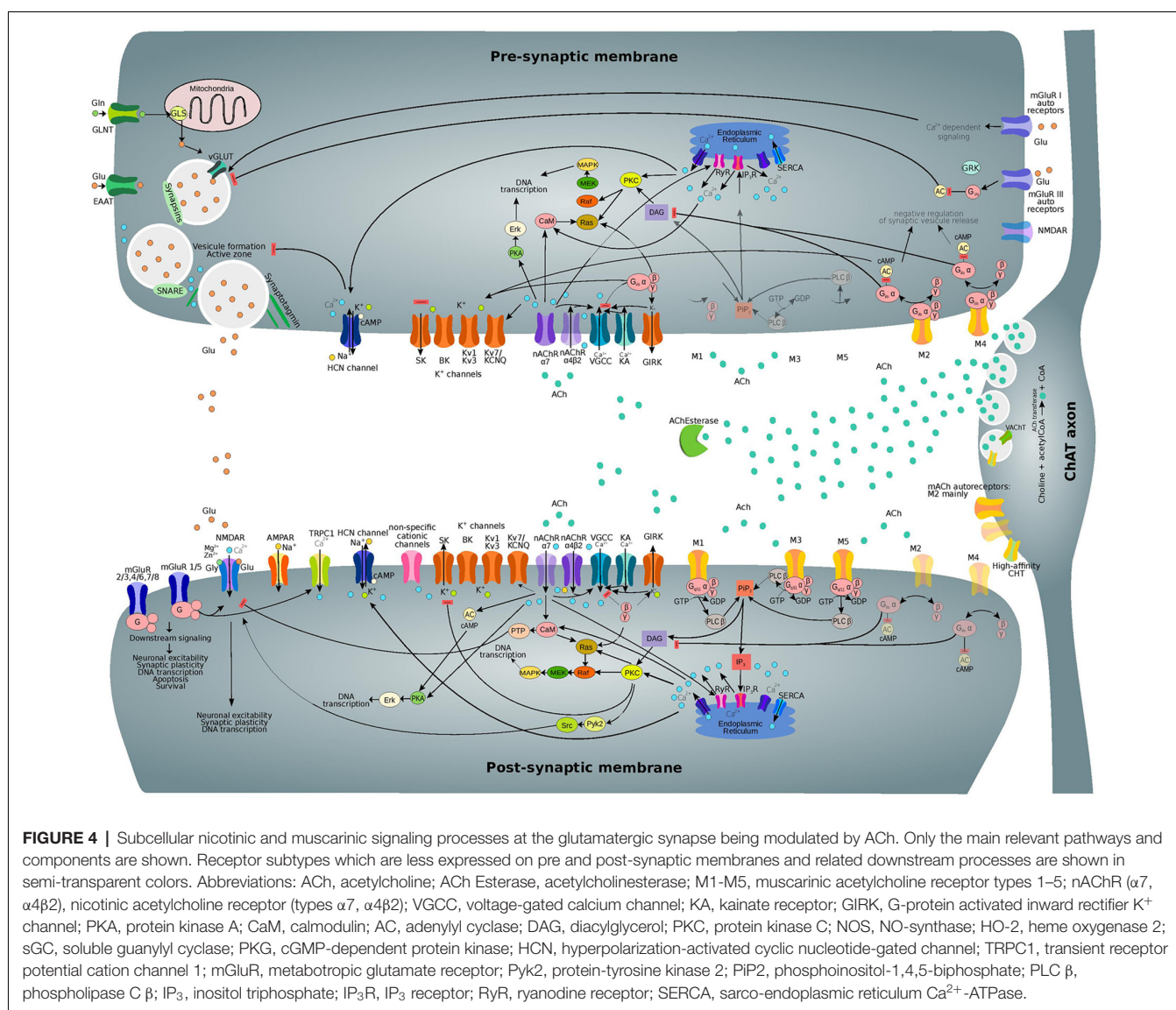
Thus, α7 and α4β2 nAChRs might exhibit differential control (Albuquerque et al., 2000).

SUBCELLULAR NICOTINIC AND MUSCARINIC PATHWAYS

ACh affects membrane conductance through several subcellular pathways, as illustrated in **Figure 4**, leading to both hyperpolarizing and depolarizing effects (**Tables 1**, **2**). ACh can act on both pre and post-synaptic membranes, binding to muscarinic and nicotinic receptors. The interplay among intracellular pathways leads to a dynamically changing outcome, such as the transient hyperpolarization and following long-term depolarization resulting from the binding of ACh to M1 mAChR (Dasari et al., 2017). When ACh interacts with M1, the exchange of coupled GDP for GTP produces the dissociation of the G-protein complex from the receptor. The released α subunit of the Gq protein then activates

the enzyme phospholipase C (PLC β) which hydrolyzes phosphatidyl-inositol 4,5 bisphosphate (PIP_2), leading to its dissociation from the membrane and the subsequent formation of diacylglycerol (DAG) and IP_3. IP_3 initiates calcium ions release from the endoplasmic reticulum (ER), serving as a trigger for this process. Refilling of the ER with Ca^{2+} ions is then obtained by the activity of the sarco-ER Ca^{2+}-ATPase (SERCA). Extracellular calcium ions are therefore crucial for the maintenance of calcium cycling. M1 activation facilitates voltage-dependent refilling of calcium stores by promoting excitation. Thus, fine-tuned calcium dynamics govern complex reciprocal relations among many different proteins contributing to changes in membrane potential. Ultimately, changes in K^+, Ca^{2+}-activated K^+-currents and non-specific cationic currents support a shift from transient hyperpolarization to a sustained excitation.

Meanwhile, DAG together with Ca^{2+} ions activate kinases such as protein kinase C (PKC), causing multiple downstream

FIGURE 4 | Subcellular nicotinic and muscarinic signaling processes at the glutamatergic synapse being modulated by ACh. Only the main relevant pathways and components are shown. Receptor subtypes which are less expressed on pre and post-synaptic membranes and related downstream processes are shown in semi-transparent colors. Abbreviations: ACh, acetylcholine; ACh Esterase, acetylcholinesterase; M1-M5, muscarinic acetylcholine receptor types 1–5; nAChR (α7, α4β2), nicotinic acetylcholine receptor (types α7, α4β2); VGCC, voltage-gated calcium channel; KA, kainate receptor; GIRK, G-protein activated inward rectifier K^+ channel; PKA, protein kinase A; CaM, calmodulin; AC, adenylyl cyclase; DAG, diacylglycerol; PKC, protein kinase C; NOS, NO-synthase; HO-2, heme oxygenase 2; sGC, soluble guanylyl cyclase; PKG, cGMP-dependent protein kinase; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; TRPC1, transient receptor potential cation channel 1; mGluR, metabotropic glutamate receptor; Pyk2, protein-tyrosine kinase 2; PiP2, phosphoinositol-1,4,5-biphosphate; PLC β, phospholipase C β; IP3, inositol triphosphate; IP3R, IP3 receptor; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase.

effects. PKC controls the function of many proteins including members of both pre and post-synaptic membranes. PKC is also involved in synaptic plasticity regulation and causes the internalization of AMPARs and NMDARs, leading to LTD phenomena (Callender and Newton, 2017).

PKC can also phosphorylate metabotropic glutamate receptor 5 (mGluR5; Hwang et al., 2005) as well as many other proteins. Moreover, PKC activates heme-oxygenase 2 (HO-2; Artinian et al., 2001) and inhibits NO-synthase (NOS), interfering with the calcium/calmodulin activation of NOS enzyme (Borda et al., 1998). These effects contribute to the downstream processes involving carbon monoxide (CO) and nitric oxide (NO) as interacting messengers (Mathes and Thompson, 1996; Artinian et al., 2001). Long-term effects of PKC activation include changes in DNA transcription that are mediated by MAPK/Erk signaling. Furthermore, there is recent evidence for the direct interaction of M3 mAChR with PLC β, which increases signaling efficiency (Kan et al., 2014).

The downstream signaling pathways of M3 and M5 receptors overlap with that of M1, and therefore they are grouped as M1-like receptors; similarly, M2-type mAChRs comprise both M2 and M4 receptors. Binding of ACh to M2-type mAChRs results in the inhibition of adenylyl cyclase (AC) by the α subunit of $G_{i/o}$ protein and in the subsequent reduction of cAMP levels (Muñoz and Rudy, 2014). However, there are some differences between the G_i and G_o mechanisms of AC regulation (Jiang and Bajpayee, 2009). The βγ-complex of the dissociated G-protein can activate the G-protein activated inward rectifier K^+ channels (GIRK) and inhibit voltage-gated calcium channels (VGCCs). Moreover, G_o proteins can also regulate Na^+ channels (Jiang and Bajpayee, 2009). Particular effects of M1 and M2 receptors on different ion channels have been already summarized by Thiele et al. (2012).

A significant increase in intracellular calcium concentration comes from the direct flow of ions due to the permeability of nAChRs to Ca^{2+}. However, nAChR activation also leads to the activation of VGCC and subsequent Ca^{2+} influx. (Dajas-Bailador and Wonnacott, 2004; Shen and Yakel, 2009). Moreover, functional cross-talk among presynaptic nAChRs has been shown to affect signal transduction (Marchi and Grilli, 2010). Therefore, the action of one receptor might depend on the function of co-existing receptor subtypes in the same cell. The interaction between presynaptic nicotinic receptors with other ionotropic or metabotropic receptors serves the purpose of producing an integrated response.

TRANSCRIPTOME CELL-SPECIFIC PREDICTION OF CHOLINERGIC RECEPTORS

In recent years, a wealth of transcriptomic data from the mouse brain has become available (Saunders et al., 2018; Zeisel et al., 2018). Many different cell types may exist; one study found 565 different cell groups, for example (Saunders et al., 2018). Since a standard classification of cortical cell types is still emerging, most articles employ different approaches to arrive at cell type specific transcriptomes.

We examined a representative data set from the somatosensory cortex in order to interpret possible cell-specific differences in cholinergic receptor expression (**Figure 5**). We chose this data set since excitatory cell types are mapped to layer-specific types, allowing the easiest comparison with the types referenced in this review. In this dataset, normalized expression of M1 receptors is highest in L4 PCs. There is a strong expression of M2 in deep layer neurons, particularly in layer 5a. M3 is highly expressed in layer 2/3 and layer 5a, while M4 is highest in layer 4. α3 nAChR subunits are highest in layer 4, but also in the deep layers. β subunit expression is highest in layer 6 and layer 6a neurons. Inhibitory interneuron expression of cholinergic receptors is definitely cell-type specific, though heterologous. PV cells express more nAChRα3 than do somatostatin-expressing interneurons (**Figure 5B**). Somatostatin expression is best correlated with M2 expression and nicotinic β subunit expression and negatively correlated with M1 expression (**Figure 5C**). VIP and Htr3a expression is correlated with nAChRα3, nAChRα4, and nAChRα5. Furthermore, ChAT expression is correlated with M1 expression. In layer 5a, the effects of the predominantly-expressed nAChR and mAChRs seemed to be synergistic.

We also examined an additional dataset for frontal cortex (**Figure 5E**; Saunders et al., 2018). M5 is expressed in a subset of interneurons, including some cholinergic and MCs. The nicotinic receptor Chrna5 is expressed in a subset of deep PCs. Chrna6 is most expressed in a particular type of layer 5 PC. This dataset illustrates that the degree of sub-classification of PCs is likely to be important. For example, there are many subtypes of L5PCs, which have different cholinergic receptor expression. Both datasets showed consistency in M3 expression in L2/3 and L5a PCs but not L4 and L5 PCs.

In addition to cell-type specific correlation, nAChR genes that encode heteromeric α/β subunits are well correlated among themselves (Zoli et al., 2015; Saunders et al., 2018). The genes encoding the α subunits correlate well with the corresponding β subunit.

Cholinergic neurons can be identified by cluster analysis (Zeisel et al., 2018). In particular, separate types have been identified in the red nucleus and habenular nucleus of the thalamus (ibid). ACh often is released in neurons releasing other neurotransmitters (Zeisel et al., 2018). In the habenular nucleus, the glutamate transporter Slc17a6, in cholinergic cells, suggesting co-release of glutamate and ACh (Mancarci et al., 2017). In the ventral midbrain, a neuron type that was both dopaminergic and cholinergic was identified (Zeisel et al., 2018). Many forebrain cholinergic neurons also are GABAergic (Mancarci et al., 2017), consistent with the co-release of these two substances (Saunders et al., 2015).

GLOBAL NETWORK EFFECT AND MODULATION OF BRAIN STATES

The transition between different brain states that occurs whenever an organism switches from one behavioral state to
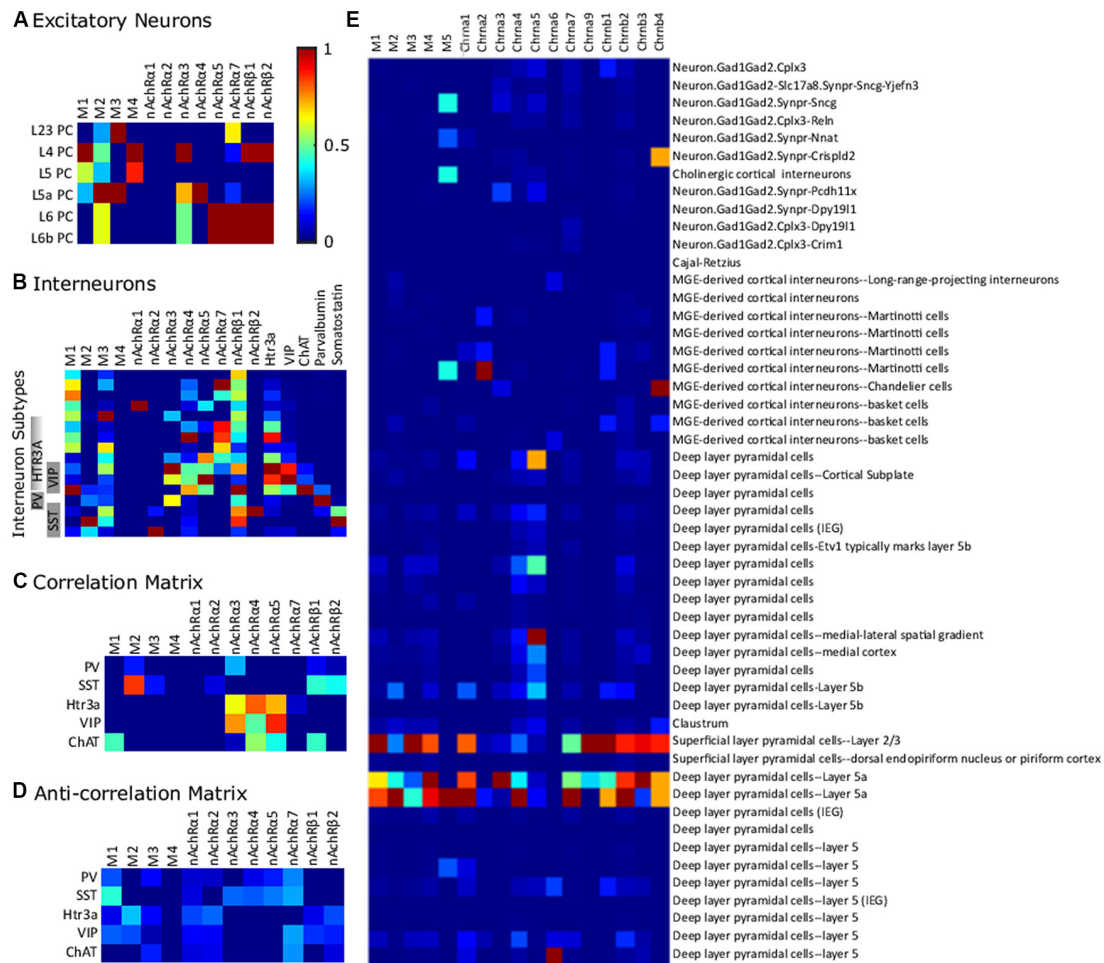
FIGURE 5 | Differential expression of cholinergic receptors in transcriptome-derived cell types. **(A)** Excitatory cell types. **(B)** Interneurons in somatosensory cortex. Gene expression is normalized to a maximum of 1 on a gene-by-gene basis. **(C)** Correlation matrix (positive values of correlation matrix Pearson correlation coefficient matrix). **(D)** Anti-correlation matrix (negative values of correlation matrix). The data is from Zeisel et al. (2018) and was collected with high-throughput single-cell RNA sequencing, a method which counts individual RNA molecules. Abbreviations: PV, parvalbumin; SST, somatostatin; VIP, vasointestinal peptide; ChAT, choline acetyltransferase. **(E)** Expression of ACh receptor genes across the Frontal cortex cell-clusters identified in Saunders et al. (2018). The data was collected using Drop-seq (a method which allows the use of older animals and elimination of certain technical artifacts) to profile the RNA expression of individual cells. Semi-supervised independent component analysis was used to group cells into the sub-clusters using network-based clustering (ibid). Expression levels were normalized to the highest expression across all the selected genes. In this data set, receptor expression was particularly high in L23 and L5a PCs.

another is associated with changes in the overall pattern of neural activity, which can be captured with EEG or LFP recordings. The pattern of EEG activity can change dramatically with the behavioral state of the animal (Lee and Dan, 2012), as can be seen in the transition from slow-wave sleep to wakefulness (or from deep sleep to REM sleep), when the EEG pattern shifts from large and synchronous waves of neural activity to a more desynchronized and short-amplitude wave pattern (Berger, 1929). Ensemble neuronal activity undergoes impressive changes during behavioral state transitions, and different brain states have been associated with different brain functions; definitive evidence for these functions although, is still lacking, and the mechanism by which these transitions are achieved in the cortical network is not yet understood. Many authors have proposed that the switch between cortical states may be driven by the action of neuromodulators like ACh (Lee and Dan, 2012). However, precisely how these neuromodulators influence global cortical processing by locally targeting specific cells is largely an unsolved mystery.

BASAL FOREBRAIN MODULATION OF BRAIN STATES

A large body of evidence suggests that the BF, a complex and heterogeneous structure classically defined by the presence of clusters of cholinergic neurons, is crucial for the maintenance of the sleep/wake cycle and for processes that underlie arousal and attentional modulation, but it is unclear which BF neurons promote each brain state and how they interact with each other to regulate transitions between states (Anaclet et al., 2015). Already

since 1930, it was known that BF lesions could cause severe insomnia (Saper et al., 2001); however, this evidence has been an object of constant challenge over the years, and the attempts to replicate this experiment would yield different results. Finally, Szymusiak and McGinty (1986) observed that sleep-active cells were confined to the ventral BF in the cat (the horizontal limb of the diagonal bands of Broca, substantia innominata, entopeduncular nucleus and ventral globus pallidus) and that these areas partially overlap with those where chemical and electrical stimulations evoke sleep, and where lesions suppress sleep. The sleep-active cells were thus considered optimal candidates for mediating some of the sleep-promoting functions attributed to the BF (Szymusiak and McGinty, 1986).

Many BF neurons are active during wake and during REM sleep (Lee and Dan, 2012), and specific lesions reduce wakefulness, in agreement with the finding that BF lesions cause significant increases in delta waves occurrence during wakefulness, and that BF stimulation induces cortical desynchronization of EEG or LFP signals, accompanied by a decrease in correlated spiking. Furthermore, the BF receives inputs from the LDT and PPT pontine nuclei; cholinergic neurons that can be found at the level of the LDT nucleus exhibit an increase in firing rate during cortical activation, just before the transition from slow-wave sleep frequencies to faster frequencies (Saper et al., 2010).

Therefore, it seems reasonable to hypothesize the existence of functionally diverse neurons in the BF: according to Duque et al. (2000), BF cells that exhibit different wake/sleep activity pattern, also express different molecular markers (Zaborszky and Duque, 2000). There are three major neuronal types in the BF: cholinergic, glutamatergic and GABAergic cells (Anaclet et al., 2015; Xu et al., 2015). There might be extensive local synaptic interactions among BF neurons mediating local reciprocal inhibition between GABAergic neurons and sleep-active and wake-active cholinergic neurons. The well-known flip-flop circuit for sleep/wake cycle control (Saper et al., 2010) could, therefore, comprise multiple loops and switches. However, some findings suggest that BF GABAergic neurons provide major contributions to wakefulness, while cholinergic and glutamatergic neurons appear to play a lesser role; chemogenetic activation of GABAergic neurons promotes wake and high-frequency EEG activity, whereas cholinergic or glutamatergic activation have a destabilizing effect on slow-wave-sleep (SWS), but has no effect on total wake (Anaclet et al., 2015).

Cholinergic neurons residing in the BF can be divided into two subpopulations, that might be involved in different functions: an early-spiking population may reflect phasic changes in cortical ACh release associated with attention, while the late-spiking group could be more suited for the maintenance of the cholinergic tone during general cortical arousal (Unal et al., 2012).

MULTI-TRANSMITTER NEURONS: ACh AND GABA CO-TRANSMISSION

Nevertheless, functional co-transmission of ACh and GABA seems to be a common feature of nearly all forebrain ACh-producing neurons (Henny and Jones, 2008; Granger et al., 2016). BF inputs to the neocortex are therefore not only constituted of different fibers, but also use a mixture of functionally diverse neurotransmitters (Kalmbach et al., 2012). This opens the question of whether there is a substantial difference between the cholinergic modulation and the BF modulation of neocortical activity. The contribution of GABA needs to be considered when studying the functional impact of ACh-producing neurons: electrical stimulation of BF fibers might evoke markedly different responses than optogenetically-evoked selective cholinergic release.

Does the co-release happen in a target-specific modality, at different terminals branching from the same axon, or is the release site the same for both transmitters? And if so, how does GABA affect the ongoing cholinergic modulation? Release of an excitatory (ACh) and inhibitory (GABA) neurotransmitter by the same axons seems to be functionally antagonistic. However, both transmitters could act in parallel, depending on the mode of co-transmission (Granger et al., 2016). If both ACh and GABA are released simultaneously onto the same post-synaptic cells, then GABA may act to shunt the (supposed) excitation generated by ACh. Otherwise, they could target different postsynaptic cells, such that GABA inhibits one cell population while ACh excites another. Given previous experimental results showing that GABA release from VIP interneurons shunts activity of Sst$^+$ interneurons, but not other VIP interneurons, it is thought that VIP/ChAT cortical interneurons may release ACh and GABA onto different post-synaptic targets, perhaps from separate synaptic vesicle populations (Granger et al., 2016). Indeed, a recent analysis of the molecular composition of the pre-synaptic terminals of cortical VIP/ChAT interneurons revealed that ACh and GABA vesicles are confined to separate boutons. At the post-synaptic level, the subset of GABAergic boutons seems to contact prevalently other inhibitory interneurons, while ACh boutons target mostly L1 interneurons and other VIP/ChAT cortical interneurons. Here, ACh evokes EPSCs that are mediated by nicotinic receptors (Granger et al., 2018). Another recent study conducted in the mPFC confirms that only 10%–20% of post-synaptic targets of VIP/ChAT cortical interneurons are contacted by both cholinergic and GABAergic inputs (Obermayer et al., 2018); here they report that VIP/ChAT neurons directly excite interneurons in layers 1–3 as well as PCs in L2/3 and L6 by fast nicotinic transmission.

Immunolabeling studies (Beaulieu and Somogyi, 1991) have shown substantial co-labeling of presynaptic cholinergic terminals for both GABA and ChAT in the neocortex, but more studies should address the functional consequences of the synaptic co-release of these neurotransmitters and try to dissect the differential impact of each transmitter on postsynaptic cells excitability. Analysing the co-localization of post-synaptic receptors or scaffolding proteins could also allow the identification of individual synapses that are sensitive to both ACh and GABA. These possibilities should be addressed systematically in order to precisely understand the contribution of each neurotransmitter to cortical processing.

ACh INVOLVEMENT IN NEUROPLASTICITY

Apart from the fine-tuning of sleep/wake transitions, cholinergic neuromodulation is tightly implicated in regulating selective attention to a given sensory stimulus by altering the activity of the sensory cortex that perceives that modality (Kim et al., 2016). ACh is known to be especially involved in cortical arousal (Saper et al., 2010) and in the state-dependent modulation of cortical activity; cholinergic neurons are active during locomotion (Buzsaki et al., 1988) and during transition to the attentive state (Kim et al., 2016). Studies have shown that the occurrence of relevant sensory events evokes a transient increase in ACh concentration in the rat PFC (Hasselmo and Sarter, 2011). Conversely, activating cholinergic transmission in the PFC determines an improvement in subject's performance during sustained attention tasks (Saper et al., 2010). It is, therefore, reasonable to hypothesize that ACh can induce long-lasting changes in neuronal excitability, and indeed this was demonstrated. Pioneering experiments showing that ablation of noradrenergic and cholinergic innervation in the striate cortex substantially impairs ocular dominance plasticity in kittens (Bear and Singer, 1986) opened the way for subsequent studies on the involvement of ACh in cortical plasticity. Some showed that when a tone is paired with NBM stimulation or ACh application, auditory cortex receptive fields change and prolonged enhanced responses to the paired frequency can be observed (Metherate and Weinberger, 1990; Rasmusson, 2000). Others discovered that co-application of muscarinic agonists with glutamate induces a prolonged increase in response to glutamate in somatosensory cortical neurons (Sugihara et al., 2016), and that these effects concern as well the somatosensory cortex and the primary visual area V1. According to Metherate and Weinberger (1990), the potentiation can be blocked by cortical application of atropine, but others (Sugihara et al., 2016) report that cholinergic antagonists cannot reverse the prolonged changes, thereby confirming that ACh is necessary for the induction, but not the maintenance of these modifications. ACh seems to act more as an instructive, rather than a permissive signal (Lin et al., 2015).

ACh is as well involved in the generation of LTD at synapses between cortical pyramidal neurons and striatal medium spiny neurons through disinhibition of Ca_v channels. Here, the activation of D_2 receptors reduces basal ACh release from cholinergic striatal interneurons and lowers M_1 receptor tone in medium spiny neurons, which leads to enhanced opening of intraspine $Ca_v1.3$ Ca^{2+} channels in response to synaptic depolarization. The calcium transient results in enhanced production of endocannabinoids (ECs) such as 2-arachidonoylglycerol, and activation of presynaptic CB_1 receptors that reduce glutamate release (Wang et al., 2006).

Furthermore, the role of several neuromodulatory systems in STDP induction (Pawlak et al., 2010) has been studied across multiple brain areas. While dopamine (DA) and NA modulation of STDP has been mostly investigated in subcortical areas, ACh's role in STDP induction has been extensively researched in neocortical sensory areas and in the PFC. In mouse mPFC,

nicotine application increases the threshold for STDP in L5PCs by reducing their dendritic calcium signals. This effect, however, is due to an enhancement in GABAergic transmission in various types of interneurons in the PFC network, that express multiple types of nAChRs (Couey et al., 2007), and not to a direct nicotinic action on PCs.

Taken together, evidence suggests that cholinergic inputs to the cortex incoming from the BF should be viewed more as teaching, rather than motivational signals. Overall, activation of the cholinergic system controls the shift from a correlated or synchronized state, to a decorrelated or desynchronized state and results in an enhancement of cortical information processing (Lee and Dan, 2012). However, exactly how the detection of relevant stimuli is enhanced and which are the mechanisms at the basis of this ACh-induced desynchronization are still a matter of open debate.

ACh ENHANCEMENT OF SENSORY PROCESSING

NBM stimulation has a differential effect on spontaneous and sensory-evoked activity. In a recent study, Meir et al. (2018) showed that NBM stimulation desynchronizes cortical LFP and increases the SNR of sensory-evoked responses while suppressing ongoing spontaneous synaptic activity. The authors recorded spontaneous PSPs occurring in L4 and showed that following NBM stimulation the frequency and amplitude of sPSPs were decreased. Moreover, the mean membrane voltage of the response became more hyperpolarized, and trial-to-trial variability was decreased, both during spontaneous and evoked activity. However, sensory stimulation did not change the amplitude of the response, whereas it caused a prominent reduction in the noise amplitude, therefore changing the SNR of the sensory response. By analyzing the coupling of Vm and LFP signals, they also showed that cholinergic activation largely reduced fluctuations in the membrane potential and caused a decorrelation in network activity.

Chen et al. (2015) were able to identify a defined microcircuit in the superficial layers of mouse V1 that supports ACh driven desynchronization. The authors measured the activity of different inhibitory interneurons while optogenetically stimulating superficial cholinergic axons, and found that cholinergic inputs facilitate Sst^+ interneurons, which in turn inhibit PV^+ interneurons and PCs. Optogenetic inhibition of Sst^+ neurons blocks desynchronization, whereas direct activation of Sst^+ neurons is sufficient to induce desynchronization (Chen et al., 2015). The observed desynchronization in cortical activity may explain the role of ACh in mediating transitions between phases of the sleep-wake cycle, but it fails to explain how ACh enhances sensory processing. A large body of evidence suggests that ACh enhances sensory inputs while simultaneously suppressing intrinsic cortical activation (Kimura et al., 1999; Disney et al., 2007; Newman et al., 2012), but a detailed understanding of this process is currently lacking. ACh's role may substantially differ across sensory areas and affect different tuning properties.

Nucleus basalis activation affects sensory responses to natural stimuli of a population of cortical neurons. Before BF stimulation, multi-unit activity (MUA) in the rat's V1 is highly correlated but poorly time-locked to the stimulus; after BF stimulation it becomes less correlated but more time-locked to the sensory event. NBM stimulation also decreases single-unit activity (SU) correlation (between cells correlation) and increases response reliability (between trials correlation coefficient) but does not induce any significant change in receptive field size, orientation tuning nor direction selectivity. Atropine application decreases NBM induced decorrelation, indicating that mAChRs support this effect (Goard and Dan, 2009). After NBM stimulation a shift in the firing modality of the LGN resembling that found at the level of the thalamus can be observed, namely a transition from burst to tonic mode (Bazhenov et al., 2002; Castro-Alamancos and Gulati, 2014). A similar study (Thiele et al., 2012) was conducted in the extrastriate cortex of the macaque and yielded opposing results: at the level of the middle temporal (MT) area it revealed how other tuning properties, like orientation and direction discriminability, are also affected by cholinergic modulation; in this case, ACh had little effect on response reliability, though it is still not clear whether these differences are attributable to differences existing between rodents and primates or to functional differences between sensory areas. In an effort to clarify the precise role of neocortical cholinergic modulation, Disney et al. (2007) concentrated on the role of nAChRs in a well-studied cortical model system, the V1 of the macaque monkey. Here they showed *in vivo* that nicotine reliably enhances the gain of responses to visual stimuli in layer 4c, but not in other layers. Having found β2-nAChR in a pre-synaptic position at the level of thalamo-cortical synapses on PV$^+$ interneurons, they prove that nicotine enhances detection of visual stimuli through enhanced TC transmission. These findings confirm that cholinergic activation causes an increase in cortical sensory responses through enhancement of thalamic synaptic transmission and suppression of intracortical inputs. A systematic effort to extend these results to other sensory areas is therefore needed in order to decipher whether the mechanism supporting cholinergic modulation is common throughout all cortical areas or if different tuning properties are affected each time.

ACh MODULATION OF THALAMO-CORTICAL TRANSMISSION

Castro-Alamanco and Gulati recorded, multi-electrode activity (MUA) and field potential from adult rat barrel cortex following multi-whisker stimulation at 0.2 Hz, while increasing concentrations of carbachol or other drugs were applied by means of micro-dialysis. The authors found that the application of 50 μM carbachol, but not norepinephrine, can stop the emergence of the 10–15 Hz oscillations that are observed during baseline recordings and that in the presence of atropine these oscillations are even enhanced (Castro-Alamancos and Gulati, 2014). The effect of carbachol on barrel cortex LFP is thus congruent with the traditionally termed desynchronization

for doses higher than 50 μM (Moruzzi and Magoun, 1949; Steriade et al., 1993). A low tone of cholinergic activation (0.5–1 μM) however, reinforces the deactivated cortical state by enhancing synchronous slow oscillations. A very high tone of cholinergic activation (250–2,500 μM) leads to a significant increase in tonic firing, without altering the overall firing rate. An interesting follow-up to this experiment would be to check whether the same effect can be observed in the whole somatosensory region, and across other sensory cortices. The group then tried to decipher whether cholinergic activation would also modulate thalamocortical activity: by recording from the VPM, they found that cholinergic cortical activation suppresses burst-firing in the thalamus and changes neuronal firing to a tonic mode. This result is fairly consistent with the outcome predicted by the model of thalamo-cortical slow-wave sleep oscillations and transition to activated states generated by Bazhenov et al. (2002). Here, the increase in ACh activity was modeled by the reduction of a K$^+$ leak current in pyramidal and thalamo-cortical cells and resulted in the abolishment of the hyperpolarizing phase of network activity and a consequent increase in the input/resistance relationship, accompanied by a switch to the tonic firing (15–20 Hz) modality. The transition from bursting to tonic firing thus seems to be a characteristic feature of relay diencephalic structures like the thalamus and the meta-thalamus.

Enhanced thalamo-cortical transmission seems to be a constant finding across a vast number of articles and reviews (Bazhenov et al., 2002; Disney et al., 2007; Hasselmo and Sarter, 2011) with the aim of revealing the mechanisms by which cholinergic neuromodulation operates. Next studies in this field should, therefore, consider the possibility that cholinergic inputs reach the cortex not only through direct BF projections but also exploiting the thalamo-cortical loop.

Voltage-sensitive dye imaging revealed that ACh application to the neocortex, upon stimulation of layer 2/3, suppresses the spread of excitation to nearby areas. Thus, ACh seems to play an important role in coding sensory stimuli by enhancing thalamocortical inputs, but at the same time, by suppressing intracortical interactions (Kimura et al., 1999).

One of the proposed models for the cholinergic mediated shift from default mode to detection mode suggests that ACh acts to enhance the glutamatergic representation of thalamic input through stimulation of nAChRs, while suppressing the cortical spread of associational input through activation of mAChRs (Hasselmo and Sarter, 2011). Minces et al. (2017) recently evaluated the effect of increases in cortical ACh following optogenetic BF stimulation on the correlation structure of the visual network and found that transient cholinergic release in the cortex decreases the slope between signal and noise correlations. The authors propose that this mechanism acts to increase the encoding capacity of the network.

Another article evaluated the impact of ACh on local circuit activation and found that cholinergic inputs exclude unreliable neurons from contributing to circuit activity while conserving neurons that were active in response to thalamic activity and showed strong correlations. Moreover, weak functional connections were pruned, thus yielding a more

modular and hierarchical circuit structure. Once again, these results highlight how ACh is able to reorganize the circuit function in a way that promotes the discriminability of thalamic inputs at the expense of weak pairwise relationships (Runfeldt et al., 2014).

SENSORY MODALITY-SPECIFIC INFORMATION PROCESSING AND ACh

Many studies (Disney et al., 2007; Minces et al., 2017) have focused on trying to understand the role played by ACh in improving stimuli detection or modifying receptor fields size in the visual cortex. While many of them have been done in primates, others have privileged the somatosensory areas and highlight the involvement of the cholinergic system in the regulation of sensory cortical processing in rodents as well, supporting the idea that cholinergic modulation of cortical microcircuits is functionally equivalent across brain areas and model organisms, even though a canonical and anatomically equivalent system is not strictly identifiable (Coppola and Disney, 2018).

The finding that distinct neuronal clusters in the BF project selectively to specific sensory areas (Kim et al., 2016) and that cholinergic inputs to sensory cortices are spatially segregated supports the idea that cholinergic release improves sensory discrimination in a modality-selective manner and with a high degree of specificity. The authors mapped BF projections to different sensory areas and found retrobead-labeled neurons from three different sensory cortices within the BF, with a clear distinction between the clusters of cells: neurons in the HDB project preferentially to V1, the posterior part of NBM projects to A1, while the aNBM preferentially projects to S1. These results were further confirmed by another experiment in which the authors optogenetically activated cholinergic neurons in the BF subnuclei and successfully induced modality-selective desynchronization in specific sensory cortices.

A similar experiment was performed by Chaves-Coira et al. (2016), who also used retrograde anatomical procedures to demonstrate the existence of specific neuronal groups in the BF implicated in the modulation of specific sensory cortices. However, here the authors found that most of the neurons located in the HDB projected to the S1 cortex, suggesting that this area is specialized in the sensory processing of tactile stimuli, and the NBM was found to have a similar number of cells projecting to S1 as to A1. Furthermore, optogenetic HDB stimulation induced a larger facilitation of tactile evoked potentials in S1 than auditory evoked potentials in A1, while optogenetic stimulation of the NBM facilitated either tactile or auditory evoked potentials equally. These results suggest that cholinergic projections to the cortex are organized into spatially segregated pools of neurons that modulate specific cortical areas; although, additional research will be needed in order to provide a clear and definitive picture of the topographical organization of the projections arising from the BF region and innervating the cortex. Despite the many attempts to clarify this issue, it remains unclear whether there exist distinct neuronal populations in the HDB, or whether

the differences observed in the outcomes of the experiments mentioned above are due to discrepancies existing in the transgenic mouse lines used or to the slightly different techniques that were employed.

ACh is thus involved both in the bottom-up attentional process that leads to a general and whole-state arousal of the cortex and in the top-down modifications of circuit activity that occur during detection of behaviorally relevant sensory stimuli. Cognitive functions of cholinergic projection systems vary according to the brain area that is being modulated. Cholinergic modulation may act as a common mechanism to improve sensory encoding in several brain areas.

SUMMARY AND OUTLOOK

ACh release in the neocortex controls transitions between brain states, such as attention, memory and wakefulness, and can occur through volume or synaptic transmission. However, it is not clear yet whether one modality prevails upon the other or if they are complementary mechanisms. Further studies are needed to establish correlations between the distribution profile of the receptor subtypes, the relative proximity and density of cholinergic varicosities to assess differences between the two modalities. Moreover, as results could vastly vary across species, a systematic effort is crucial to be able to compare quantitative measurements.

The expression of muscarinic and nicotinic cholinergic receptors—the two main types—varies according to the cell-type and the pattern of receptor localization varies across cortical layers. A detailed knowledge of the subcellular localization of cholinergic receptors is, however, currently lacking. The detection of cholinoceptive structures such as the receptor protein has become easier with the advent of polyclonal antibodies targeting different subtypes. Future investigations should, therefore, converge on systematically measuring the amount of each receptor subtype across cellular compartments.

In this review, we have endeavored to determine, in a quantitative manner, the cellular and synaptic effects of ACh release in the neocortex. While the cholinergic modulation of excitatory PCs has been extensively researched, its effect on inhibitory interneurons is still largely unknown. For example, the effect of ACh on BCs (fast-spiking, PV$^+$ interneurons) remains unclear. This could be due to the lack of a thorough classification of diverse morphological types of BCs where a differential distribution of cholinergic receptors could modulate divergent cellular and synaptic effects. Furthermore, it is not clear whether bath-application of cholinergic agonists is comparable to a physiological activation of the cholinergic system. Applied concentrations of cholinergic agonists vary substantially (up to three orders of magnitude) across electrophysiological studies, which seldom use more than one concentration. To obtain carefully designed dose-response curves of the effects of cholinergic agonists is paramount to dissect the consequences of physiological ACh release in the neocortex. The advent of optogenetics holds promise in designing physiological protocols of ACh release. Future experiments should not only merely

employ traditional bath-application of cholinergic agonists but also exploit optogenetics to reconcile how doses of agonists directly map to effects of endogenous, physiological release of ACh.

The effects of ACh on synaptic connections can vary drastically according to the identity of the presynaptic terminal and its postsynaptic partner. Additionally, the magnitude of the postsynaptic response also depends on the receptor subtype being activated. Therefore, there is a clear requirement for systematic investigations of the effects of ACh on different synapse-types, combined with knowledge of implicated cell-types and receptor subtypes to unravel the effects of ACh release on necortical synaptic transmission.

ACh is involved in the induction of synaptic plasticity mechanisms, which could support its role in cortical learning and memory. In addition, ACh enhances sensory processing by affecting receptor fields size and tuning properties. It is not clear, however, if the effects of ACh are modality-specific or can be generalized to all sensory processing, nor exactly which tuning properties are affected. Many studies point to a role of ACh in increasing the SNR of a sensory response, and others describe how ACh suppresses cortico-cortical interactions in favor of thalamic transmission. Therefore, further clarification is required on the matter. Moreover, special attention must be paid in integrating data from primates and rodents: neuromodulatory systems are commonly the object of evolutionary modifications, even though they might maintain some functional similarity throughout species.

The mechanisms of ACh-induced changes in the physiology of neocortical neurons and their synapses, and how these changes shape the emergence of global network states still remains elusive. The impact of ACh on global cortical computations sustains cognitive functions such as attention, learning and memory, which are characterized by desynchronized network activity. Cholinergic inputs mainly originate in the BF, a structure comprising distinct multi-transmitter neuronal populations. The functional relevance of neuronal subpopulations in the BF and the co-release of two potentially antagonistic transmitters to the desynchronization of cortical activity is unknown. Furthermore, recent work identifies that a sub-population of VIP$^+$ cortical interneurons co-release ACh and GABA with potentially differing functions across species. Future research should, therefore, focus on dissecting the impact of each transmitter on cellular excitability. In addition, analyzing the co-localization of post-synaptic receptors could also allow the identification of individual synapses that are sensitive to multiple neurotransmitters. All these possibilities should be addressed systematically in order to precisely understand the contribution of each neurotransmitter to ACh-induced effects on the emergence of cortical network states in health and disease.

AUTHOR CONTRIBUTIONS

CC, DK, PS and SR wrote the manuscript and drafted the figures and tables. SR, DK and HM reviewed and edited the manuscript and the figures. SR conceived the idea and supervised the study.

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A Novel Type of Neuron Within the Dorsal Striatum

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The striatum is predominantly composed of medium spiny projection neurons, with the remaining neurons consisting of several types of interneurons. Among the interneurons are a group of cells that express tyrosine hydroxylase (TH). Although the intrinsic electrical properties of these TH-expressing interneurons have been characterized, there is no agreement on the number of TH-expressing cell types and their electrical properties. Here, we have used transgenic mice in which YFP-tagged channelrhodopsin-2 (ChR2) was expressed in potential TH-expressing cells in a Cre-dependent manner. We found that the YFP$^+$ neurons in the striatum were heterogeneous in their intrinsic electrical properties; unbiased clustering indicated that there are three main neuronal subtypes. One population of neurons had aspiny dendrites with high-frequency action potential (AP) firing and plateau potentials, resembling the TH interneurons (THIN) described previously. A second, very small population of labeled neurons resembled medium-sized spiny neurons (MSN). The third population of neurons had dendrites with an intermediate density of spines, showed substantial AP adaptation and generated prolonged spikes. This type of striatal neuron has not been previously identified in the adult mouse and we have named it the Frequency-Adapting Neuron with Spines (FANS). Because of their distinctive properties, FANS may play a unique role in striatal information processing.

Keywords: interneurons, neuron diversity, dorsal striatum, tyrosine hydroxylase, intrinsic electrical properties, neuron classification

INTRODUCTION

The striatum is a major component of the basal ganglia circuit and is involved in a number of functions such as motor planning, motivation and reward perception. Medium-sized spiny neurons (MSN) are the most abundant cell type in the striatum and constitute about 95% of all striatal neurons in rodents (Graveland and DiFiglia, 1985; Kreitzer, 2009). MSN are GABAergic and are the only known projection neurons of the striatum. The remaining striatal neurons are made up of several kinds of interneurons that differ in their neurochemical and electrophysiological characteristics: (1) fast-spiking (FS) parvalbumin (PV)-expressing GABAergic interneurons; (2) low-threshold Ca^{2+} spiking (LTS) somatostatin (SOM)/neuropeptide Y (NPY)/nitric oxide synthase (NOS)-expressing GABAergic interneurons; and (3) spontaneously active cholinergic interneurons. In addition,

calretinin-expressing GABAergic interneurons have also been observed in immunohistochemical studies but their electrophysiological properties are unknown (Rymar et al., 2004; Tepper et al., 2010). More recently, the use of transgenic mice expressing neuron-specific markers has revealed still other types of striatal interneurons. These include a NPY-expressing interneuron that differs from the NPY-LTS cell (Ibáñez-Sandoval et al., 2011; Assous et al., 2017), as well as a heterogeneous population of interneurons that express 5HT3a receptors (Faust et al., 2015, 2016; Muñoz-Manchado et al., 2016).

An additional population of tyrosine hydroxylase (TH)-immunoreactive (TH^+) interneurons has been found in the striatum of several species, including primates (Dubach et al., 1987), rat (Tashiro et al., 1989), mouse (Mao et al., 2001) and human (Ikemoto et al., 1997; Cossette et al., 2005). These neurons are up-regulated after denervation of dopaminergic input from the midbrain (Nakahara et al., 2001; Tandé et al., 2006; Darmopil et al., 2008) and their morphology, electrophysiological properties and distribution have been characterized (Ibáñez-Sandoval et al., 2010; Masuda et al., 2011; Ünal et al., 2011). These characterizations have yielded somewhat inconsistent conclusions; specifically, it is currently unclear whether there are two (Masuda et al., 2011) or four (Ibáñez-Sandoval et al., 2010) subtypes of TH-expressing neurons.

Here, we have extended the genetic approach to characterize striatal neurons in transgenic mice in which YFP-tagged Channelrhodopsin-2 (ChR2) expression was driven by Cre expression in a TH-Cre mouse line. We found that YFP^+ striatal neurons were quite heterogeneous in their intrinsic electrical properties and could be sorted, by an unsupervised clustering method, into three main groups. More than 20% of the neurons resembled the TH-expressing interneurons (THIN) described previously (Ibáñez-Sandoval et al., 2010), while a small fraction of neurons closely resembled typical MSN. A third group of cells had unique electrophysiological properties that did not resemble those of THIN, MSN, or any other striatal cell that has previously been described. These cells showed substantial action potential (AP) adaptation, generated prolonged spikes in response to depolarizing currents and possessed dendrites with an intermediate density of spines. We have named these novel neurons Frequency-Adapting Neurons with Spines (FANS) and here we compare the properties of FANS to those of the other YFP^+ cells within the dorsal striatum in our transgenic mice.

MATERIALS AND METHODS

Animals

Transgenic mice expressing ChR2 fused to YFP in TH-Cre expressing neurons were generated by mating a line of TH-Cre transgenic mice (Lindeberg et al., 2004) with another line (Ai32) expressing YFP-tagged ChR2 behind a floxed stop cassette (Madisen et al., 2012). Both mouse lines were obtained from Jackson Labs. All mice used in the experiments were of either sex and age 6–14 weeks; all animal procedures were approved by the Institutional Animal Care and Use Committee of Biopolis.
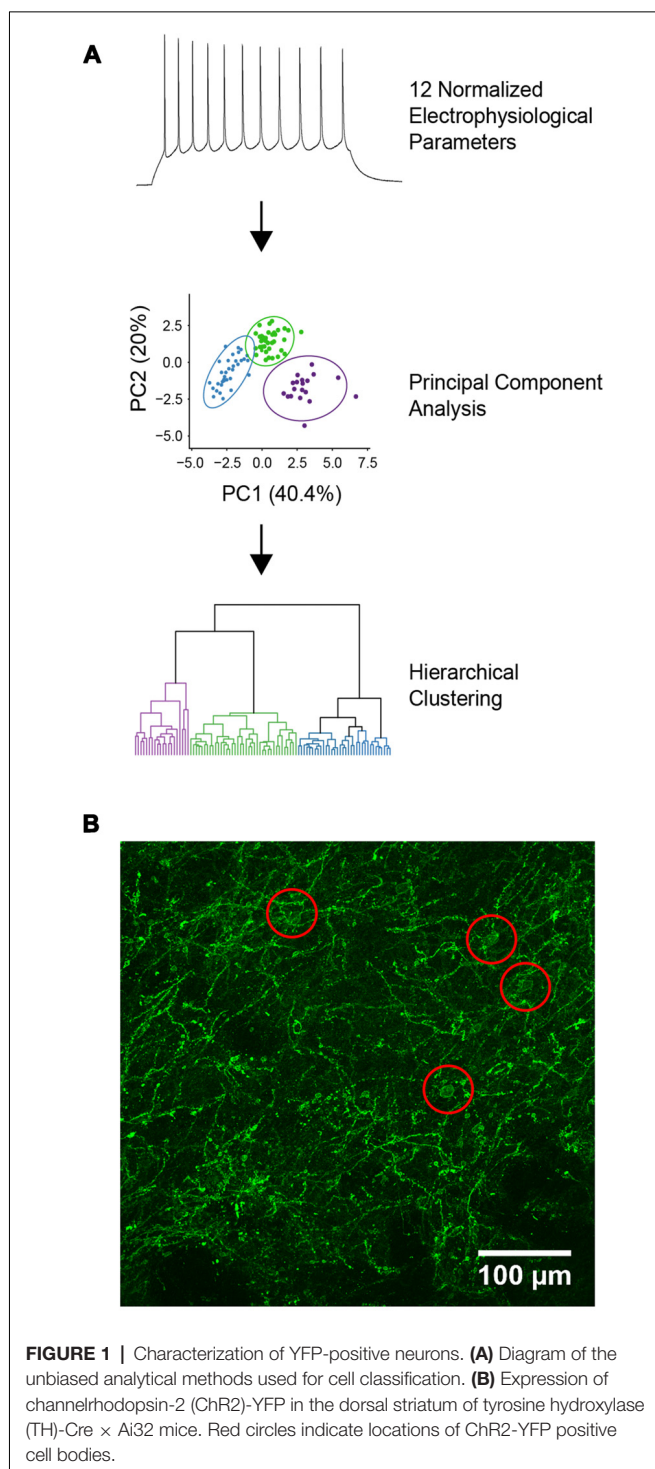
Preparation of Brain Slices

Mice were anesthetized with isoflurane and euthanized *via* decapitation. Brains were removed quickly and placed in carbogen-saturated ice-cold cutting solution containing the following (in mM): 250 sucrose, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 1.25 $HaH_2PO_4 \cdot H2O$, 3 myoinositol, 2 sodium pyruvate, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.5 ascorbic acid and 1 kynurenic acid. 300 μm thick parasagittal slices containing the dorsal striatum were cut with a vibratome and then transferred to a recovery chamber containing carbogen-saturated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 126 NaCl, 24 $NaHCO_3$, 10 glucose, 2.5 KCl, 1 $HaH_2PO_4 \cdot H2O$, 2 $MgCl_2$, 2 $CaCl_2$, 0.4 ascorbic acid. The brain slices were incubated at 34°C for 1 h before being returned to room temperature for recording in ACSF.

Electrophysiology

ChR2-YFP expressing cells in the dorsal striatum were visualized using a 25× objective (NA = 1.05) on an Olympus FV-1000 two-photon microscope. Neurons expressing ChR2-YFP were activated by blue light (BP470-495 excitation filter, Olympus) from a Mercury arc lamp. The duration of the light flashes was controlled by an electronic shutter (Uniblitz Model T132, Vincent Associates). Whole-cell patch clamp recordings were made using a MultiClamp 700B amplifier (Molecular Devices) and were performed under visual guidance. Recording pipettes (6–8 MΩ resistance) were filled with an internal solution containing (in mM, unless otherwise stated): 130 potassium gluconate, 10 KOH, 2.5 $MgCl_2$, 10 HEPES, 4 Na_2ATP, 0.4 Na_3GTP, 5 EGTA, 5 disodium phosphocreatine, 0.025 Alexa fluor 594 (Invitrogen) and 0.2% (w/v) neurobiotin (Vector Labs). All reagents were obtained from Sigma-Aldrich unless otherwise indicated. Series resistance and whole-cell compensation were performed immediately after establishing the whole-cell recording configuration, while bridge balance was performed in current-clamp mode. Recordings with series resistance greater than 25 MΩ, resting membrane potential (RMP) more depolarized than −50 mV or fluctuations greater than 10% were excluded from analysis. Electrophysiological data were acquired with a Digidata 1330a interface and pClamp 10.4 software (both from Molecular Devices); data were analyzed using Clampfit 10.4 (Molecular Devices), OriginPro (OriginLab) and/or GraphPad Prism 7 (GraphPad). Numerical values presented here indicate mean ± SEM. Liquid-liquid junction potentials were estimated from the compositions of ionic species in the external and internal solutions, using the junction potential calculator in pClamp; membrane potentials stated in this paper include this correction.

Electrophysiological Classification of Neuron Types

To identify functionally related YFP^+ neurons, intrinsic electrical properties were recorded from each cell and unsupervised hierarchical clustering was used to cluster these data (**Figure 1A**). A total of 12 electrical properties (**Table 1**) were determined from responses to a series of 1 s long current steps, ranging from −200 pA to 200 pA. Input resistance was calculated

FIGURE 1 | Characterization of YFP-positive neurons. **(A)** Diagram of the unbiased analytical methods used for cell classification. **(B)** Expression of channelrhodopsin-2 (ChR2)-YFP in the dorsal striatum of tyrosine hydroxylase (TH)-Cre × Ai32 mice. Red circles indicate locations of ChR2-YFP positive cell bodies.

TABLE 1 | Electrophysiological properties used for unbiased classification of YFP-positive neurons.

Property	Description
C_m (pF)	Membrane capacitance measured after whole-cell configuration was achieved.
R_{in} (MΩ)	Slope of linear fit between current-voltage response curve.
RMP (mV)	Y-intercept of linear fit between current-voltage response curve.
Sag amplitude (mV)	Difference between most hyperpolarized voltage and steady-state voltage, measured in response to hyperpolarizations to approximately −100 mV.
AHP (mV)	Difference in voltage from AP threshold to maximum negative deflection during repolarization.
AP amplitude (mV)	Difference between AP threshold and peak amplitude of 1st AP.
AP frequency adaptation	Ratio between instantaneous frequencies of the first two APs and last three APs.
AP amplitude adaptation	Ratio between amplitudes of first and last AP during AP train.
AP Half-width (ms)	Time difference between rising phase and decaying phase of AP at half-maximum amplitude.
Decay rate of AP (mV/ms)	Maximum rate of rise of membrane voltage during AP rising phase.
Rise rate of AP (mV/ms)	Maximum rate of rise of membrane voltage during AP falling phase.
Current threshold (pA)	Smallest current that evoked at least one AP.

amplitude, duration (half-width), and maximum rise and fall rates of APs. AP adaptation properties were measured from responses to the smallest current step that elicited at least 10 AP. These properties were extracted from raw data using custom scripts written in Matlab (Mathworks).

From the 12 intrinsic properties, data dimensionality was reduced using principal component (PC) analysis. PCs were calculated using singular value decomposition, performed using the pcaMethods package in the R programming language (Stacklies et al., 2007; R Core Team, 2013). The first three PCs, which accounted for 69.2% of the total variance in the data, were used for unsupervised agglomerative hierarchical clustering using Euclidean distance as a measure of linkage distance. Ward's (1963) minimum variance was used to combine clusters, which reduced the total within-cluster variance. This was implemented using the online webtool Clustvis, which uses R packages such as pheatmap, FactoMineR and ggplot2 internally to generate dendrograms of the hierarchically clustered data (Metsalu and Vilo, 2015). The optimal number of clusters was determined using silhouette analysis which measures the quality of clustering by calculating the separation between identified clusters (Rousseeuw, 1987). Silhouette analysis was performed using the cluster package in R with Euclidean distance used for calculating silhouette width (Maechler et al., 2018). To visualize the identified clusters, a dendrogram was constructed where the horizontal lines represent joined clusters. Radar plots were generated in R using the packages ggplot2 and radarchart after normalizing population feature means to ranges of 0–1.

after constructing a current-voltage relationship from responses to hyperpolarizing current pulses. These responses were also used to determine other passive properties, such as membrane time constant, voltage sag during hyperpolarization, and input conductance. Responses to depolarizing current pulses were used to determine active properties, such as threshold current for evoking an AP, AP amplitude, after-hyperpolarization (AHP)

Immunohistochemistry and Imaging

Following electrical recordings, neurobiotin-filled cells were visualized by fixing brain slices in 4% paraformaldehyde overnight at 4°C followed by permeabilization in phosphate-buffered saline containing 0.25% Triton X-100 (PBST) for 1 h at room temperature. Slices were then incubated in streptavidin-Alexa 633 (1:1,000 diluted in PBST) for 48 h at 4°C before washing in PBST three times for 20 min each. Brain slices were mounted on glass slides and imaged using the same microscope system described above. A Z-stack of images was obtained for each labeled cell and cell morphology was digitally reconstructed as described below. Maximum projection images of such reconstructions are shown here unless otherwise stated.

To analyze and compare the morphology of neurobiotin-labeled neurons, cells were digitally reconstructed using AdReconstructor, a software suite for reconstruction of neuronal morphology (Nair et al., 2018). This program first applies pre-processing algorithms to remove common sources of noise, such as non-specific background staining and neurobiotin leakage. Cells were then reconstructed using the All Path Pruning algorithms developed by Xiao and Peng (2013) and post-processing measures were applied to ensure accuracy of reconstructions. The automated traces obtained in the open source SWC format were then quantified using L-Measure to obtain parameters such as surface area against path distance and spine density (Scorcioni et al., 2008). Sholl analysis was performed to analyze dendritic branching using the Simple Neurite Tracer plugin in ImageJ (Ferreira, 2016).

ChR2-YFP expressing cells in the dorsal striatum were also visualized by immunolabeling, using an anti-YFP/GFP antibody. The dendrites of YFP$^+$ cells were dispersed throughout the dorsal striatum and the somata of these cells seemed to be evenly dispersed throughout the striatum (**Figure 1B**). Brain slices were fixed in 4% paraformaldehyde overnight at 4°C followed by permeabilization and blocking of the endogenous epitopes by applying 10% normal goat serum in PBST for 1 h at room temperature. Chicken anti-GFP antibody (Abcam) was applied at 1:1,000 in PBST for 48 h at 4°C and then washed three times in PBST for 20 min each. Goat anti-chicken Alexa 488 secondary antibody was applied at 1:500 in PBST for 2 h at room temperature followed by washing three times in PBST for 20 min each. All antibodies were obtained from Life Technologies unless otherwise stated. All image processing and analyses were carried out using FIJI, except those used for morphological reconstruction as described above.

Morphological Classification of Neuron Types

To determine the relationship between electrophysiologically-defined cell types and their morphological properties, we also performed unsupervised hierarchical clustering of morphological features extracted from digital reconstructions of 15 neurobiotin-labeled neurons. The morphological properties extracted were based on definitions used in Scorcioni et al. (2008) and Gouwens et al. (2018), and are described in detail in **Table 2**. A total of 23 properties were extracted from digital reconstructions saved in the open source SWC format using the "compute global feature" plugin in Vaa3D (Peng et al., 2014). Dimensionality reduction was then performed using PC analysis, calculated using singular value decomposition and implemented using pcaMethods in R (Stacklies et al., 2007). Agglomerative hierarchical clustering was then performed on the first three PCs which account for 69.7% of the total variance in the dataset using the online tool Clustvis with correlation as a distance measure and Ward's distances as a clustering method (Metsalu and Vilo, 2015). To identify the optimal number of clusters, silhouette analysis was again used-using Euclidean distance to calculate silhouette width- and implemented using the cluster package in R (Maechler et al., 2018). Radar plots were created in R using the packages ggplot2 and radarchart after normalizing population means to a range of 0–1.

RESULTS

Three Types of YFP$^+$ Striatal Neurons

In an attempt to identify TH-expressing neurons in the striatum, we used double-transgenic mice with ChR2 expression driven by the TH promoter. Positive striatal cells could be identified, in live brain slices prepared from these mice, by the fluorescence of YFP fused to the ChR2. Expression of ChR2 was confirmed by the APs that were evoked when brief (5 ms duration) laser light flashes (458 nm; 0.006–0.018 mW) were used to activate the ChR2 (**Figure 2A**). Due to the possibility of ectopic expression of ChR2-YFP in neurons that do not express TH in adulthood (Lammel et al., 2015; Stuber et al., 2015), we do not assume that neurons expressing ChR2-YFP actually express TH and instead will conservatively refer to these cells as YFP$^+$ neurons.

Applying depolarizing and hyperpolarizing current pulses to such YFP$^+$ cells revealed a diversity of cellular responses (**Figure 2B**). Unsupervised hierarchical clustering of these cell responses, based on the 12 intrinsic electrical properties listed in **Table 1**, revealed that there were three different populations of YFP$^+$ cells (**Figure 3A**). Comparing AP half-width, AP amplitude adaptation during prolonged depolarizations, and cell input resistance also showed a clear separation into the same three cell types identified by the clustering approach (**Figure 3B**). Indeed, all 12 intrinsic electrical properties considered differed significantly between the three different types of neurons (**Figure 3C**).

One type of cell resembled the THIN described previously (Ibáñez-Sandoval et al., 2010); these cells exhibited high input resistance and prominent voltage sag during responses to hyperpolarizing currents (**Figure 2B**, left and **Figure 3C**). THIN often exhibited a rebound depolarization following a hyperpolarizing current pulse (**Figure 2B**, green arrowhead), which sometimes evoked rebound APs. Large depolarizing

TABLE 2 | Morphological properties used for unbiased classification of YFP-positive neurons.

Property	Description
Number of nodes	The total number of nodes in the given digital reconstruction. A node in a reconstruction represents a single sample point of the neuron defined by its X, Y and Z coordinates, a radius, and its connectivity to other nodes in the neuron.
Soma surface area (μm^2)	The surface of the spherical node representing the soma in the digital reconstruction.
Number of stems	The number of nodes attached to the soma.
Number of bifurcations	The number of points which have two daughter nodes for the given reconstruction.
Number of branches	The number of compartments that lie between two branching points or between one branching point and a termination point in the given reconstruction.
Number of tips	The number of terminal tips for the given input neuron.
Neuronal height (μm)	Height is the difference of minimum and maximum y-values after eliminating the outer points on either end by using the 95% approximation of the y-values of the given reconstruction.
Neuronal width (μm)	Width is the difference of minimum and maximum x-values after eliminating the outer points on either end by using the 95% approximation of the x-values of the given reconstruction.
Neuronal depth (μm)	Depth is the difference of minimum and maximum z-values after eliminating the outer points on either end by using the 95% approximation of the z-values of the given reconstruction.
Average diameter (thickness; μm)	The average diameter of all compartments of the neuron.
Total length (μm)	The total length of the neuron is computed as the sum of distances between two connected nodes for all branches.
Total volume (μm^3)	The total volume of the entire neuron.
Maximum Euclidean distance to root (μm)	The maximum Euclidean distance of all nodes. Euclidean distance is the straight line distance from the soma to the node.
Maximum path distance to root (μm)	The maximum path distance of all nodes. The path distance is the sum of lengths of all connected nodes from the soma, ending with that node.
Maximum branch order	The maximum order of the branch. A branch's order is defined with respect to the soma where the soma has a branch order = 0 and ever bifurcation has an increasing branch order.
Average contraction	The average ratio between Euclidean distance of a branch and its path length.
Average fragmentation	The average number of compartments that constitute a branch between two bifurcation points or between a bifurcation point and a terminal tip.
Average parent-daughter ratio	The average ratio between the diameter of a daughter branch and its parent branch
Average local amplitude angle (degrees)	The average angle between the first two compartments at a bifurcation
Average remote amplitude Angle (degrees)	The average angle between two bifurcation points or between bifurcation point and terminal point or between two terminal points.
Intersection at 30 μm	The number of Sholl plot intersections at a distance of 30 μm from the soma.
Intersection at 50 μm	The number of Sholl plot intersections at a distance of 50 μm from the soma.
Spine density (μm^{-1})	Total number of spines in all branches of a given neuron
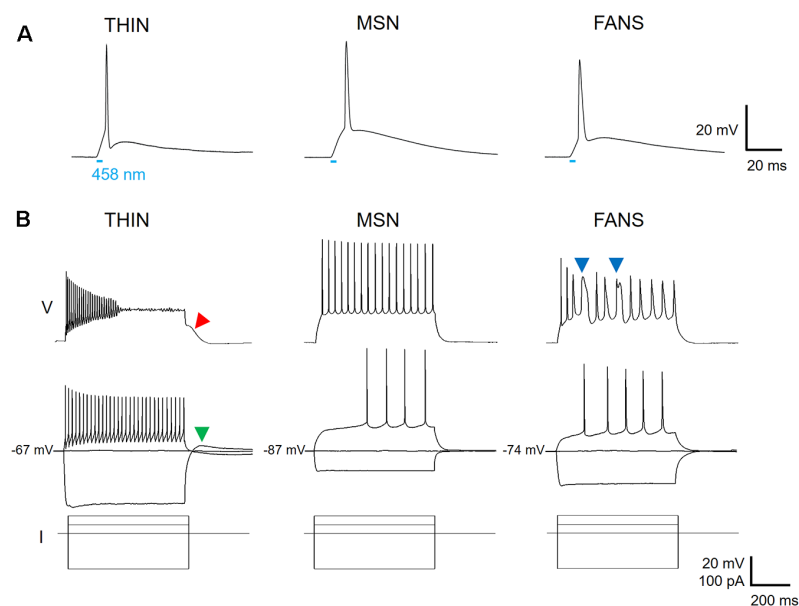
FIGURE 2 | Three types of ChR2-YFP expressing cell in the dorsal striatum. **(A)** Action potentials could be evoked in response to a 5 ms laser light flash (458 nm, at bar), confirming expression of ChR2 in the three cell types. **(B)** Voltage responses to hyperpolarizing and depolarizing current pulses reveal different firing patterns for the three cell types (top two rows, current pulses shown at bottom).
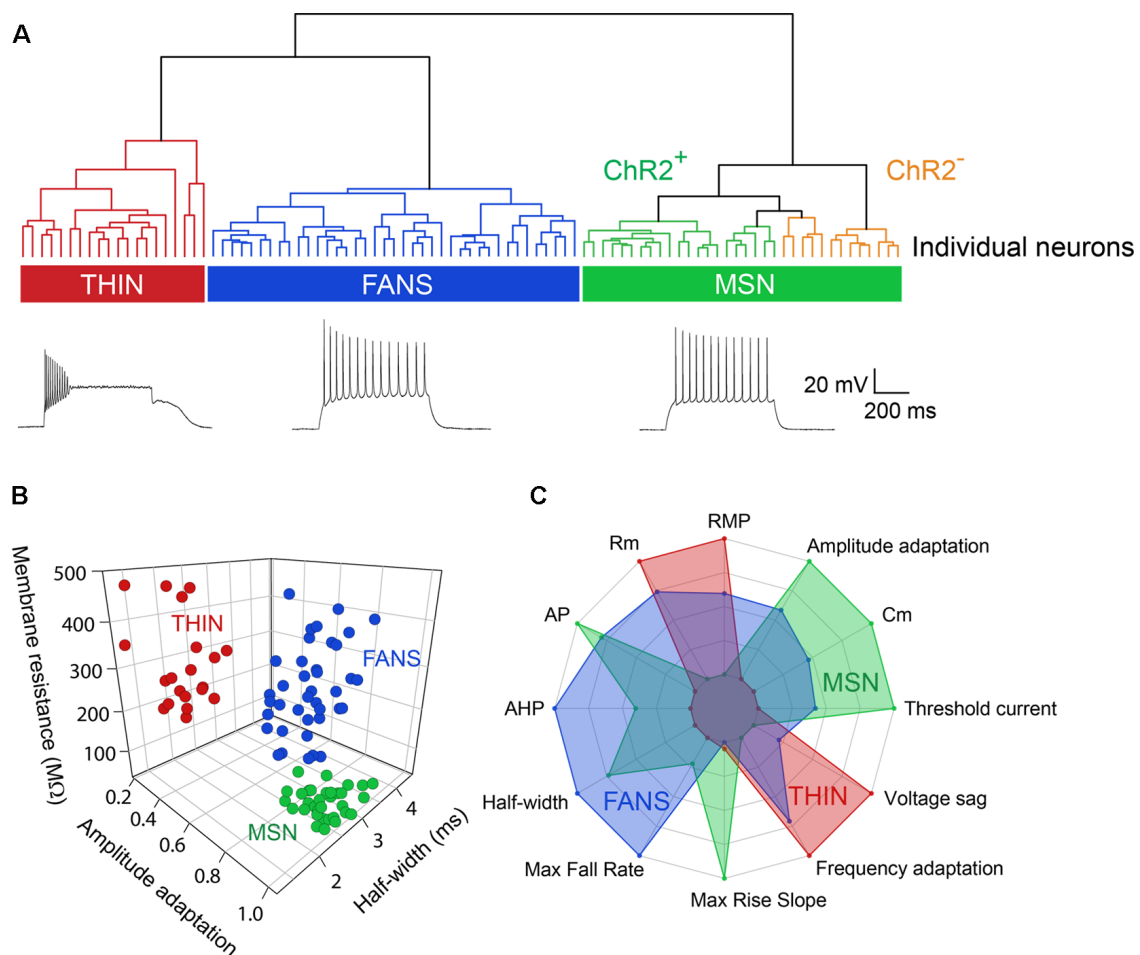
FIGURE 3 | Unbiased hierarchical analysis revealed significant differences between the three types of YFP⁺ neurons. **(A)** A dendrogram showing the unbiased classification of the three types of YFP⁺ neurons (top) and spiking pattern of a representative cell from each type (bottom). **(B)** 3D scatter plots showing the clustering of the three YFP⁺ cell types. **(C)** Radar plots revealing substantial differences between the three cell types, with indicated properties displayed using normalized population means in the range of 0–1.

current pulses often evoked damped voltage oscillations during such depolarizations, as well as a plateau potential following the end of depolarization (**Figure 2B**, red arrow). Four subtypes of THIN, with subtle differences in their electrophysiological properties, have been reported (Ibáñez-Sandoval et al., 2010), and these are evident in the subclusters of THIN shown in **Figure 3A**. To simplify our analyses, we have grouped together recordings made from all of these THIN subtypes.

Unexpectedly, a small proportion of the YFP⁺ cells unambiguously exhibited the intrinsic electrical properties of MSN, specifically low input resistance and delayed spiking with depolarization (**Figure 2B**, middle and **Figure 3C**). While these cells exhibited some small differences in population means when compared to ChR2⁻ MSN, such as their higher RMP and input resistance, as well as smaller AP and AHP amplitudes (see **Figure 4**), overall in our analysis they clustered together with MSN that did not express ChR2 (**Figure 3A**). This indicates that the two populations of MSN are much

more similar to each other than they are to the other YFP⁺ cell types.

In addition, a third population of cells displayed properties distinct from those of any striatal cell described previously. These cells were characterized by intrinsic electrical properties that were intermediate between those of THIN and MSN (**Figures 3B,C**). Although their responses to small depolarizing currents were more similar to those of MSN than THIN (**Figure 2B**, right), they exhibited significantly more AP adaptation during prolonged depolarizations (see **Figures 6–8** below). Further, in response to large depolarizations, these cells exhibited APs that broadened substantially, often yielding plateau potentials (**Figure 2B**, blue arrows). As will be established in more detail below, these cells are unique in their properties, most notably their large range of AP frequency adaptation (see **Figure 6**) and the presence of spines on their dendrites (see **Figure 9**). We have therefore named these cells Frequency-Adapting Neurons with Spines (FANS).
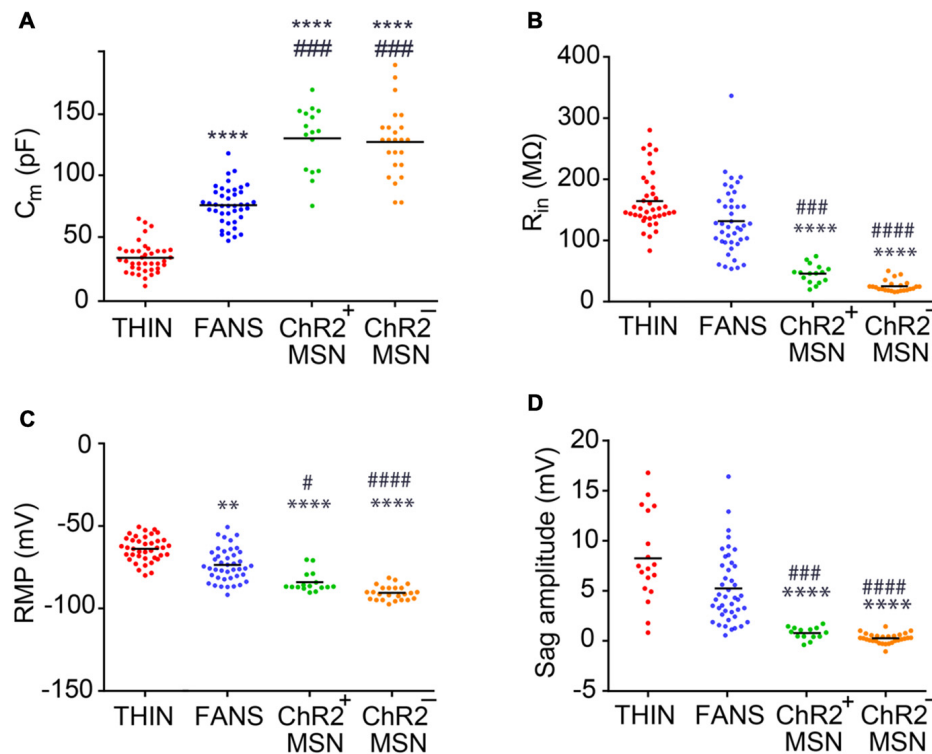
FIGURE 4 | Passive electrical properties of the YFP$^+$ neurons in the dorsal striatum. **(A)** Membrane capacitance. **(B)** Input resistance. **(C)** Resting membrane potential (RMP). **(D)** Amplitude of voltage sag during responses to hyperpolarizing current. One-way ANOVA with Dunn's multiple comparison test; $^#p < 0.05$, $^{**}p < 0.01$, $^{###}p < 0.001$, $^{****}/^{####}p < 0.0001$, where *and $^#$indicate comparisons with THIN and FANS, respectively. Each horizontal line indicates mean value.

Intrinsic Electrical Properties of Three YFP$^+$ Cell Types

We characterized the three types of YFP$^+$ neurons more extensively by comparing their passive electrical properties (**Figure 4**). We first measured the apparent membrane capacitance (C$_m$), which is proportional to cell surface area and, hence, the overall size of the cell. The C$_m$ of the three YFP$^+$ cell types were significantly different from each other (**Figure 4A**; one-way ANOVA, $p < 0.0001$; Dunn's multiple comparison test), with THIN being smallest, followed by FANS and MSN. The C$_m$ of YFP$^+$ MSN was very similar to that of YFP$^-$ MSN. As expected, cell input resistance—which is inversely related to surface area—followed the reverse order, with THIN and FANS significantly different from MSN but not from each other (**Figure 4B**; $p < 0.0001$; one-way ANOVA with Dunn's multiple comparison test). Again, there were no significant differences between the input resistances of MSN that did or did not express ChR2-YFP.

Striatal MSN are characterized by hyperpolarized RMPs and this was evident both in MSN that did or did not express ChR2-YFP (**Figure 4C**). In contrast, THIN and FANS had RMPs that were significantly more depolarized than those of MSN ($p < 0.0001$; one-way ANOVA with Dunn's multiple comparison test). In addition, the amplitude of the depolarizing sag in membrane potential in response to hyperpolarizing current

pulses—when measured at similar membrane potentials to take into account differences in input resistance (see **Figure 2A**, left)—was largest in THIN (Ibáñez-Sandoval et al., 2010) while FANS had an intermediate degree of sag and MSN exhibited the smallest amount of sag (**Figure 4D**; $p < 0.0001$; one-way ANOVA with Tukey's multiple comparison test). In summary, the passive electrical characteristics (and amount of depolarizing sag) of THIN, FANS and MSN were distinctively different from each other.

We next compared the waveforms of APs evoked in the three types of YFP$^+$ cells in response to depolarizing current pulses (**Figure 5A**). FANS had the broadest APs (**Figure 5A**, left), measured as their width at half-maximum amplitude, while THIN had the narrowest APs (**Figure 5B**; $p < 0.0001$; one-way ANOVA with Holm-Sidak's multiple comparison test). The reverse was true for the AHP that followed the APs (**Figure 5A**, right): AHP magnitudes were greatest for THIN while FANS and MSN were similar (**Figure 5C**; $p < 0.0001$; one-way ANOVA with Holm-Sidak's multiple comparison test). Peak AP amplitude, measured for the first AP evoked during a depolarizing current pulse, was largest for FANS and smallest for THIN (**Figure 5A**, left); the APs of MSN were similar in amplitude to those of FANS (**Figure 5D**; $p = 0.0002$; one-way ANOVA with Holm-Sidak's multiple comparison test).
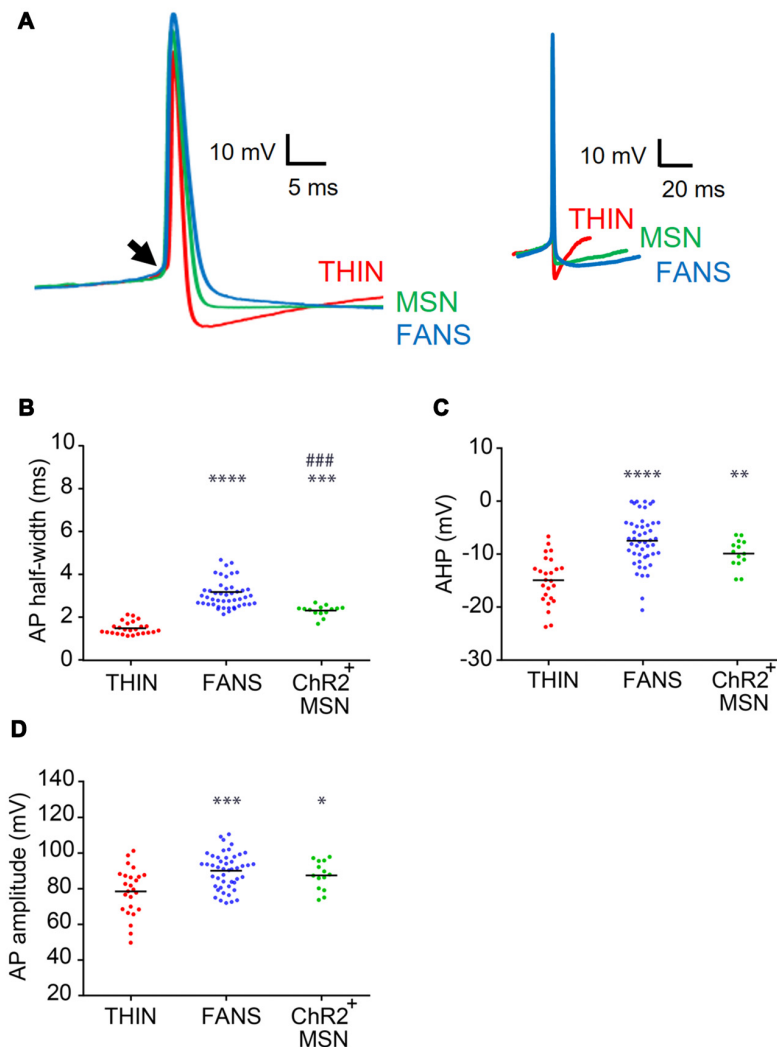
FIGURE 5 | AP characteristics of YFP$^+$ neurons in the dorsal striatum. **(A)** Representative APs recorded from each cell type, shown at fast (left) and slow (right) time scales. Arrow indicates the point of inflection of APs, which was used as the reference point when measuring the amplitudes of APs and their after-hyperpolarizations (AHPs). **(B)** AP duration as measured at half-width. **(C)** AHP amplitude. **(D)** AP peak amplitude. One-way ANOVA with Holm-Sidak's multiple comparisons; $*p < 0.05$, $**p < 0.01$, $***/####p < 0.001$, $****p < 0.0001$, where $*$ and $\#$ indicate comparisons with THIN and FANS, respectively. Each horizontal line indicates mean value.

Action Potential Adaptation During Sustained Depolarization

During trains of APs, AP properties dynamically changed due to adaptation (**Figure 6A**). We quantitatively compared AP adaptation in response to sustained depolarizations (1 s duration) of all three types of YFP$^+$ striatal cells. Because adaptation properties vary with the degree of depolarization, we measured adaptation in different cells at a standard criterion current level of 50 pA above the minimum current required to evoke a single AP.

Frequency Adaptation

The frequency of APs declined during sustained depolarizations (**Figure 6A**). Plots of normalized AP instantaneous frequency (IF), defined as the inverse of the interval between successive APs, illustrate the differences in the AP frequency adaptation properties of these three cell types (**Figure 6B**). While both FANS and THIN showed substantial AP frequency adaptation, evident as a progressive decline in IF during a train of APs, MSN exhibited less frequency adaptation. The degree of decline during these trains was quantified by the ratio of IF of APs measured at the beginning and end of the depolarizing current pulse (**Figure 6C**). MSN showed significantly less AP frequency adaptation in comparison to THIN or FANS ($p < 0.0001$; one-way ANOVA with Dunn's multiple comparison test).

In most THIN and FANS, the time course of the decline in AP frequency during adaptation could be described by a double exponential function, with a fast initial phase followed by a second, more gradual component (**Figure 6D**). When a plateau potential occurred in FANS, AP frequency adaptation was interrupted and appeared to reset. For this reason, responses
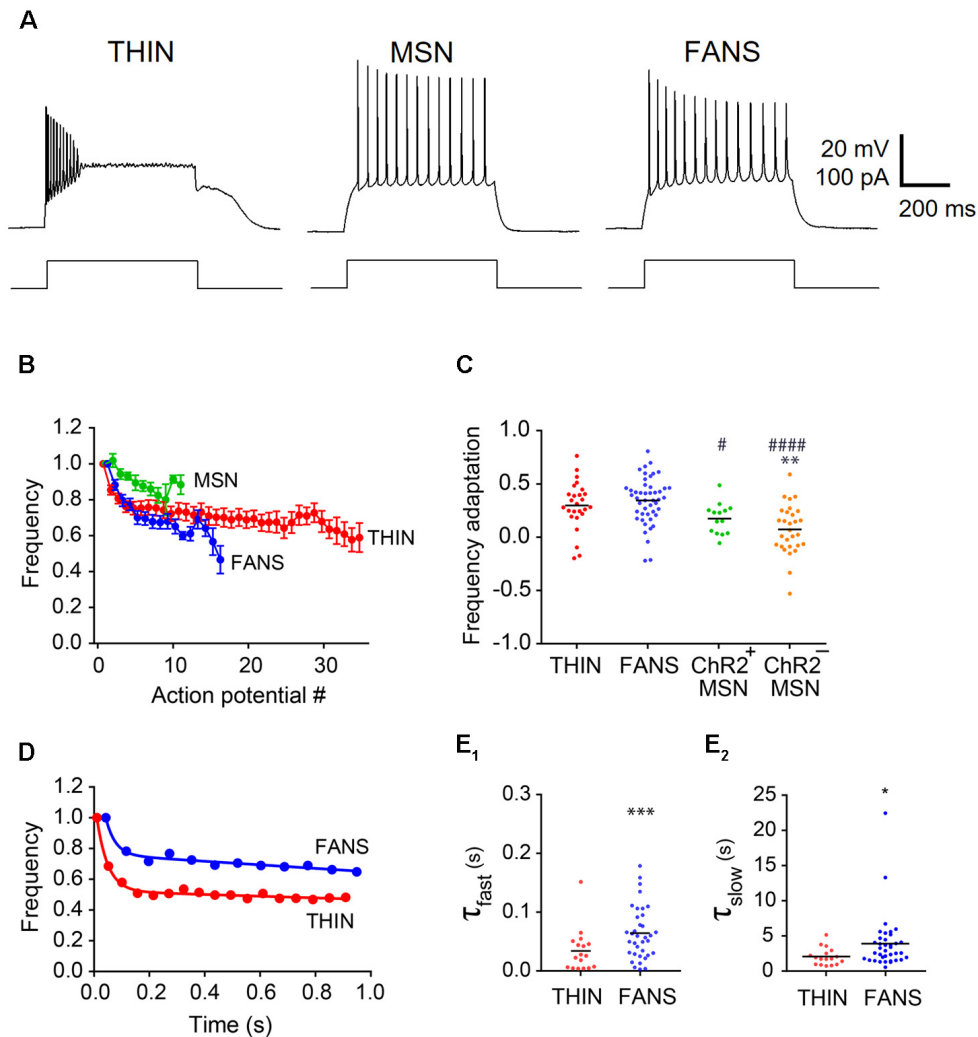
FIGURE 6 | Adaptation of AP frequency in YFP$^+$ neurons. **(A)** Representative traces of APs evoked by 1 s current pulses. **(B)** Normalized mean instantaneous frequency (IF); error bars indicate SEM. **(C)** Frequency adaptation calculated as (1-last AP/first AP). One-way ANOVA with Dunn's multiple comparisons; */#$p < 0.05$, **$p < 0.01$ and ####$p < 0.0001$, where *and #indicate comparisons with THIN and FANS, respectively. **(D)** Normalized IF vs. time plots fitted by two-component exponential curve from representative THIN and FANS neurons. **(E)** Exponential fit time constants (τ) of the fast **(E1)** and slow **(E2)** components of THIN and FANS calculated for IF (unpaired t-tests; ***$p < 0.001$; ****$p < 0.001$). Each horizontal line indicates mean value.

that included plateau potentials were excluded from our analysis. MSN were also excluded, because they exhibited relatively little AP frequency adaptation. The mean time constants of both the fast and slow components for the remaining FANS were significantly larger than those of THIN, indicating slower frequency adaptation in FANS (**Figures 6E1,E2**; $p < 0.001$, unpaired t-test).

Amplitude Adaptation
In all three cell types, the peak amplitudes of APs also progressively decreased during prolonged depolarization (**Figures 6A**, **7A**). The kinetics of this adaptation of AP amplitude is illustrated for all three cell types in **Figure 7B**. By calculating the ratio of the amplitudes of the last and first

APs, we could compare the amount of this adaptation of AP amplitude across the three cell types (**Figure 7C**). THIN exhibited the greatest amount of AP amplitude adaptation, while MSN had the least (**Figure 7C**; $p < 0.0001$; one-way ANOVA with Dunn's multiple comparison test). For all three cell types, AP amplitude adaptation followed a bi-exponential time course (**Figure 7D**). Calculation of the time constants for these exponential functions indicated that the initial rate of adaptation was similar in THIN and FANS, while MSN had significantly slower adaptation (**Figure 7E1**; $p < 0.0001$, one-way ANOVA with Tukey's multiple comparison test). The three cell types showed no significant differences in the kinetics of the slow phase of amplitude adaptation, although there was a tendency for THIN to have slightly faster adaptation than
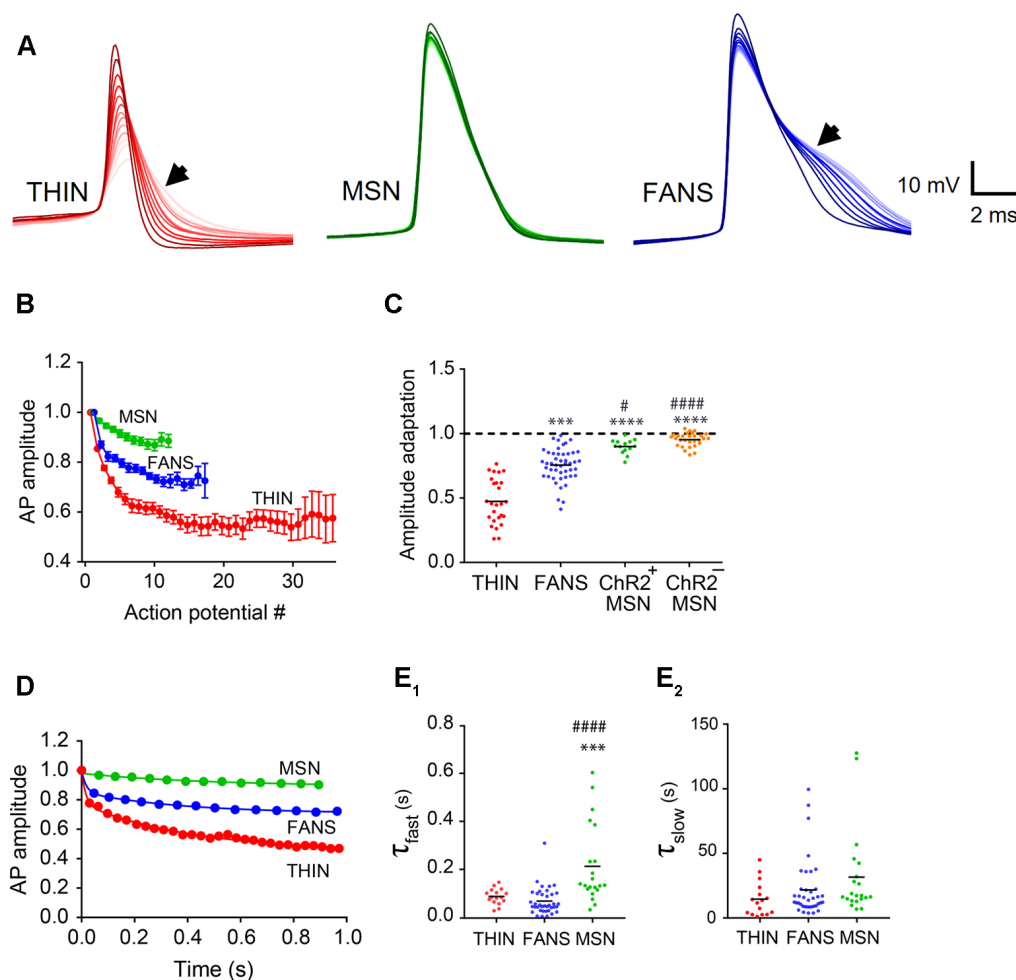
FIGURE 7 | AP amplitude adaptation in YFP$^+$ neurons of the dorsal striatum. **(A)** Representative traces of APs. Dark to light colors indicate AP progression. Arrows indicate AP broadening evident in THIN and FANS. **(B)** Normalized mean AP peak amplitude, mean ± SEM. **(C)** AP amplitude change calculated as the ratio of last AP/first AP. One-way ANOVA with Dunn's multiple comparisons; $^{\#}p < 0.05$, $^{***}p < 0.001$ and $^{****/\#\#\#\#}p < 0.0001$, where * and $^{\#}$ indicate comparisons with THIN and FANS, respectively. **(D)** Normalized amplitude vs. time plots fitted by two-phase exponential curve from representative cells. **(E)** Exponential fit time constants (τ) of the fast **(E1)** and slow **(E2)** components of THIN, FANS and ChR2$^+$MSN calculated for AP amplitude (one-way ANOVA with Tukey's multiple comparisons; $^{***}p < 0.001$, $^{\#\#\#\#}p < 0.0001$). Each horizontal line indicates mean value.

FANS and for FANS to be slightly faster than MSN (**Figure 7E2**; $p = 0.1158$, one-way ANOVA).

AP Broadening

During prolonged depolarizations, AP duration also progressively broadened in both FANS and THIN (**Figure 7A**, black arrows). Two temporal patterns for AP broadening were observed for different THIN: accelerating (positive curvature; **Figure 8A1**) or decelerating (negative curvature; **Figure 8A2**) changes in the rate of adaptation, indicated by the slopes of the plots. FANS also tended to exhibit decelerating broadening of APs (**Figure 8B**). The degree of AP broadening measured at half-width was on average larger for THIN than for FANS (**Figure 8C**). In contrast, MSN exhibited little, if any, AP broadening ($p < 0.0001$; one-way ANOVA with Dunn's multiple comparison test). Due to the difference in the kinetics of AP

broadening in THIN, only THIN with negative curvature were compared with FANS. In these cells, the time course of AP broadening could be described by single exponential functions. The rate of adaptation of AP duration in THIN was slower than in FANS, as demonstrated by the larger time constants for AP broadening in THIN (**Figure 8D**; unpaired *t*-test, $p = 0.0009$).

In summary, FANS exhibit distinctive electrophysiological properties that are uniquely different from the properties of both THIN and MSN (**Table 3**). Therefore, FANS appear to be a novel, previously unrecognized cell type within the striatum.

Unique Morphology of FANS

To examine their morphology, YFP$^+$ neurons also were filled with neurobiotin during whole-cell patch clamp recordings (**Figure 9**, left). Neurobiotin-filled neurons were then fixed,

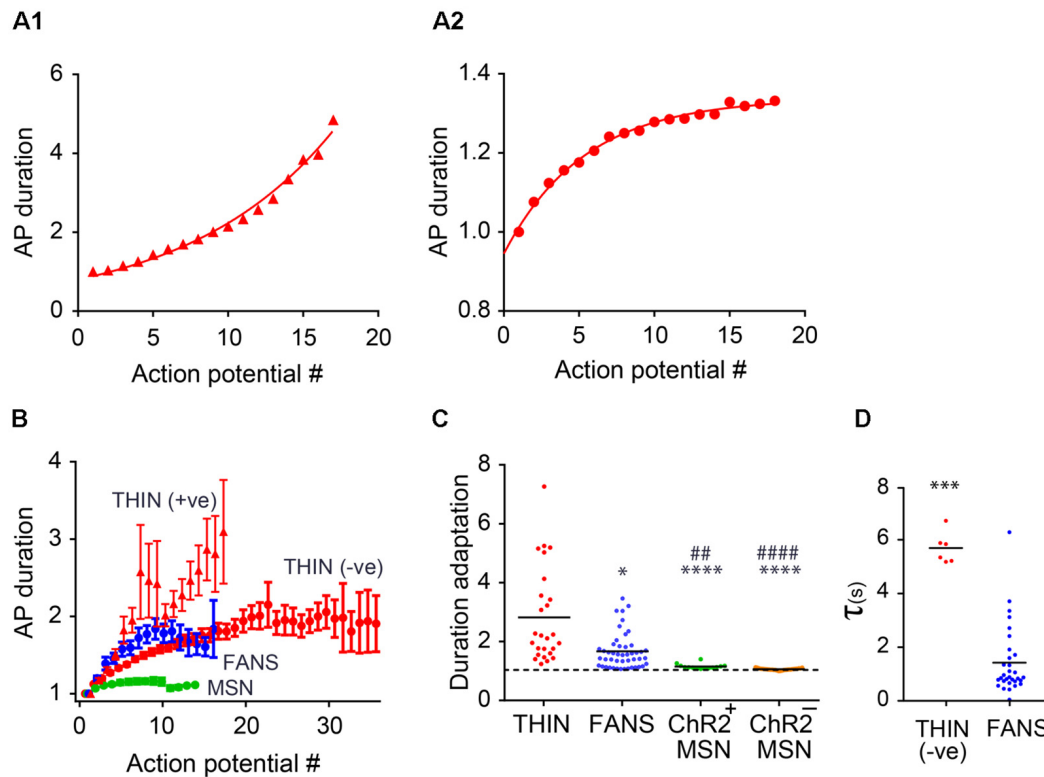**FIGURE 8 |** AP duration adaptation in YFP$^+$ neurons in the dorsal striatum. **(A)** Changes in AP duration (measured at half-width) from a representative THIN showing positive **(A1)** and negative **(A2)** curvatures. **(B)** Normalized mean AP duration, mean ± SEM. **(C)** AP duration adaptation calculated as the ratio of last AP/first AP. One-way ANOVA with Dunn's multiple comparisons; *$p < 0.05$, ##$p < 0.01$ and ****/####$p < 0.0001$, where *and #indicate comparisons with THIN and FANS, respectively. **(D)** Time constants (τ) of AP broadening for THIN and FANS (unpaired t-test; ***$p = 0.0009$). Each horizontal line indicates mean value.

TABLE 3 | Electrical and morphological properties of TH interneuron (THIN), frequency-adapting neurons with spines (FANS) and medium-sized spiny neuron (MSN).

Property	THIN	FANS	MSN (ChR2$^+$)	MSN (ChR2$^-$)
C_m (pF)	34.7 ± 2.0	76.6 ± 2.4	131 ± 6.5	128 ± 5.7
R_{in} (MΩ)	328 ± 14.5	262 ± 17.0	90.8 ± 7.6	49.6 ± 3.8
RMP (mV)	−63.9 ± 1.2	−73.6 ± 1.5	−84.1 ± 1.5	−90.6 ± 0.9
Sag amplitude (mV)	4.4 ± 0.8	1.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
AP duration (ms)	1.47 ± 0.06	3.20 ± 0.14	2.34 ± 0.07	
AHP (mV)	−14.9 ± 0.9	−7.4 ± 0.7	−10.0 ± 0.7	
AP amplitude (mV)	78.7 ± 2.6	90.2 ± 1.5	87.6 ± 2.1	
AP frequency adaptation	0.30 ± 0.04	0.34 ± 0.03	0.18 ± 0.04	0.08 ± 0.04
AP duration adaptation	2.82 ± 0.31	1.68 ± 0.09	1.14 ± 0.02	1.01 ± 0.01
AP amplitude adaptation	0.47 ± 0.03	0.76 ± 0.02	0.90 ± 0.01	0.95 ± 0.01
Soma diameter (μm)	13.7 ± 0.5	15.5 ± 0.8	13.8 ± 1.0	
No. primary dendrites	3.3 ± 0.3	3.9 ± 0.3	5.5 ± 0.2	
Spine density (μm^{-1})	0	0.52 ± 0.04	0.57 ± 0.06	

Values indicate mean ± SEM.

processed and imaged on a two-photon microscope. Stacks of cell images obtained at different z-axis planes were then digitally reconstructed in an automated fashion to obtain bias-free representations of neuronal morphology (**Figure 9**, right; see "Materials and Methods" section). THIN had somata that were medium-sized (13.7 ± 0.5 μm, long axis diameter) and oval or round in shape (**Figure 9A**). The primary and secondary dendrites of THIN appeared to branch sparsely

and were often varicose; most notably, their dendrites lacked spines (**Figure 9A**, inset). These morphological features are very similar to those previously reported for THIN (Ibáñez-Sandoval et al., 2010). The morphology of YFP$^+$ MSN also resembled typical MSN (Gertler et al., 2008; Kreitzer, 2009): they had medium-sized somata (13.8 ± 1.0 μm, long axis diameter) and dendrites that branched extensively and were densely covered with spines (**Figure 9B**). Occasionally the main

FIGURE 9 | Morphology of neurobiotin-filled YFP$^+$ striatal neurons. Panel **(A)** shows a THIN, while **(B)** shows a MSN and **(C)** is a FANS. Left—Green-on-black images are maximum-projection fluorescence images. Right—Black-on-white images are 3D-reconstructed neurons. Red lines indicate axons, distinguished by their smaller diameters and absence of spines. Regions in blue boxes are enlarged in the insets to reveal the presence of dendritic spines in both MSN and FANS, but not in THIN.

axon of these cells could be observed for a sufficient length to visualize its projection towards the posterior of the brain. The morphology of FANS was different from that of THIN or MSN: the dendrites of FANS were also covered with spines, similar to MSN, but their dendritic branching was much less extensive (**Figure 9C**). The somata of FANS were also medium-sized (15.5 $\pm$ 0.8 μm, long axis diameter) and were mostly oval in shape.

Several of the morphological properties of the three types of YFP$^+$ neurons are quantified in **Figure 10**. All three cell types had similar-sized soma (**Figure 10A**; $p = 0.25$, one-way ANOVA with Dunn's multiple comparison test). MSN had significantly more primary dendrites than either FANS or THIN (**Figure 10B**; $p = 0.0031$, one-way ANOVA with Dunn's multiple comparison test). MSN also had the highest density of dendritic spines, as revealed by a 3D analysis of spine density from the reconstructed neurons (**Figure 10C**). FANS had a lower density of spines compared to MSN, while THIN had no spines ($p \leq 0.001$, one-way ANOVA with Tukey's multiple comparison test). Our measured values of spine density for MSN are similar to those previously reported (Shen et al., 2008).

Dendritic branching in the three types of cells first was compared by a two-dimensional analysis described by Sholl (1953). This analysis revealed that dendritic branching was

greatest in MSN and least in THIN (**Figure 10D**). Although the extent of branching was similar in MSN and FANS within the first 10 μm from their somata, MSN branched more extensively at 20–130 μm from their somata (**Figure 10D**; $p < 0.05$; multiple t-tests). As an alternative way to compare dendritic branching in the three cell types, a three-dimensional variant of the Sholl analysis was also performed (Scorcioni et al., 2008). This analysis measured neurite surface area as a function of path distance from the cell body and captured the spatial extent of the neuron in three-dimensions, as opposed to the planar projections used in the Sholl (1953) analysis. Furthermore, measurement of surface area increases the functional relevance of the analysis because surface area should be more proportional to the number of synapses. This analysis (**Figure 10E**) revealed the same differences between the MSN and FANS that were evident in the Sholl (1953) analysis (**Figure 10D**), with MSN having the most dendritic branching, THIN having the least and FANS exhibiting branching intermediate between MSN and THIN over the first 100 μm from the cell body ($p < 0.05$, multiple t-tests).

To systematically examine the relationship between the morphological properties of the three types of YFP$^+$ neurons, we extracted 23 features from digital reconstructions of these neurons and then performed an unsupervised clustering analysis. FANS clustered separately from both THINs and MSNs in our analysis (**Figure 11A**); remarkably, although the intrinsic electrical properties of these neurons were not considered in the morphological clustering analysis of **Figure 11A**, the clustering nonetheless completely segregated the neurons in a way that was consistent with the distinctive electrical properties of these neurons. This indicates that FANS represent a unique cell type both in regard to their morphological properties and their intrinsic electrical properties. Comparing dendritic branching within the first 50 μm from the soma, total length and spine density also showed a clear separation into the same three cell types identified by electrophysiology (**Figure 11B**). In fact, 11 of the morphological properties that we considered differed considerably between the three different types of YFP$^+$ neurons, with a fairly consistent trend that MSNs possessed the highest means for each of these features, followed by FANS and then THIN (**Figure 11C**).

Heterogeneity of FANS

Although FANS were distinctly different from THIN and MSN in regard to their intrinsic electrical properties and morphology, there was considerable heterogeneity between individual FANS. FANS were diverse both in terms of their dendritic field orientation and in their AP firing patterns. For example, their dendritic fields could be oriented along the long axis of the cell body, towards one side of the cell body, or could radiate in numerous directions around the soma (**Figure 12**).

Hierarchical clustering, based on intrinsic electrical properties, revealed three subtypes of FANS (**Figure 13A**). We refer to these as Types I, II and III. The three subtypes of FANS showed differences in their AP amplitude, RMP and amount of AP amplitude adaptation (**Figure 13B**). These FANS subtypes also showed differences in most of the 12 properties used for classification (**Figure 13C** and
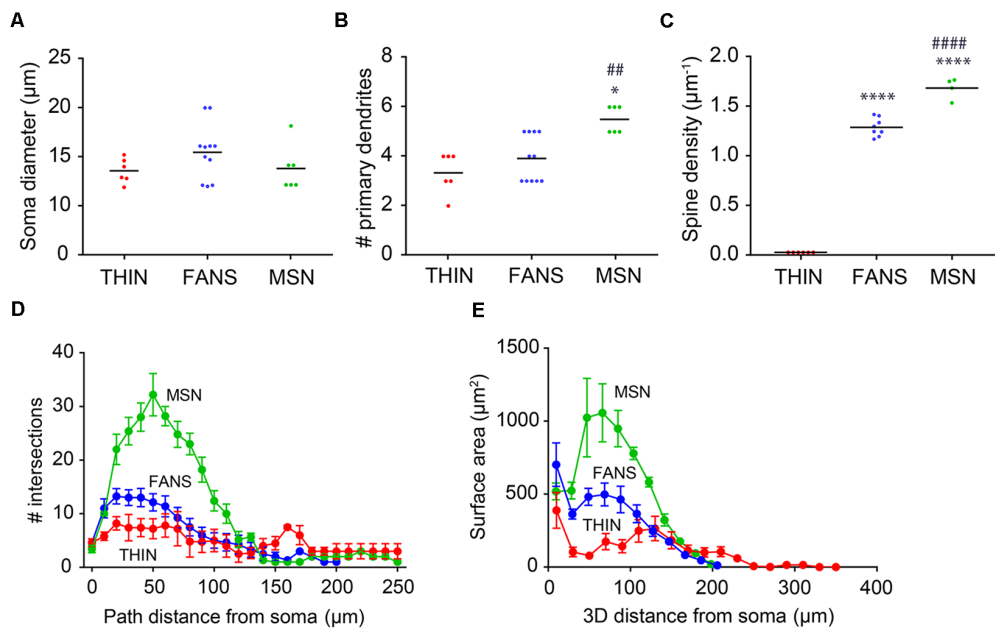
FIGURE 10 | Morphological analysis of YFP^{+} striatal neurons. **(A)** Soma diameter (one-way ANOVA, $p = 0.2458$; Dunn's multiple comparisons). **(B)** Number of primary dendrites (one-way ANOVA, $p = 0.0031$; Dunn's multiple comparisons; $*p < 0.05$ to THIN, $^{\#\#}p < 0.01$ to FANS). **(C)** Spine density (one-way ANOVA, $p < 0.0001$; Tukey's multiple comparisons; $****p < 0.0001$ to THIN, $^{\#\#\#\#}p < 0.0001$ to FANS). **(D)** Sholl analysis indicating dendritic arborization, with points indicating means and error bars indicating ±SEM. **(E)** Surface area vs. path distance plot to indicate dendritic branching, with points indicating means and error bars indicating ±SEM.
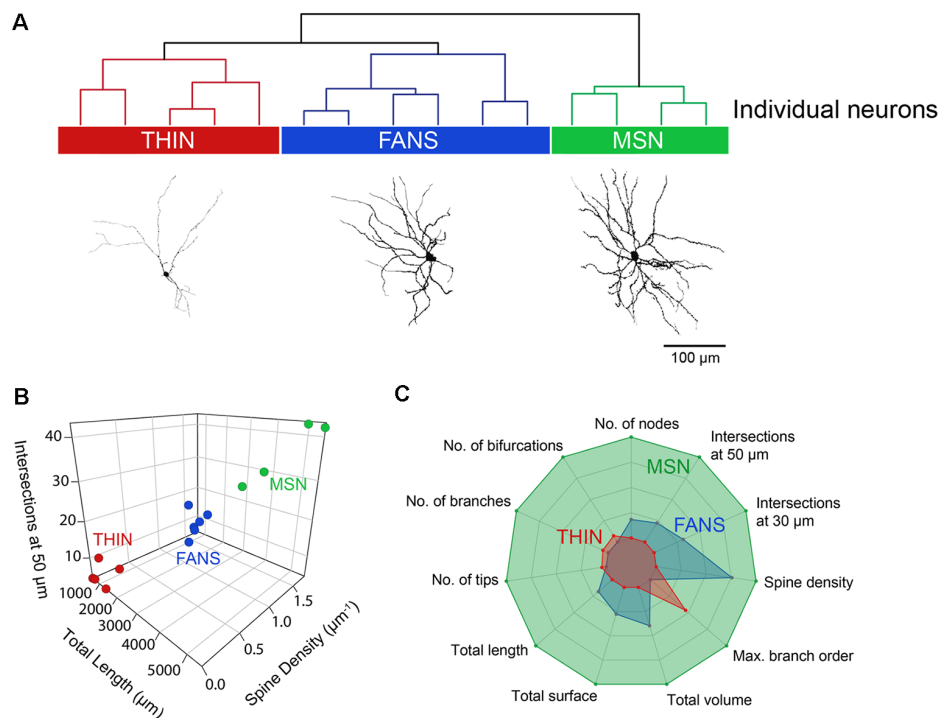
FIGURE 11 | Unsupervised hierarchical clustering of morphology recapitulates electrophysiologically defined cell types. **(A)** A dendrogram showing the unbiased classification of the three types of YFP^{+} neurons (top) and digital reconstruction of a representative cell from each type (bottom). **(B)** 3D scatter plots showing the clustering of the three YFP[+] cell types. **(C)** Radar plots revealing substantial differences between the three cell types, calculated using normalized population means in the range of 0–1.

FIGURE 12 | (A–J) A gallery of 10 neurobiotin-filled FANS showing their diverse morphology. Light lines indicate axons, wherever traceable; dark lines indicate dendrites.

DISCUSSION

Here, we describe a heterogeneous population of YFP$^+$ neurons in the striatum of transgenic mice with ChR2-YFP expression driven by the TH promoter.

Heterogeneity of YFP$^+$ Striatal Neurons

Three main subtypes of YFP$^+$ striatal neurons were found with distinct morphological and electrophysiological properties (**Figures 2**, **3**; **Table 3**). One subtype resembled the THIN described previously (Ibáñez-Sandoval et al., 2010), confirming the existence of these cells *via* a different genetic targeting strategy. Most THIN we examined were of the Type I or III described previously (Ibáñez-Sandoval et al., 2010). In fact, these two THIN subtypes are very similar to each other in terms of their firing patterns; they mainly differ in their RMP and input resistances. A second, small proportion of neurons resembled MSN. While this could arise from ectopic expression of Cre recombinase, as has been previously reported in some TH-Cre lines (Lammel et al., 2015; Stuber et al., 2015), it is also possible that these MSN might express TH at some point during their development. A third distinct population of YFP$^+$ striatal neurons has not been observed previously in mature animals; these neurons have morphological and electrophysiological properties that uniquely distinguish them from all other YFP-expressing neurons (**Figure 3**). We have named these cells FANS. FANS have passive membrane properties (C$_m$, R$_{in}$, RMP and sag amplitude) that are intermediate between THIN and MSN (**Figure 4**) and their AHPs are longest lasting and smallest in amplitude (**Figure 5**). Similar to THIN, FANS show substantial adaptation of AP frequency, 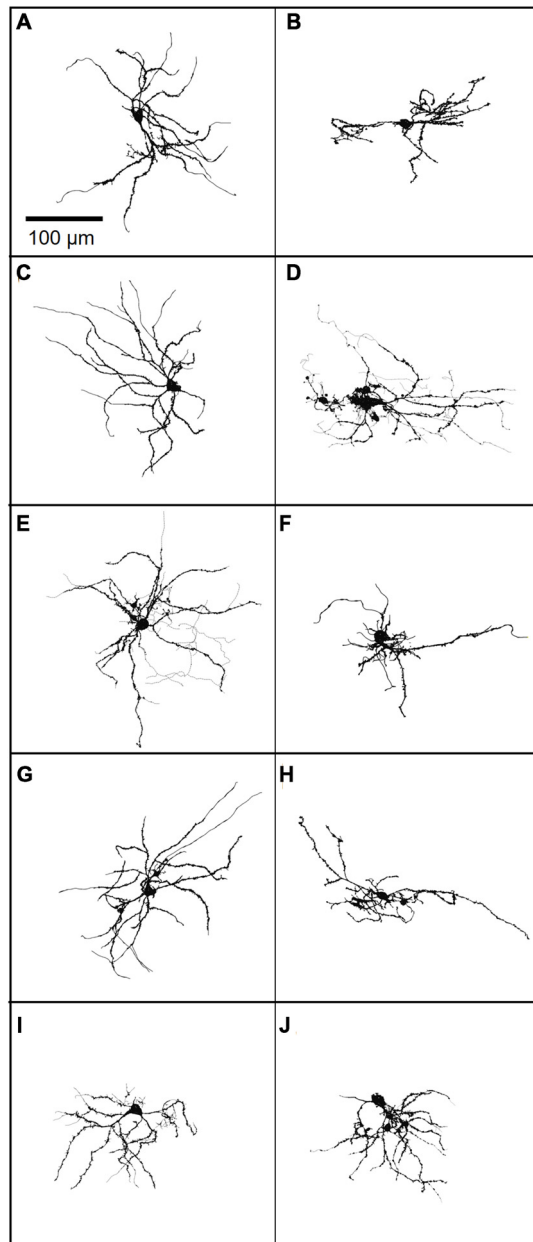duration and peak amplitudes during AP trains, but differ from THIN in the amount or rate of adaptation (**Figures 6–8**). Morphologically, FANS have dendritic spines; while MSN are the only striatal neurons previously described to have dendritic spines, our quantitative analysis indicates that FANS spine density and dendritic branching are significantly lower than that of MSN (**Figures 9–11**). Finally, FANS may represent a heterogeneous population because they have diverse morphological and electrophysiological properties (**Figures 12**, **13**).

Unique Properties of FANS

Our discovery of FANS is surprising because they were not reported in previous descriptions of THIN in TH-EGFP mice (Grace and Bunney, 1983; Ibáñez-Sandoval et al., 2010; Ünal et al., 2011, 2015) or in TH-Cre mice with virally expressed ChR2-EYFP (Xenias et al., 2015). The reason why FANS were not observed previously is unknown but could be related to their relatively low numbers and the absence of specific markers to identify them. In addition, transgene expression patterns may differ with the same promoter due to positional effect variegation. Finally, it could be that TH-expressing cells could have been underreported in previous experiments; for example, in the experiments of Xenias et al. (2015), not all dopamine-expressing neurons in the midbrain expressed EYFP. The electrophysiological properties of FANS are somewhat similar, but not identical, to those of the type A TH-expressing

Table 4). The temporal patterns of AP firing varied between the different classes of FANS, with depolarization-induced plateau potential present mostly in Type III FANS. Due to the relatively small number of FANS successfully filled with neurobiotin, it is not yet clear whether the differences in morphology and intrinsic electrical properties of FANS are correlated. However, based on an analysis of the 10 neurobiotin-filled FANS shown in **Figure 12**, it appeared that Type II FANS had less dendritic arborization than the other two FANS subtypes (**Figure 13A**, bottom).

FIGURE 13 | Heterogeneous morphological and functional properties of FANS. **(A)** Dendrogram revealing three subtypes of FANS. A representative cell's morphology and spiking pattern are shown for each subtype. **(B)** 3D scatter plot showing the three most distinct electrophysiological properties between the three subtypes. **(C)** Radar plot summarizing the overall differences between the three subtypes, calculated using normalized population means in the range 0–1.

TABLE 4 | Electrical properties of Type I, II and III FANS.

Property	Type I	Type II	Type III
C_m (pF)	76.8 ± 3.4	81.7 ± 7.0	73.3 ± 3.1
R_{in} (MΩ)	275 ± 21	164 ± 18	312 ± 29
RMP (mV)	−74.0 ± 1.7	−82.1 ± 2.3	−66.2 ± 2.1
Sag amplitude (mV)	1.8 ± 0.5	1.4 ± 0.3	1.3 ± 0.1
AP duration (ms)	3.3 ± 0.1	3.0 ± 0.2	2.8 ± 0.1
AHP (mV)	−3.7 ± 0.6	−5.1 ± 1.7	−3.1 ± 0.9
AP amplitude (mV)	100 ± 1.3	83.6 ± 2.9	83.1 ± 2.1
Maximum AP frequency adaptation (Hz)	45.8 ± 6.3	26.6 ± 14	46.3 ± 6.7
AP amplitude adaptation	0.75 ± 0.02	0.63 ± 0.04	0.82 ± 0.02
Current threshold (pA)	43.5 ± 2.5	27.5 ± 6.4	35.0 ± 4.6

Values indicate mean ± SEM.

striatal neurons described in neonatal mouse striatum by Masuda et al. (2011). Likewise, THIN are similar to what these authors call type B neurons. Thus, it is possible that these two types of TH-expressing neurons in neonatal striatum

are the developmental precursors of FANS and THIN in the adult.

FANS are not a subtype of THIN, because their firing patterns do not resemble any previously described THIN

subtypes. In addition, FANS have dendrites with a high density of spines, while THIN are largely aspiny; only a very small proportion of Type I THIN reportedly have spine-like appendages and the density of these structures still is much lower than the density of dendritic spines we observed in FANS (Ibáñez-Sandoval et al., 2010).

Two lines of evidence clearly indicate that FANS also are not MSN. First, none of the 12 electrophysiological and 11 morphological properties of FANS clustered with MSN in our unbiased clustering analyses (see **Figures 3** and **11**). Second, we did not encounter any FANS amongst non-YFP$^+$ neurons of the striatum; most non-YFP$^+$ neurons we examined were MSN while we occasionally encountered PV and cholinergic interneurons. With MSN making up $\sim$95% of the total striatal population, we would expect to see FANS relatively frequently among non-YFP$^+$ neurons if they are indeed a subtype of MSN. Finally, in contrast to the axons of MSN, we never observed the axons of FANS projecting out of the striatum. Therefore, we are confident that FANS are distinctly different from MSN.

FANS are also unlikely to be the other types of striatal interneurons that have been previously identified (see Tepper et al., 2010 for a review of striatal interneurons). For example, FANS do not exhibit the rapid APs that characterize PV interneurons. They also lack the low-threshold calcium spikes observed in SOM/NPY/NOS-positive interneurons and have much lower input resistance than these interneurons. Further, cholinergic interneurons have very large cell bodies, tend to fire spontaneous APs and have prominent hyperpolarization-induced sag, all properties that differ from those of FANS. Most importantly, unlike striatal interneurons, FANS have spines. We therefore conclude that FANS are a unique type of striatal neuron.

The plateau spikes generated in FANS in response to large depolarizing currents resemble the complex spikes seen in cerebellar Purkinje cells and cartwheel cells of the dorsal cochlear nucleus, two electrical signals that arise from activation of voltage-dependent calcium channels (Llinás and Sugimori, 1980; Kim and Trussell, 2007; Portfors and Roberts, 2007). Based on these similarities, we speculate that the depolarization-induced plateau spikes, as well as the other distinctive intrinsic properties, may allow FANS to provide input with a unique temporal structure in comparison to other striatal neurons. In addition, because FANS have dendritic spines, it is likely that they receive glutamatergic input from the cortex and/or dopaminergic input from the midbrain; both of these types of input contact dendritic spines on MSN (Freund et al., 1984; Dubé et al., 1988).

Several questions remain regarding the identity of FANS in the striatum. First, although we used TH-Cre mice as a tool for genetic access to FANS, it is not clear whether or not FANS express TH. It is possible that FANS do express TH at some time during their development or FANS may not express TH at all; as mentioned above, ectopic expression has been reported in TH-Cre mouse lines (Lammel et al., 2015; Stuber et al., 2015). Future work will be needed to understand

why Cre (and ChR2-YFP) is expressed in FANS in our mice. Second, it is not known what neurotransmitter(s) FANS use. For the case of GABAergic THIN, despite the expression of TH these neurons are not dopaminergic because they do not release dopamine and lack other molecular components required for dopamine synthesis, such as L-amino acid decarboxylase, dopamine transporter and vesicular monoamine transporter-2 (Xenias et al., 2015). Third, it is unclear whether FANS are projection neurons or interneurons. For the 10 FANS we successfully labeled with neurobiotin (**Figure 12**), axonal arborizations were visible and observed to ramify locally; they were never observed to send long-distance projections beyond their dendritic fields (see **Figures 12D,E**). This observation suggests that FANS, like THIN (Ibáñez-Sandoval et al., 2010), are likely to be interneurons.

CONCLUSIONS

We have discovered FANS, a novel type of neuron in the mouse dorsal striatum. The unique electrical and morphological properties of these neurons add a novel cellular element to striatal local circuitry; future work will be needed to determine the functions of these cells.

ETHICS STATEMENT

All mice used in the experiments were 6–14 weeks old of either sex and all procedures were approved by the Institutional Animal Care and Use Committee of Biopolis, Agency of Science and Technology, Singapore.

AUTHOR CONTRIBUTIONS

MM and GA designed the experiments. MM performed the experiments. MM and AN analyzed the data. MM, AN and GA wrote the article.

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Neural Correlates of Anesthesia in Newborn Mice and Humans

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Monitoring the hypnotic component of anesthesia during surgeries is critical to prevent intraoperative awareness and reduce adverse side effects. For this purpose, electroencephalographic (EEG) methods complementing measures of autonomic functions and behavioral responses are in use in clinical practice. However, in human neonates and infants existing methods may be unreliable and the correlation between brain activity and anesthetic depth is still poorly understood. Here, we characterized the effects of different anesthetics on brain activity in neonatal mice and developed machine learning approaches to identify electrophysiological features predicting inspired or end-tidal anesthetic concentration as a proxy for anesthetic depth. We show that similar features from EEG recordings can be applied to predict anesthetic concentration in neonatal mice and humans. These results might support a novel strategy to monitor anesthetic depth in human newborns.

Keywords: development, anesthesia, LFP, EEG, mouse, human, machine learning, network dynamics

INTRODUCTION

Reliable monitoring of anesthesia depth is critical during surgery. It allows for loss of consciousness, analgesia and immobility without incurring the risk of side effects and complications due to anesthetic misdosing. Typically used measures to monitor anesthesia depth are inspired and end-tidal anesthetic concentrations as well as physiologic parameters, including respiratory rate and depth (in the absence of neuromuscular blockade or controlled ventilation), heart rate, blood pressure, and responses to noxious stimuli (Punjasawadwong et al., 2014). These measures all respond to spinal and brainstem reflexes and are not specific for arousal or cortical responses to noxious events.

Anesthesia-induced changes in brain activity can be measured with electroencephalographic (EEG) recordings. Specific algorithms have been developed to predict anesthesia depth in adults (Glass et al., 1997; Prichep et al., 2004; Kreuzer, 2017). The most commonly used of such methods, the Bispectral Index, has been shown to significantly reduce intraoperative awareness, amount of anesthetic used, recovery time and post-anesthesia care unit stay in a recent Cochrane meta-analysis (Punjasawadwong et al., 2014), but see (Kalkman et al., 2011; Hajat et al., 2017). However, evidence of similar benefits in infants and younger children is sparse, as recently shown (Cornelissen et al., 2015, 2017, 2018a). EEG in anesthetized infants changes dramatically depending on postnatal age. Slow oscillations are present from birth on, whereas theta and alpha oscillations

occur 3 months after birth, but lack the frontal dominance typically seen in adults (Davidson et al., 2005; Lo et al., 2009; Hayashi et al., 2012; Cornelissen et al., 2015; Koch et al., 2017).

EEG recordings mainly monitor neocortical activity. Converging evidence from animal and human studies has shown that most anesthetics slow EEG oscillations (Alkire et al., 2008; Chauvette et al., 2011; Purdon et al., 2015). While power at high frequency oscillations is reduced (>40 Hz), power at slower frequencies (<15 Hz) is enhanced (Purdon et al., 2015). The computations underlying proprietary indexes such as the Bispectral index or Narcotrend are thought to take advantage of these phenomena (Kertai et al., 2012). However, in preterm and term neonates for the first weeks of life, EEG during sleep-wake cycles is weakly correlated with behavioral states and shows characteristic bursts or spontaneous activity transients (Milh et al., 2007; O'Toole et al., 2016). Anesthesia-induced theta and alpha oscillations have been reported to emerge around 3–4 months of age, albeit with less frontal predominance than in older children and adults (Cornelissen et al., 2015, 2018a). Moreover, high concentrations/doses of anesthetics have been reported to depress brain activity and enhance signal discontinuity in both human and rodent neonates (Chang et al., 2016; Cornelissen et al., 2017; Stolwijk et al., 2017). However, to our knowledge, a comprehensive algorithmic approach identifying EEG parameters that robustly correlate with anesthetic depth during early postnatal development is still lacking.

Here, we developed a novel strategy to model anesthesia depth by using common electrophysiological features that correlate with inhaled anesthetic concentrations during early development of mice and humans at similar stage of brain development. We performed intracranial electrophysiological recordings to study the temporal and dose-dependent dynamics of brain activity in neonatal mice [postnatal day (P) 8–10] during bolus urethane administration, and during dose-titrated isoflurane general anesthesia, respectively. Dominant local field potential (LFP) features of anesthetic state were identified and used to develop a machine-learning algorithm that distinguishes non-anesthetized from deeply anesthetized states and predicts anesthetic concentration as a proxy for anesthetic depth. Using a similar approach, we used multielectrode EEG recordings to study the dose-dependent dynamics of brain activity in a secondary analysis of a combined new and previously reported data set (Cornelissen et al., 2018a) of human infants 0–6 months of age during induction, maintenance and emergence from general anesthesia (sevoflurane, isoflurane, or desflurane) administered for routine surgical care. Dominant EEG features of anesthetic state were identified and used to develop a machine-learning algorithm to predict end-tidal volume anesthetic concentration (an indirect measure of anesthetic concentration in the brain, and anesthetic depth).

MATERIALS AND METHODS

Animals
All experiments were performed in compliance with the German laws and the guidelines of the European Community for the

use of animals in research and were approved by the local ethical committee (G132/12, G17/015, N18/015). Experiments were carried out on C57Bl/6J mice of both sexes. Timed-pregnant mice from the animal facility of the University Medical Center Hamburg-Eppendorf were housed individually at a 12 h light/12 h dark cycle, with *ad libitum* access to water and food. Day of birth was considered P0.

In vivo Electrophysiology in Neonatal Mice
Multisite extracellular recordings were performed in the prefrontal cortex (PFC) and HP, or lateral entorhinal cortex (LEC) and olfactory bulb (OB) of P8–10 mice. Pups were on a heating blanket during the entire procedure. Under isoflurane anesthesia (induction: 5%; maintenance: 2.5%), craniotomies were performed above PFC (0.5 mm anterior to Bregma, 0.1–0.5 mm right to Bregma) and HP (3.5 mm posterior to Bregma, 3.5 mm right to Bregma), or LEC (0 mm anterior to lambda, 6.5 mm right to lambda) and OB (0.5–0.8 mm anterior from the frontonasal suture, 0.5 mm right from internasal suture). Pups were head-fixed into a stereotaxic apparatus using two plastic bars mounted on the nasal and occipital bones with dental cement. Multisite electrodes (NeuroNexus, MI, USA) were inserted into PFC (four-shank, A4x4 recording sites, 100 μm spacing, 2.0 mm deep) and HP (one-shank, A1x16 recording sites, 50 μm spacing, 1.6 mm deep, 20° angle from the vertical plane), or LEC (one-shank, A1x16 recording sites, 100 μm spacing, 2 mm deep, 10° angle from the vertical plane) and OB (one-shank, A1x16 recording sites, 50 μm spacing, 1.4–1.8 mm deep). A silver wire was inserted into the cerebellum and served as ground and reference electrode. Pups were allowed to recover for 30 min prior to recordings. Extracellular signals were band-pass filtered (0.1–9,000 Hz) and digitized (32 kHz) with a multichannel extracellular amplifier (Digital Lynx SX, Neuralynx, Bozeman, MO, USA).

In vivo Electrophysiology in Juvenile Mice
Multisite extracellular recordings were performed in the PFC of P24–39 mice. Under isoflurane anesthesia (induction: 5%; maintenance: 2.5%), a metal head-post (Luigs and Neumann) was attached to the skull with dental cement and 2-mm craniotomies were performed above PFC (0.5–2.0 mm anterior to Bregma, 0.1–0.5 mm right to Bregma) and protected by a customized synthetic window. A silver wire was implanted in the cerebellum as ground and reference electrode. Surgery was performed at least 5 days before recordings. After recovery mice were trained to run on a custom-made spinning-disc. For recordings, craniotomies were uncovered and multisite electrodes (NeuroNexus, MI, USA) were inserted into PFC (one-shank, A1x16 recording sites, 50 μm spacing, 2.0 mm deep). Extracellular signals were band-pass filtered (0.1–9,000 Hz) and digitized (32 kHz) with a multichannel extracellular amplifier (Digital Lynx SX, Neuralynx, Bozeman, MO, USA).

Recordings Under Urethane
Activity was recorded for 15 min without anesthesia before intraperitoneally injecting urethane (1 mg/g body weight; Sigma-Aldrich, St. Louis, MO, USA). Activity was recorded for further 45 min. Animals were transcardially perfused after recordings,

brains were sectioned coronally, and wide field images were acquired to verify recording electrode positions.

Recordings Under Isoflurane

Mouth piece of an isoflurane evaporator (Harvard Apparatus, Holliston, MA, USA) was placed in front of the pups in the recording setup until animals accustomed to it. Activity was recorded for 15 min 0% isoflurane, but with the evaporator running (1.4 l/min). Afterward, isoflurane was added to the airflow and increased every 15 min (1%, 2%, 3%). Animals were transcardially perfused after recordings, brains were sectioned coronally, and wide field images were acquired to verify recording electrode positions.

Electroencephalographic Recordings in Human Neonates and Young Infants

Neonates and infants who were scheduled for an elective surgical procedure were recruited from the pre-operative clinic at Boston Children's Hospital from 12/2012 to 08/2018 (under Institutional Review Board P-3544, with written informed consent obtained from parents/legal guardians). Subjects required surgery below the neck, were clinically stable on the day of study and American Society of Anesthesiologists' physical status I or II. Exclusion criteria were born with congenital malformations or other genetic conditions thought to influence brain development, diagnosed with a neurological or cardiovascular disorder, or born at <32 weeks post-menstrual age. Datasets from previously published work ($n = 25$; Cornelissen et al., 2018a) and new subjects ($n = 10$) were included in the analysis. Data are presented from 35 subjects aged 0–6 months.

Anesthetic Management

Each patient received anesthesia induced with sevoflurane (32 subjects), isoflurane (two subjects) or desflurane (one subject) alone or a combination of one of the previous and nitrous oxide. Epochs used for analysis were comprised of sevoflurane, isoflurane or desflurane administration with air and oxygen, titrated to clinical signs; end-tidal anesthetic concentration was adjusted per the anesthetist's impression of clinical need, not a pre-set end-tidal anesthetic concentration.

EEG Recording

EEG data were acquired using an EEG cap (WaveGuard EEG cap, Advanced NeuroTechnology, Netherlands). Thirty-three- or 41-recording electrodes were positioned per the modified international 10/20 electrode placement system. Reference and ground electrodes were located at Fz and AFz respectively. EEG activity from 0.1 to 500 Hz was recorded with an Xltek EEG recording system (EMU40EX, Natus Medical Inc., Canada). Signals were digitized at a sampling rate of 1,024 Hz and a resolution of 16-bit. The EEG recording was started prior to anesthetic induction to capture the loss of consciousness and stopped once the participant reached the Post-Anesthesia Recovery to capture recovery of consciousness. For some infants, the EEG recording was started after anesthetic induction.

Clinical Data Collection

Demographics and clinical information were collected from the electronic medical records and from the in-house Anesthesia Information Management System (AIMS; **Supplementary Table S1**). None of the pathologies causing the need of surgery presented a risk for brain maturation. End-tidal sevoflurane, oxygen, and nitrous oxide concentrations were downloaded from the anesthetic monitoring device (Dräger Apollo, Dräger Medical Inc., Telford, PA, USA) to a recording computer in real-time using ixTrend software (ixcellence, Germany). Signals were recorded at a 1 Hz sampling rate.

Data Analysis

In vivo data were analyzed with custom-written algorithms in the Matlab environment. Data were processed as following: band-pass filtered (500–5,000 Hz) to analyze multi-unit activity (MUA) and band-pass filtered (2–100 Hz) using a third-order Butterworth filter before downsampling to analyze LFP. Filtering procedures were performed in a phase preserving manner.

Multi-Unit Activity

MUA was detected as the peak of negative deflections exceeding five times the standard deviation of the filtered signal and having a prominence larger than half the peak itself. Firing rates were computed by dividing the total number of spikes by the duration of the analyzed time window.

Detection of Oscillatory Activity

Discontinuous active periods were detected with a modified version of a previously developed algorithm for unsupervised analysis of neonatal oscillations (Cichon et al., 2014). Briefly, deflections of the root mean square of band-pass filtered signals (1–100 Hz) exceeding a variance-depending threshold were considered as network oscillations. The threshold was determined by a Gaussian fit to the values ranging from 0 to the global maximum of the root-mean-square histogram. If two oscillations occurred within 200 ms of each other they were considered as one. Only oscillations lasting >1 s were included, and their occurrence, duration and amplitude were computed.

Power Spectral Density

For power spectral density analysis, 1 s-long windows of full signal or network oscillations were concatenated, and the power was calculated using Welch's method with non-overlapping windows.

Imaginary Coherence

The imaginary part of coherence, which is insensitive to volume-conduction-based effects (Nolte et al., 2004), was calculated by taking the absolute value of the imaginary component of the normalized cross-spectrum.

Pairwise Phase Consistency

Pairwise phase consistency (PPC) was computed as previously described (Vinck et al., 2010). Briefly, the phase in the band of interest was extracted using Hilbert transform and the mean of the cosine of the absolute angular distance among all pairs of phases was calculated.

1/f Slope

1/f slope was computed as previously described (Gao et al., 2017). We used robust linear regression (MATLAB function *robustfit*)

to find the best fit over 20–40 Hz frequency range of the power spectral density, in 1-min bins.

Sample Entropy

Sample Entropy was computed using the SampEn function (MATLAB File Exchange) in 1.5 s windows and in 2 Hz frequency bins. Tolerance was set to 0.2 * std (signal), and tau to 1.

EEG Data Analysis

EEG signal was visually inspected to detect and reject channels with low signal to noise ratio and re-referenced to a common average reference. The signal was automatically scored in 5 s epochs, and channels in which signal was significantly contaminated by artifacts (patient handling, surgical electrocautery etc.) were discarded. Epochs were rejected if signal was saturated due to electrocautery, signal exceeded 150 μV, or the median signal across all EEG channels exceeds 30 μV (**Supplementary Figure S5**). Minutes containing more than 10 s of contaminated signal were removed from further analysis. On average $14 \pm 9\%$ (median ± median absolute deviation) of the signal was discarded. To compute EEG amplitude, we smoothed the absolute value of the signal, using a moving average filter with a window of 1,024 points (1 s). If more than one volatile anesthetic was used, we retained only epochs in which the main anesthetic was used in isolation. Subjects with epidural anesthesia halfway through the surgery ($n = 2$ subjects), or with less than 20 min of artifact-free signal ($n = 5$ subjects) were excluded from further analysis.

Feature Engineering

Features to predict anesthetic concentration in neonatal mice were calculated in 1-min bins. LFP power in the 1–100 Hz range in 10 Hz bins, the percentage of active periods, median length and number of oscillations, median and maximum signal amplitude were computed. All features were computed for both PFC and HP and were normalized to their median value in the non-anesthetized 15 min of recordings. Features to predict anesthetic concentration in human infants were also calculated in 1-min bins. The median amplitude of the smoothed EEG signal and the percentage of the EEG envelope that fell into each amplitude quartile was computed. Amplitude quartiles were computed on the entire EEG trace, averaged over channels. All features were calculated for unfiltered signal, and in the 1–50 Hz range in 5 Hz bins, averaged over channels. Features were normalized to their median value in the non-anesthetized portion of the recording, or lowest anesthetic concentration, if no artifact-free minute was available.

Regressors

Machine-learning analyses were performed using Python (Python Software Foundation, NH, USA) in the Spyder (Pierre Raybaut, The Spyder Development Team) development environment. Model training and performance evaluation were carried out using the scikit-learn toolbox. The set was iteratively ($n = 100$) divided between a training (2/3 of the set) and a cross-validation (1/3) set. Hyper-parameter of the model was tuned on the training set, which was further split using the standard three-fold cross-validation split implemented by the function "GridSearchCV," to which a "pipeline" object was passed. The "pipeline" object was built using the "Pipeline" function, and concatenating quantile transformation of the input features ("Quantile Transformer," tuning the number of quantiles), feature selection ("Select Percentile," using mutual information and tuning the percentage of features to select) and Radial Basis Function (RBF) kernel support-vector classification/regression (tuning the regularization parameters C and epsilon (regression only), and the kernel coefficient gamma). The classifier input was fed to the regressor as an additional feature. Performance assessment was then computed on the cross-validation set. Regressor decision space was reduced and plotted with t-sne. The decision space was approximated by imposing a Voronoi tessellation on the 2d plot, using k-nearest regression on the t-sne coordinates (Migut et al., 2015).

Statistics

Statistical analyses were performed using R Statistical Software (Foundation for Statistical Computing, Austria). Data were tested for significant differences (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) using non-parametric one- and two-way repeated-measures ANOVA (ARTool R package) with Bonferroni corrected *post hoc* analysis (emmeans R package). Correlations were computed using Spearman's rank correlation coefficient (rho). No statistical measures were used to estimate the sample size since the effect size was unknown. For the main experimental results, more information about tests used, values and parameters are provided in the supplementary material (**Supplementary Table S2**).

Data Availability

Electrophysiological data for hippocampus and prefrontal cortex mouse recordings, under both urethane and isoflurane condition, is available at the following open-access repository: https://web.gin.g-node.org/mchini/Neural_correlates_of_anesthesia_in_newborn_mice_and_humans.

RESULTS

Anesthesia Affects the Occurrence but Not the Spectral and Temporal Structure of Oscillatory Events in Neonatal Mice

We monitored the impact of anesthesia on immature brain activity in several cortical areas [PFC, hippocampus (HP), and LEC] as well as in a sensory area OB. For this, multi-site extracellular recordings of LFP and MUA were performed from P8 to 10 mice before and for 45 min after induction of anesthesia by intraperitoneal urethane injection (**Figure 1A**), an anesthetic commonly used in rodents (Khazipov et al., 2004; Colonnese and Khazipov, 2010).

The recorded network activity had a highly fragmented structure (defined as discontinuous activity) in all investigated areas (PFC, HP, LEC and OB). The full signal (i.e., entire LFP trace) consisted of transient episodes of oscillatory discharges with mixed frequencies (from here referred to as "active periods"), alternating with periods of relative electrical silence
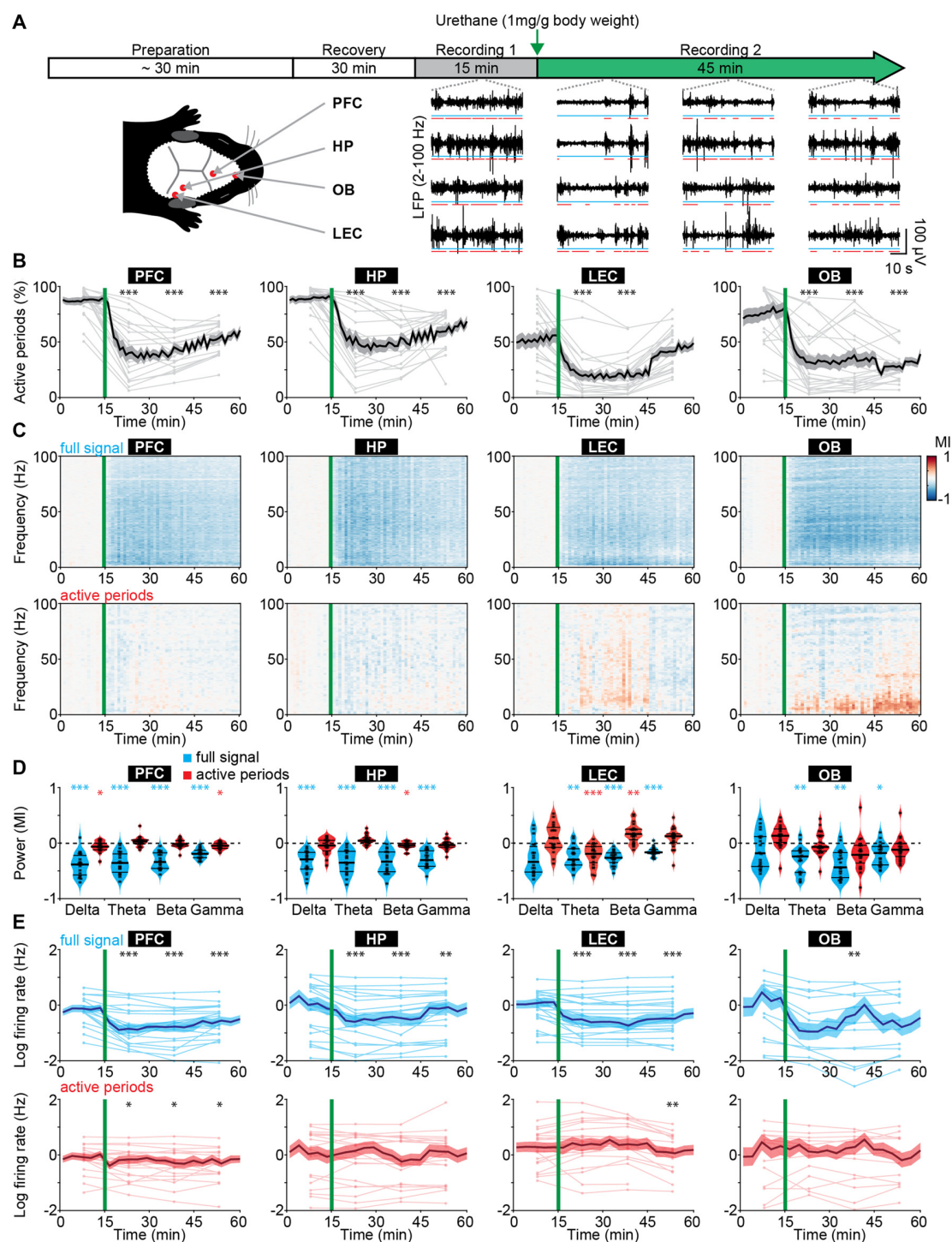
FIGURE 1 | Frequency-unspecific dampening of neuronal activity during urethane anesthesia in neonatal mice. (A) Schematic representation of experimental paradigm and recording sites as well as characteristic local field potential (LFP) recordings of discontinuous activity in the prefrontal cortex (PFC), HP, lateral entorhinal cortex (LEC), and olfactory bulb (OB) of neonatal mice (P8–10) during non-anesthetized and urethane-anesthetized state. Time windows of active periods are marked by red lines. (B) Line plots displaying the relative occurrence of active periods normalized to total recording time in PFC, HP, OB and LEC before and after urethane injection. (C) Color-coded MI of power spectra for full signal (top) and active periods (bottom) recorded in PFC, HP, LEC and OB of neonatal mice before and after urethane injection. (D) Violin plots displaying the MI of power in delta (2–4 Hz), theta-alpha (4–12 Hz), beta (12–30 Hz) and gamma (30–100 Hz) frequency bands for full signal (blue) and active periods (red) recorded in the PFC, HP, LEC and OB. (E) Line plots displaying multi-unit activity (MUA) rates during full signal (blue) and active periods (red). In (B,C,E) green lines correspond to the time point of urethane injection.

and suppressed activity (from here referred to as "silent periods"; **Figure 1A**; Khazipov et al., 2004; Hanganu et al., 2006; Brockmann et al., 2011; Bitzenhofer et al., 2017; Gretenkord et al., 2019). The prevalence of active periods decreased rapidly and robustly over time in all investigated brain areas upon urethane injection (**Figure 1B**). The most prominent reduction was observed 5–15 min after urethane injection. A partial recovery towards baseline levels during the following 30 min was detected in cortical areas, and to a lesser extent in OB (**Figure 1B**). The temporal sequence of events likely reflects the pharmacokinetics of urethane and is line with the previously reported quick onset (few minutes) and long-lasting effects of urethane anesthesia (2–4 h; Huh and Cho, 2013).

The anesthesia-induced reduced occurrence of active periods was reflected in a broadband (1–100 Hz) decrease in oscillatory power shown as modulation index (MI) defined as $(\text{power}_{\text{post}} - \text{power}_{\text{pre}})/(\text{power}_{\text{post}} + \text{power}_{\text{pre}})$. In contrast, power spectra during active periods were largely unaffected (**Figure 1C**). Of note, for OB, delta and theta power during active periods increased over time after urethane injection and might be related to an observed augmentation of respiration amplitude. Spectral properties of full signal and active periods were quantified for delta (2–4 Hz), theta-alpha (4–12 Hz), beta (12–30 Hz) and gamma (30–100 Hz) frequency bands for the first 15 min post urethane administration, the time window of strongest reduction of active periods. In contrast to the significant reduction of full signal power in all frequency bands, the power during active periods was only marginally affected by anesthesia (**Figure 1D**). Thus, urethane anesthesia affected network activity in the immature rodent brain predominantly by decreasing the amount of active periods without perturbing the frequency structure of active periods. This is in stark contrast with the well-characterized switch from a low-amplitude high-frequency regime to a high-amplitude low-frequency regime of electrical activity that has been reported for the adult rodent and human brain (Voss and Sleigh, 2007; Alkire et al., 2008).

Anesthesia was shown to induce alterations of long-range network interactions in adult rodents (Bettinardi et al., 2015) and humans (Ferrarelli et al., 2010; Lewis et al., 2012; Sarasso et al., 2015). We examined whether similar alterations are present in the immature mouse brain. Simultaneous recordings of HP and PFC, as well as OB and LEC were analyzed to assess the effects of anesthesia on long-range functional coupling. We previously showed that at the end of the first postnatal week hippocampal theta bursts drive the oscillatory entrainment of local circuits in the PFC, whereas discontinuous activity in OB controls the network activity in LEC (Brockmann et al., 2011; Ahlbeck et al., 2018; Gretenkord et al., 2019). Urethane did not modify these interactions. The synchrony within networks quantified by HP-PFC and OB-LEC coherence was similar during baseline (no urethane anesthesia) and in the presence of urethane (**Supplementary Figure S1A**). These data indicate that the core features of long-range functional coupling are retained under anesthesia in neonatal mice.

Anesthesia modified neuronal firing in all investigated areas. Firing rates in PFC, HP, LEC and OB decreased after urethane

injection and only partially recovered during the following 45 min (**Figure 1E**). However, firing rates during active periods were only marginally affected. To examine whether the timing of neuronal firing to the phase of oscillatory activity was altered by anesthesia, we calculated PPC, a firing rate-independent measure of spike-LFP phase locking (Vinck et al., 2010). All four brain regions showed similar frequency-resolved phase locking profiles before and after urethane injection (**Supplementary Figures S1B,C**).

Anesthetics have been shown to alter the excitation/inhibition balance in the adult brain through their action on specific ion channels involved in synaptic transmission (Gao et al., 2017). Such alteration is usually monitored by changes in the 1/f slope of power spectral density. Further, signal complexity and information content measured by sample entropy have been correlated with behavioral states of adults, such as consciousness, sleep/wake states and anesthesia (Liang et al., 2015; Liu et al., 2018). For neonatal mice, we observed similar values of 1/f slope and sample entropy before and during urethane anesthesia (**Supplementary Figures S1D–F**), suggesting that urethane does not perturb cortical excitation/inhibition balance and signal complexity at this early age. The findings provide additional evidence to the hypothesis that anesthesia has unique effects on the immature brain.

To add additional evidence for this hypothesis, we extended the time window of investigation and performed extracellular recordings from the PFC of juvenile mice (P24–39). At this age oscillatory activity is continuous, thus a distinction between active and inactive periods is not possible. In contrast to the frequency-unspecific reduction of active periods in neonates, urethane anesthesia increased the oscillatory power in the delta frequency band and suppressed power in beta and gamma frequency bands (**Supplementary Figure S2**), confirming the anesthetic effects in the adult brain (Alkire et al., 2008; Chauvette et al., 2011; Purdon et al., 2015).

Taken together, these results indicate that urethane anesthesia dampened neonatal brain activity mainly by augmenting the discontinuity of network activity, i.e., reducing the proportion of time the brain spent in active periods. However, the active periods were largely unaffected in their temporal structure and firing dynamics. In contrast, urethane anesthesia in older mice led to frequency-specific changes. Thus, urethane anesthesia differently impacts neonatal and adult brain activity in mice.

Suppression of Active Periods Predicts Anesthetic Concentration in Neonatal Mice

To test whether the effects of urethane on neonatal brain activity generalize to other anesthetics, we performed LFP and MUA recordings from HP and PFC of P8–10 mice at increasing doses of isoflurane-induced anesthesia (0, 1, 2 and 3%; 15 min per concentration; **Figure 2A**). Urethane hyperpolarizes neurons by potentiating a resting potassium conductance (Pagliardini et al., 2013), whereas most other anesthetics, such as isoflurane and sevoflurane, mainly act by potentiating $GABA_A$ receptor-mediated transmission (Franks, 2006). Isoflurane reduced the incidence of active periods in a dose-dependent manner (**Figure 2B**). Accordingly, the broadband reduction of LFP power
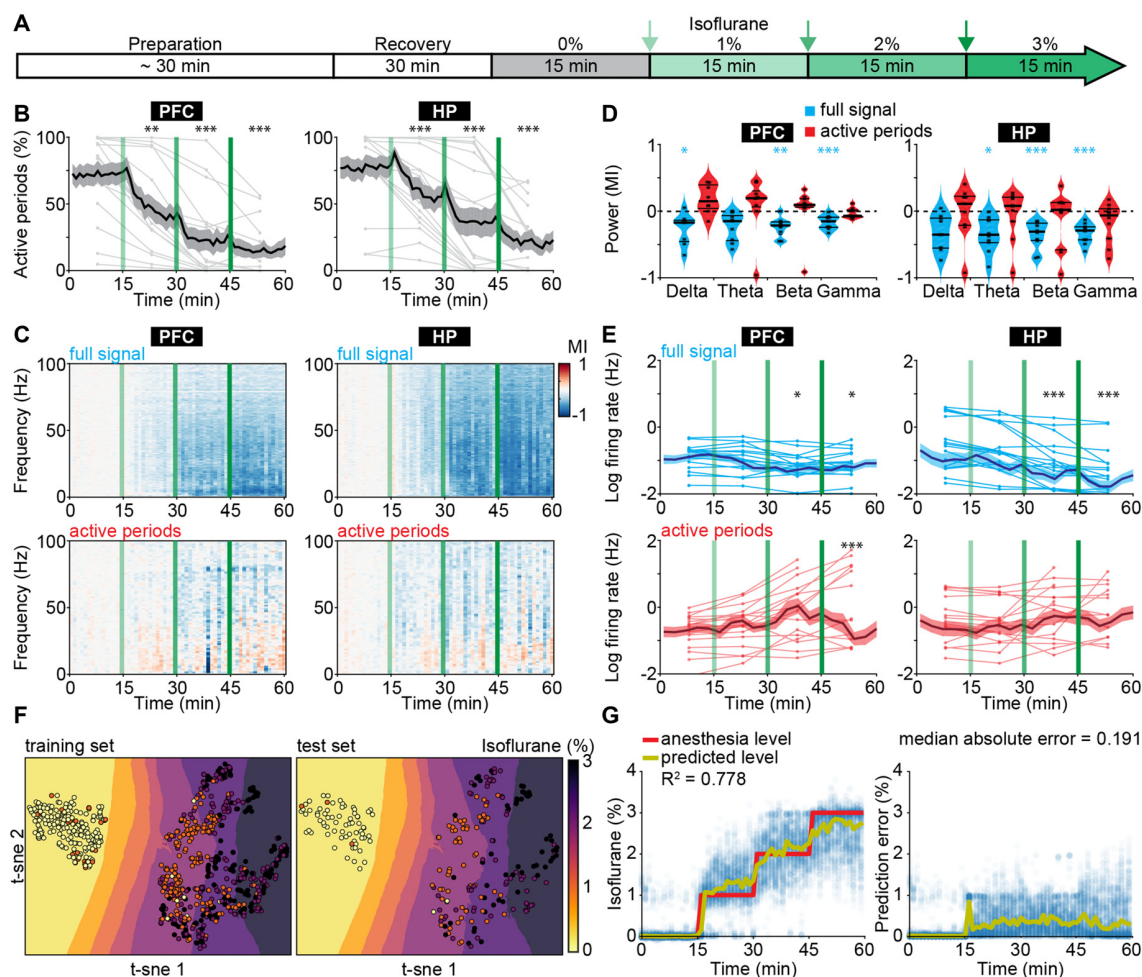
FIGURE 2 | Suppression of active periods in relationship with the depth of isoflurane anesthesia in neonatal mice. **(A)** Schematic representation of experimental protocol for LFP recordings without anesthesia and during increasing levels of isoflurane anesthesia in neonatal mice (P8–10). **(B)** Line plots displaying the relative occurrence of active periods in PFC and HP during increasing levels of isoflurane anesthesia. **(C)** Color-coded MI of power spectra for full signal (top) and active periods (bottom) during increasing levels of isoflurane anesthesia. **(D)** Violin plots displaying the MI of power in delta (2–4 Hz), theta (4–12 Hz), beta (12–30 Hz) and gamma (30–100 Hz) frequency bands for full signal (blue) and active periods (red). **(E)** Line plots displaying MUA firing rates during full signal (blue) and active periods (red). In **(B,C,E)** green lines correspond to the time points of increasing isoflurane anesthesia. **(F)** Visualization of anesthesia depth prediction by t-sne plots. Background color codes for predicted anesthesia depth, while the color of the dots represents the actual anesthesia level in the training (left) and test set (right). **(G)** Scatter plots displaying anesthesia depth predictions with support vector regression (left) and absolute errors between anesthesia depth prediction and actual anesthesia depth (right).

was also dependent on isoflurane concentration (**Figures 2C,D**). Power spectra of active periods remained largely unaffected in the presence of isoflurane, similarly to the urethane effects (**Figures 2C,D**). MUA rates during active periods in PFC and HP were hardly modified in the presence of isoflurane, yet the overall firing decreased corresponding to the reduced occurrence of active periods (**Figure 2E**). Together, these findings identify the suppression of active periods as the main effect of bolus urethane injection and isoflurane anesthesia in the neonatal mouse brain.

The development-specific response of the immature brain to anesthesia might represent the main obstacle when trying to predict anesthesia depth in infants using algorithms based on the mature brain activity of adults. Therefore, we next aimed to use electrophysiological properties specific for anesthetized

neonatal mice to predict the concentration of administered isoflurane. We used support vector regression (**Supplementary Figure S3**), with the following input features: median amplitude of broadband LFP, percent of time spent in active periods, and spectral power from 1 to 100 Hz in 10 Hz bins for both hippocampal and prefrontal activity. An additional feature was the output of a support vector classifier that received the same features as for the support vector regression, and that was designed to predict whether the animal was under anesthesia or not. The algorithm accurately predicted anesthesia depth across all levels of isoflurane concentration (**Figures 2F,G**). Estimation of information content of the different features identified the median amplitude of broadband LFP as the most informative feature (**Supplementary Figure S4A**). As the power of active

periods was only marginally affected by anesthesia, this feature mainly mirrors the suppression of active periods. Interestingly, the algorithm was also able to distinguish non-anesthetized from anesthetized recordings from neonatal mice under urethane, even though it had not been exposed to this dataset during training (**Supplementary Figure S4B**).

Thus, features of electrophysiological activity that capture the particularities of immature neuronal networks can predict anesthetic concentration in neonatal mice. The generalization of the classifier to a different anesthetic indicates that it can identify general anesthesia-related features of brain activity in neonatal mice.

Frequency-Unspecific Suppression of Activity in Anesthetized Human Neonates and Young Infants

To test if human neonates and infants, similarly to mice, respond to anesthesia with a broadband decrease of periods of oscillatory activity, we examined EEG recordings from humans aged 0–6 months postnatal age, who received general anesthesia with volatile anesthetics (sevoflurane 32 subjects, isoflurane two subjects, desflurane one subject) for surgery (**Supplementary Table S1**).

In neonatal mice, the median LFP amplitude of broadband activity was identified as the most informative feature to predict anesthetic depth. We, therefore, applied the same data analysis approach to human EEG data (**Supplementary Figure S5**). We found the median amplitude of broadband EEG activity

(averaged across all recording electrodes across the scalp) was negatively correlated with end-tidal anesthetic concentration (etAnesthetic) in human neonates from birth until 2 months postnatal age (**Figures 3A,B**). For older human infants, the correlation of the median EEG amplitude with the anesthetic concentration switched to a positive correlation, in agreement with adult human data (Hagihira, 2015). This relationship was even stronger using expected birth age, corrected for conceptional age (**Supplementary Figure S6A**). This switch from negative to positive correlation was also visible in the normalized median EEG amplitude when averaged for age-grouped babies (0–2, 2–4, 4–6 months; **Figure 3C**).

Quantification of median EEG amplitude across frequencies revealed a broadband suppression of EEG activity in human neonates of 0–2 months (**Figure 3D**). In contrast, the relationship between activity amplitude and etAnesthetic indicated frequency-specificity in human infants of 2–4 and 4–6 months, as previously reported (Cornelissen et al., 2017). Frontal activity has been shown to be particularly sensitive to age-varying anesthesia-related effects in human neonates (Cornelissen et al., 2015). Analysis of only frontal electrodes (Fp1, Fp2, F3, F4, F7, F8, Fpz) showed the same age-dependent anesthesia-induced changes as analysis of full scalp electrodes (**Supplementary Figures S6B–D**).

Thus, analogous to what we found in neonatal mice, general anesthesia in human infants younger than 2 months suppressed neuronal population activity, as reported previously (Cornelissen et al., 2015), while at older age anesthesia induced frequency-specific effects.

FIGURE 3 | Age-dependent switch from broadband suppression to frequency-specific effects of general anesthesia on electroencephalographic (EEG) activity in human neonates and infants. **(A)** Scatter plots displaying the median EEG amplitude as a function of anesthetic concentration for representative examples of 0–2, 2–4 and 4–6 months of age. **(B)** Scatter plot displaying the correlation coefficient of median EEG amplitude and anesthetic concentration in relation to birth age for sevoflurane (black), isoflurane (red), and desflurane (blue). **(C)** Line plots displaying normalized EEG amplitude as a function of anesthetic concentration. **(D)** Color-coded MI of median EEG amplitudes in different frequency bands as a function of anesthetic concentration for human babies of 0–2 months (left), 2–4 months (middle) and 4–6 months (right).

A Model to Predict End-Tidal Volume of Sevoflurane Anesthesia in Human Neonates and Infants

The correlation of EEG activity with etAnesthetic as well as the similar effects of anesthesia in neonatal mice and in humans from birth to 2 months old, suggests that anesthetic depth in babies might be predicted using similar features to those used in neonatal mice. To test this, we used a machine-learning algorithm with a similar architecture as the one we developed for neonatal mice (**Supplementary Figure S3**). The algorithm was modified to account for the developmental switch from broadb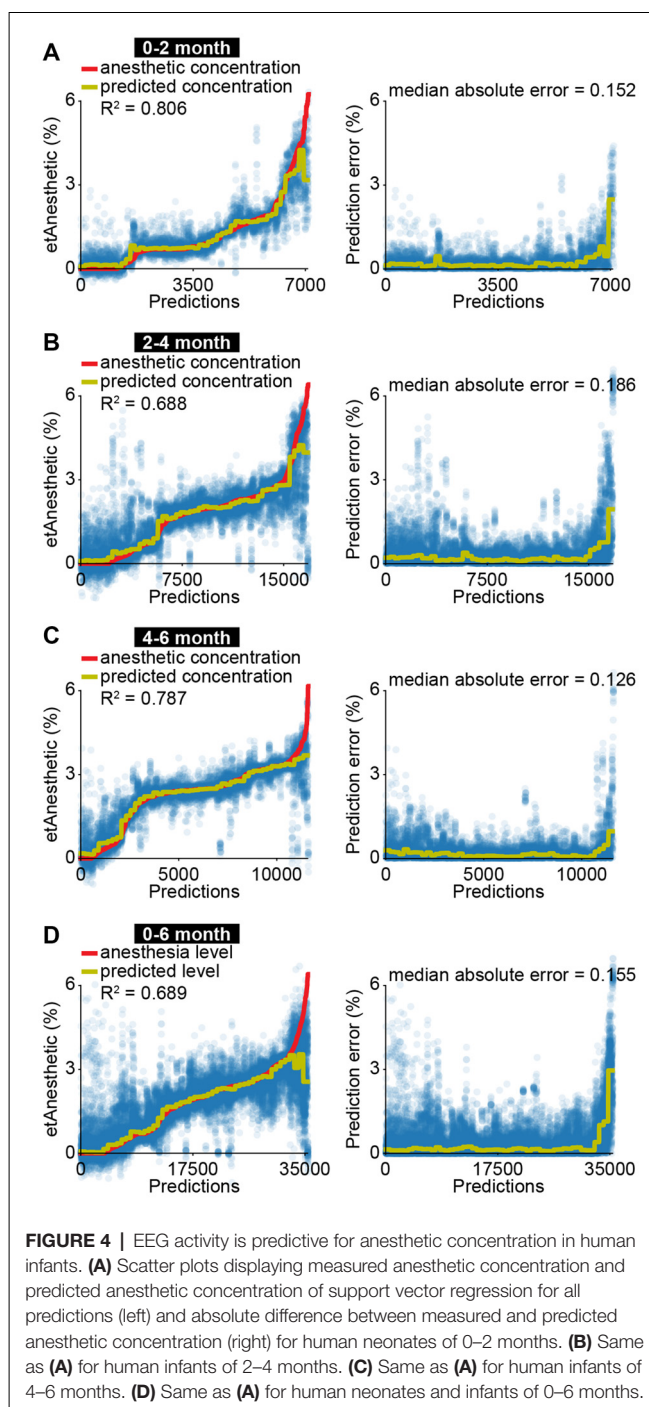and suppression to frequency-specific modulation by training three different regressors using 2 and 4 months as cut-offs. All regressors received the same input features (see "Materials and Methods" section and **Supplementary Figure S5**). Features derived from EEG activity were able to predict etAnesthetic with high accuracy for all age groups (0–2 months $R^2 = 0.806$, 2–4 months $R^2 = 0.688$, 4–6 months $R^2 = 0.787$; **Figures 4A–C**). In line with the frequency-specific alterations observed only in the older age groups, frequency-related features were rated more important for prediction of anesthesia depth in infants of 2–4 and 4–6 months than in neonates of 0–2 months (**Supplementary Figures S7A–C**). Predicting anesthesia depth for all ages with a single classifier considering age as an input feature performed with high accuracy (0–6 months $R^2 = 0.689$; **Figure 4D**, **Supplementary Figure S7D**). Training the predictor with only 20% of the data and testing it on the remaining 80% resulted in a reduced, but still solid prediction ($R^2 = 0.512$, median absolute error = 0.209) indicating the robustness of the predictor (**Supplementary Figure S8A**). This result confirms the age-varying effects of anesthesia on the brain and stresses the importance of considering age when developing algorithms aiming to assess anesthetic depth. Predicting anesthesia depth with a reduced number of four EEG channels (F4, P4, F3, P3) or two EEG channels (P4, P3) often used for long-term monitoring yielded similar results (four channels $R^2 = 0.713$, median absolute error = 0.134; two channels $R^2 = 0.671$, median absolute error = 0.161; **Supplementary Figures S8B,C**).

Thus, mouse and human neonates show similar changes in network activity in response to anesthesia. These results highlight how neurophysiological activity could be beneficial for future attempts at predicting anesthetic depth in clinical settings.

DISCUSSION

Monitoring brain function during anesthesia is desirable to avoid intraoperative awareness and side effects resulting from unnecessarily high doses of anesthetics. Since consciousness is an elusive concept and cannot be directly measured, EEG features have been used to guide anesthesia delivery during human surgery. Monitoring methods developed for adults perform poorly in human neonates and infants, particularly during the first months of life (Davidson et al., 2005; Hayashi et al., 2012; Poorun et al., 2016; Koch et al., 2017). Age-specific effects of anesthetics on immature brain activity are considered

FIGURE 4 | EEG activity is predictive for anesthetic concentration in human infants. **(A)** Scatter plots displaying measured anesthetic concentration and predicted anesthetic concentration of support vector regression for all predictions (left) and absolute difference between measured and predicted anesthetic concentration (right) for human neonates of 0–2 months. **(B)** Same as **(A)** for human infants of 2–4 months. **(C)** Same as **(A)** for human infants of 4–6 months. **(D)** Same as **(A)** for human neonates and infants of 0–6 months.

the main reason for such poor performance. Implementation of neonate- and infant-specific anesthesia monitors requires elucidation of distinct anesthesia-induced EEG features during early development. We took advantage of a translational approach to address this open question. We first carried out an in-depth investigation of anesthetic effects on brain activity in neonatal mice, and then applied this knowledge to develop features that would correlate with an anesthetic concentration in human neonates.

In contrast to the continuous EEG signal observed in adults, neonatal EEG around birth is characterized by a highly discontinuous and fragmented temporal organization, with bursts of cerebral activity (active periods) alternating with interburst intervals lacking activity (silent periods; Anderson et al., 1985; Connell et al., 1987; Stockard-Pope et al., 1992; Lamblin et al., 1999; Vecchierini et al., 2003, 2007; Stevenson et al., 2017). Neonatal mice show a similar discontinuous organization of cortical activity (Khazipov et al., 2004; Hanganu et al., 2006; Brockmann et al., 2011). In accordance with the similar organization of early activity patterns in age-matched mouse pups and human infants, we found comparable effects of anesthesia on LFP and EEG signals, respectively.

It is well established that in the adult rodent and human brain most anesthetics favor slow oscillations at the expense of faster ones, thereby slowing the EEG rhythm (Alkire et al., 2008; Chauvette et al., 2011; Purdon et al., 2015). This principle is thought to underlie most algorithms that are clinically used to predict anesthesia depth (Davidson et al., 2005). Indeed, such algorithms perform poorly with anesthetics, such as ketamine, that do not share this mechanism of action (Hans et al., 2005). In line with previous studies (Ackman et al., 2014; Kirmse et al., 2015), we report that both urethane and isoflurane anesthesia affect brain activity in a different way in neonatal mice. Instead of favoring slow oscillations at the expense of faster ones, anesthesia in neonatal mice broadly suppresses activity in a frequency-unspecific manner. The dampening of cortical activity for human infants of 0–2 months suggests a development specific effect of anesthesia on immature brain activity that translates between mice and humans.

In rodents, the switch from activity suppression to frequency-specific modulation of neuronal activity by anesthesia has been reported to occur around P12 (Ackman et al., 2014). This coincides with the emergence of slow oscillations during sleep, suggested to depend on the maturation of thalamocortical networks (Steriade et al., 1993; Ackman et al., 2014). Consistent with our previous studies evaluating EEG properties of this data set, we found that theta and alpha oscillatory activity under anesthesia emerges in humans at around 4 months postnatal age (Cornelissen et al., 2015, 2017, 2018a). Developmental changes in the brain explain the changes in EEG dynamics that occur with postnatal age. In humans, gross brain development occurs in a caudal to rostral direction, with myelination of the medulla, pons, and thalamus starting within the first few postnatal weeks, and frontal cortex myelination starting around 3–4 postnatal months (Brody et al., 1987; Kinney et al., 1988). Regional differences in the rate of synaptogenesis, glucose metabolism and myelination across the cortex occur between subcortical and cortical regions, and between different regions of the cortex during the first 12 postnatal months in human infants (Huttenlocher and Dabholkar, 1997; Tau and Peterson, 2010; Dehorter et al., 2012; Catts et al., 2013; Semple et al., 2013; Murata and Colonnese, 2019). A key role in brain development is played by subplate neurons, the first neurons generated in the cerebral cortex, which guide the formation of thalamocortical connections (Kanold and Luhmann, 2010; Kostović and Judas, 2010). The subplate cells form the first functional connections

and relay oscillatory activity in the developing brain (Kanold and Luhmann, 2010). Alpha oscillations in the anesthetized brain are postulated to be produced by thalamocortical circuits, and the gradual emergence of highly powered alpha oscillations at 4-months of age suggests an important developmental milestone has been reached in the processes guided by the subplate neurons. Future studies with a wider age range in mice and humans, including data of human infants studied at preterm, and children in older than 6 months of age, may deepen the understanding of anesthetic effects on brain activity throughout development.

The anesthetics evaluated across species in this study were comparable but not identical in terms of mechanism of action. Moreover, anesthetic management practices used in mice were simplified compared to commonly-used anesthetic practices in the clinic. Multimodal anesthesia requires the use of low-dose anesthetics in combination with analgesic and neuromuscular blocking agents to provide optimal anesthesia and reduce the side effect. These agents act on different drug targets in the nervous system and may have subtle but different effects on brain oscillatory activity (Brown et al., 2018).

In adult human volunteers, the correlation with anesthetic depth and EEG parameters can be performed using verbal reports to establish a threshold for unconsciousness (Purdon et al., 2015). However, in non-verbal populations such as human infants, one must rely on indirect behavioral measures which are more readily performed on emergence rather than induction and incision (Cornelissen et al., 2018b). Future investigations need to include surgical incision and other stimuli into the mouse models to understand with greater granularity the anesthetic titration around the minimal concentrations required to suppress movement, autonomic, and cortical responses to noxious stimuli.

In summary, we report that the suppression of brain activity in mouse and human neonates correlates with anesthetic concentration. The detailed understanding of anesthesia effects on network activity in mice allowed us to identify features and develop a machine-learning algorithm that is able to predict anesthetic concentration from EEG recordings in human neonates. We propose that, after appropriate training, an algorithm based on what we introduce here could learn to associate specific EEG effects with certain anesthetic doses. Eventual mismatches between administered and predicted anesthetic dose would then identify patients that are particularly sensitive/insensitive to an anesthetic, thus helping the anesthetist in administering appropriate levels of anesthetics. By these means, the risk of adverse neurodevelopmental outcome might be mitigated.

ETHICS STATEMENT

All animal experiments were performed in compliance with the German laws and the guidelines of the European Community for the use of animals in research and were approved by the local ethical committee (G132/12, G17/015, N18/015). Human neonates and infants who were scheduled for an elective surgical procedure were recruited from the pre-operative clinic at Boston Children's Hospital from 12/2012 to 08/2018 (under Institutional

Review Board P-3544, with written informed consent obtained from parents/legal guardians).

AUTHOR CONTRIBUTIONS

MC, SB and IH-O designed the experiments. MC, SB, SG, JK, JP, and LC carried out the experiments. MC, SB, SG, JK and JP analyzed the data. MC, SB, LC, CB and IH-O interpreted the data and wrote the article. All authors discussed and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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Microglia Regulate Pruning of Specialized Synapses in the Auditory Brainstem

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The assembly of uniquely organized sound localization circuits in the brainstem requires precise developmental mechanisms. Glial cells have been shown to shape synaptic connections in the retinogeniculate system during development, but their contributions to specialized auditory synapses have not been identified. Here we investigated the role of microglia in auditory brainstem circuit assembly, focusing on the formation and pruning of the calyx of Held in the medial nucleus of the trapezoid body (MNTB). Microglia were pharmacologically depleted in mice early in development using subcutaneous injections of an inhibitor of colony stimulating factor 1 receptor, which is essential for microglia survival. Brainstems were examined prior to and just after hearing onset, at postnatal days (P) 8 and P13, respectively. We found that at P13 there were significantly more polyinnervated MNTB neurons when microglia were depleted, consistent with a defect in pruning. Expression of glial fibrillary acidic protein (GFAP), a mature astrocyte marker that normally appears in the MNTB late in development, was significantly decreased in microglia-depleted mice at P13, suggesting a delay in astrocyte maturation. Our results demonstrate that monoinnervation of MNTB neurons by the calyx of Held is significantly disrupted or delayed in the absence of microglia. This finding may reflect a direct role for microglia in synaptic pruning. A secondary role for microglia may be in the maturation of astrocytes in MNTB. These findings highlight the significant function of glia in pruning during calyx of Held development.

Keywords: microglia, calyx of Held, MNTB, pruning, auditory brainstem, CSF1R inhibitors, depletion

INTRODUCTION

Binaural cues used for sound localization are initially processed in auditory brainstem circuits that require speed and reliability (Trussell, 1999). Accordingly, these pathways display specialized synapses. A key component is the calyx of Held (1893), the large, encapsulating excitatory axon terminal of globular bushy cells (GBC) in the anteroventral cochlear nucleus (AVCN) that terminates in the contralateral medial nucleus of the trapezoid body (MNTB; Morest, 1968; Smith et al., 1991; Kandler and Friauf, 1993). This synapse possesses several hundred active sites and

a large readily releasable pool of synaptic vesicles that allow for fast, sustained, and precise signaling to MNTB neurons (Borst and Sakmann, 1996; Taschenberger et al., 2002).

Medial nucleus of the trapezoid body neurons also receive other excitatory inputs such as collaterals of spherical bushy cells (Cant and Casseday, 1986), GBCs (Hamann et al., 2003), or descending input from higher auditory structures (Kuwabara et al., 1991) as well as inhibitory inputs (Albrecht et al., 2014). The MNTB is a sign-inverting relay nucleus that sends glycinergic inhibitory projections to several auditory brainstem nuclei, including the lateral superior olive (LSO; Kuwabara and Zook, 1991; Tollin, 2003) and medial superior olive (MSO; Spangler et al., 1985; Kuwabara and Zook, 1992) on the same side. The LSO also receives frequency-matched excitatory input from the ipsilateral AVCN (Sanes, 1990; Glendenning et al., 1992) and uses the balance of inhibitory MNTB and excitatory VCN inputs to compute interaural intensity differences used in sound localization (**Figure 1A**; Tollin, 2003; Kandler and Gillespie, 2005; Grothe et al., 2010). In the MSO, inhibitory input is needed for computation of interaural time differences, which provide sound localization cues for lower frequencies (Brand et al., 2002; Pecka et al., 2008; Myoga et al., 2014). The MNTB is thus a key relay nucleus for many aspects of auditory processing.

The calyx of Held undergoes major changes during development. In early postnatal development, axonal collaterals form multiple small "protocalyces" on MNTB neurons (Kandler and Friauf, 1993; Hoffpauir et al., 2006). These synapses are then refined and excess synapses are pruned until a single calyx remains on each MNTB neuron (Morest, 1968; Satzler et al., 2002; Hoffpauir et al., 2006; Holcomb et al., 2013). This pruning results in a largely one-to-one innervation pattern from the AVCN to the MNTB (Harrison and Warr, 1962; Friauf and Ostwald, 1988; Bergsman et al., 2004; Wimmer et al., 2004).

Communication between neurons and glia is required for the establishment of functional neural circuits (Reemst et al., 2016; Neniskyte and Gross, 2017). Microglia, the resident immune cells of the brain, are critical for brain wiring (Hammond et al., 2018) and synaptic pruning during development (Paolicelli et al., 2011; Ji et al., 2013). Their gene expression profiles, and likely their functions, vary with brain region (Grabert et al., 2016; De Biase and Bonci, 2018; Masuda et al., 2019). Microglia are most diverse in the developing mouse brain, with more homogenous populations present in the adult brain (Hammond et al., 2019; Li et al., 2019). We tested whether brainstem microglia contribute to the assembly of specialized auditory pathways. In the brainstem, microglia appear in the AVCN by postnatal day 0 (P0) and by P6 they are seen in the MNTB. Microglia peak in number during the second postnatal week, coinciding with the maturation of the calyx of Held. Several studies have shown that microglia influence expression of astrocytic proteins (Elmore et al., 2014; Spangenberg et al., 2016; Jin et al., 2017). Both astrocytes and microglia are found in close proximity to the developing calyx, indicating their possible involvement in its growth and maturation (Holcomb et al., 2013; Dinh et al., 2014).

Here, we treated animals during postnatal development with an inhibitor of colony stimulating factor 1 receptor (CSF1R), a tyrosine kinase receptor essential for microglia survival

(Stanley et al., 1997; Erblich et al., 2011; Elmore et al., 2014). We demonstrated that this treatment effectively eliminated microglia during the postnatal period. We found that microglia depletion was associated with reduced expression of the mature astrocytic marker, glial fibrillary acidic protein (GFAP), suggesting a delay in astrocyte maturation. Significantly, elimination of microglia prevented the pruning of polyinnervated MNTB neurons. These results suggest that microglia are needed for synaptic pruning in the MNTB.

MATERIALS AND METHODS

Animals

We used 42 postnatal day 8 (P8) and 46 P13 wild-type C57BL/6 mice of both sexes. All procedures were approved by the University of California, Irvine Institutional Animal Care and Use Committee.

Treatment

Animals were treated with BLZ945 (MW: 398.48, MedChem Express HY-12768/CS-3971), a small molecule inhibitor of CSF1R (Pyonteck et al., 2013), to eliminate microglia. BLZ945 was dissolved in dimethylsulfoxide (DMSO; D136-1; Thermo Fisher Scientific) by gradually adding it to the vial with BLZ945 and briefly vortexing. 0.01 mL of solution was administered subcutaneously (sc) at a dose of approximately 200 mg/kg at P2. The same volume of drug was administered at P4 and P6 for the P8 endpoint. Additional injections were delivered at P8 and P10 for the animals perfused at P13. DMSO-only injections were used as controls (**Figure 1B**). At P8 or P13 mice were weighed and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3 (PBS). Brainstems were dissected and postfixed in PFA solution. After 2–3 h, brains were equilibrated in a 30% sucrose solution in 0.1 M PBS then sectioned coronally at 18 μm using a cryostat (CM 1850-3-1; Leica Microsystems). Sections were mounted on chrome-alum coated glass slides in a 1-in-5 series.

Neuronal Tracing

In 11 animals at P8 and 13 animals at P13 we performed neuronal tracing to fill calyces of Held in the MNTB. Mice were perfused transcardially with artificial cerebrospinal fluid (aCSF; 130 mM NaCl, 3 mM KCl, 1.2 mM KH$_2$PO$_4$, 20mM NaHCO$_3$, 3 mM HEPES, 10 mM glucose, 2 mM CaCl$_2$, 1.3 mM MgSO$_4$ perfused with 95% O$_2$ and 5% CO$_2$). Brains were quickly dissected and placed in a chamber with oxygenated aCSF.

The brain was temporarily transferred to a Petri dish with aCSF and axonal projections from the AVCN to the MNTB were filled using a rhodamine dextran amine (RDA; MW 3000, Invitrogen) solution (6.35% RDA with 0.4% Triton-X100 in PBS). A pulled glass micropipette was filled with RDA and pulses of RDA were delivered close to the midline in the ventral acoustic stria (VAS) using an Electro Square Porator (ECM830; BTX) at a rate of 5 pulses per second (pps) at 55 V for 50 ms. These pulses resulted in sparse labeling of GBC axons and calyces of Held on both sides of the brainstem (**Figures 1C,D**). In some
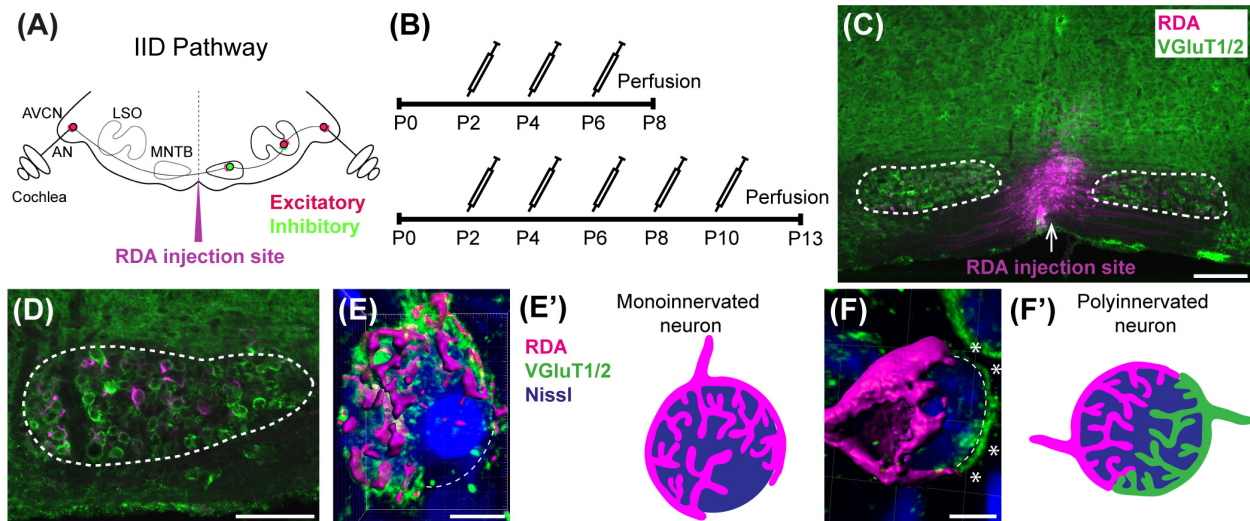
FIGURE 1 | Calyx labeling, reconstruction in 3D, and analysis. **(A)** A schematic of the interaural intensity differences (IID) pathway and RDA injection site in the VAS to sparsely label calyces of Held in the MNTB. GBCs in the AVCN receive direct input from the AN. In turn, GBCs synapse on inhibitory neurons in the contralateral MNTB. MNTB neurons then innervate excitatory neurons in the LSO. LSO neurons also receive tonotopically matched excitatory terminals from SBCs in the ipsilateral AVCN. LSO neurons compute the balance of frequency-matched inhibition and excitation arising from both ears to determine the sound source location in the horizontal plane. **(B)** Timeline of injections. Two age cohorts were included in the study. The P8 mice group received vehicle or BLZ945 injections at P2, P4, and P6, and then were perfused at P8. Animals in the P13 age group were injected every 2 days from P2 until P10, and then perfused at P13. **(C)** A photograph of an RDA injection site (magenta) in the VAS and sparsely labeled calyces in the mouse brainstem. VGluT1/2 labeling is shown in green. **(D)** A higher magnification of the left MNTB with sparsely RDA-labeled calyces of Held. **(E)** Reconstructed calyx area used for size and volume measurements (magenta). MNTB neurons were classified into mono- or polyinnervated based on the presence of PV or VGluT1/2 labeling outside the RDA-labeled calyx. Shown is an example of a monoinnervated neuron. VGluT1/2 labeling is present and is co-localized to the RDA-labeled calyx. Dashed line indicates approximate boundary of the Nissl-stained neuron that is contacted by the RDA-filled calyx of Held. **(E')** Schematic illustration of a monoinnervated MNTB neuron contacted by an RDA-labeled calyx. **(F)** An example of a polyinnervated MNTB neuron. It is contacted by an RDA-filled calyx and an additional VGluT1/2 positive calyceal input (indicated by white asterisks) around the remaining surface of the same neuron (dashed line). **(F')** Schematic illustration of a polyinnervated MNTB neuron. Both the RDA-labeled calyx and one or more additional inputs terminate on the same MNTB neuron. Scale bar in panel **(C)** = 200 μm; scale bar in panel **(D)** = 100 μm; scale bar in panel **(E)** = 6 μm; scale bar in panel **(F)** = 5 μm. Abbreviations: AN – auditory nerve, AVCN – anterior ventral cochlear nucleus, GBC – globular bushy cell, LSO – lateral superior olive, MNTB – medial nucleus of the trapezoid body, SBC – spherical bushy cell, VAS – ventral acoustic stria. Panel **(A)** has been adapted from Joris and Yin (1998).

cases, sequential labeling was performed with Alexa 488 dextran amine (DA488; 6.35% with 0.4% Triton-X100 in PBS) so that a single brainstem would have AVCN axons sparsely labeled with two different fluorophores. The brain was then placed back into the aCSF chamber for approximately 2 h under continuous oxygenation to allow for dye transport. The tissue was then transferred to 4% PFA solution for another 2 h followed by incubation in 30% sucrose solution in 0.1 M PBS. Brainstems were cryosectioned in the coronal plane at 18 μm and mounted on an alternating series of four slides. Slides with adequately labeled axons and calyces of Held were immunolabeled for parvalbumin (PV) at P8 and for vesicular glutamate transporters 1 and 2 (VGluT1/2) at P13.

Immunofluorescence

We performed immunofluorescence for a calcium binding protein specific for microglia (IBA1), VGluT1/2, vesicular GABA transporter (VGAT), synaptophysin1 (Syn), PV, and astrocytic markers – S100 calcium-binding protein B (S100β), aldehyde dehydrogenase 1 family member L1 (ALDH1L1), and GFAP.

Mounted sections were surrounded with a PAP pen hydrophobic barrier and rinsed in 0.1 M PBS for 10 min. For antigen retrieval, tissue was incubated for 5 min in 0.1%

sodium dodecyl sulfate in 0.1 M PBS solution followed by washes in 0.1 M PBS. Sections were then blocked with normal goat blocking solution containing 5% normal goat serum (NGS; Vector Laboratories S-1000) and 0.3% Triton X-100 (Acros 9002-93-1) in 0.1 M PBS for 1 h in a humidity chamber at room temperature followed by overnight incubation in primary antibodies. The tissue was then rinsed in 0.1 M PBS and incubated for 1 h in goat anti-rabbit, anti-guinea pig, or anti-chicken secondary antibody tagged with an Alexa (Invitrogen) fluorophore. Sections were washed in 0.1 M PBS and incubated in red or blue fluorescent Nissl stain (NeuroTrace 530/615 or 435/455, Life Technologies N21482 or N21479) diluted 1:200 in 0.3% Triton X-100 in 0.1 M PBS. After 1 h, tissue was rinsed in 0.1 M PBS and coverslipped with Glycergel mounting medium (Dako C0563).

For PV labeling, tissue was incubated for 3 h in a blocking solution made of 10% bovine serum albumin (Sigma 1002552698), 5% NGS, and 0.1% Triton X-100 in 0.1 M PBS followed by a 48-h incubation with mouse anti-PV primary antibody at 4°C. Sections were then allowed to equilibrate at room temperature for 30 min and rinsed 5 times in 0.1 M PBS. Tissue was placed in the blocking solution for 30 min then incubated in goat anti-mouse secondary antibody tagged

with an Alexa 488 fluorophore. Three hours later, slides were rinsed and incubated for 1 h in fluorescent Nissl stain diluted 1:200 in 0.3% Triton X-100 in 0.1 M PBS, washed 3 times and coverslipped with Glycergel mounting medium. The list of primary and secondary antibodies used in this study and their concentrations are listed in **Table 1**.

Fluorescent Microscopy

We acquired 10× and 20× magnification images of sections throughout the rostro-caudal extent of the MNTB using a Zeiss Axioskop-2 microscope, an Axiocam camera, and Axiovision software. We analyzed the MNTB on both sides of the midline, including only sections with an intact MNTB. For each animal in our analysis, at least three sections were included per primary antibody.

Microglial, Astrocytic, and Synaptic Protein Areal Coverage Analysis

For each specimen, a series of 20× multichannel fluorescent photographs spanning the rostro-caudal extent of the MNTB was assembled as an image stack in FIJI (Schindelin et al., 2012). For each section, the channel corresponding to Nissl-staining was used to outline the boundary of the MNTB, which was stored as a region-of-interest (ROI) polygon in FIJI. All subsequent operations were performed on the image channel corresponding to the immunolabeling of interest (e.g., VGAT). To correct for minor irregularities in overall illumination and/or labeling intensity across a set of sections, a level adjustment was applied such that the mean pixel intensity within each MNTB ROI was equivalent across all sections of an image stack without causing over- or undersaturation. Only global (section-wide) adjustments of pixel values were permitted; local manipulations were not made.

To identify positive immunolabeling, a histogram of pixel intensity was constructed based on all pixels within all MNTB ROIs in the image stack. This histogram was used as the input for automatic image thresholding in FIJI where a threshold value was computed using the *Default* algorithm and applied to all images in the stack. Label/optical density for each section was evaluated and the areal coverage ratio was quantified as the total number of thresholded pixels within a given MNTB ROI.

Microglia Counts

Each section used for IBA1 areal coverage analysis was examined for microglial cell counts. Microglial cell bodies were identified by a small cell body with processes radiating out of it. Microglia were not counted if only IBA1-positive processes but no somata were present. If a microglial cell body was on the edge of the MNTB ROI (drawn with reference to Nissl staining), it was excluded if <50% of the soma was inside of the ROI. IBA1-positive cells were counted using FIJI's "Cell Counter" plugin. The average number of cells per MNTB slice was calculated by dividing the microglia count by the number of MNTB slices examined.

Confocal Microscopy

Rhodamine dextran amine-labeled and PV- (P8) or VGluT1/2-immunolabeled (P13) tissue was used for confocal microscopy (Leica SP8, 63× oil objective, zoom: 1.5, pinhole: 1) and subsequent analysis. Nissl, RDA, and PV or VGluT1/2 z-stack images of calyces of Held were acquired at a resolution of 1024 × 1024 pixels, with a z-step size of 0.5 μm (Grande et al., 2014; Wang et al., 2018). Gain and offset were set for each fluorescent channel and each slide separately. In a few cases settings were adjusted to account for differences in labeling intensity between sections on the same slide. Image stacks were exported and the surface area and volume of calyces of Held were analyzed using the surface module in Imaris software (v9.2.1; Bitplane). Calyces throughout the MNTB were randomly labeled with RDA and, thus, the entire medio-lateral extent of the MNTB was analyzed. Surfaces of RDA-labeled calyces of Held were reconstructed using the default mode and then manually adjusted to achieve as accurate RDA fill of the calyx of Held as possible (**Figure 1**). Sections were coded and analyzed blind to treatment group. We did not change the intensity of

TABLE 1 | List of antibodies used in the project.

Antigen	Host	RRID	Cat. no.	Source	Dilution
Primary antibodies					
ALDH1L1	Rabbit	N/A	ab177463	Abcam	1:500
IBA1	Rabbit	AB_839504	019-19741	Wako	1:500
GFAP	Chicken	AB_304558	ab4674	Abcam	1:1000
GlyT2	Rabbit	AB_2619997	272 003	Synaptic Systems	1:200
S100β	Rabbit	AB_882426	ab52642	Abcam	1:500
Synaptophysin	Guinea Pig	AB_1210382	101 004	Synaptic Systems	1:500
VGAT	Rabbit	AB_2492282	2100-VGAT	PhosphoSolutions	1:200
VGluT1/2	Rabbit	AB_2285905	135 503	Synaptic Systems	1:200
Secondary antibodies					
Alexa 488	Chicken	AB_2534096	A11039	Thermo Fisher Scientific	1:200
Alexa 488	Guinea Pig	AB_2534117	A11073	Thermo Fisher Scientific	1:500
Alexa 488	Rabbit	AB_2633280	A32731	Thermo Fisher Scientific	1:500
Alexa 647	Rabbit	AB_2535812	A21244	Thermo Fisher Scientific	1:500
Alexa 647	Guinea Pig	AB_2735091	A21450	Thermo Fisher Scientific	1:500

confocal stacks to avoid affecting surface area and volumetric results. Only complete or near complete RDA-filled calyces of Held with visible labeled preterminal axon segments were included in the analysis. However, the preterminal axon itself was cropped out of the images where possible. Additionally, RDA-labeled calyces that were too close to one another to be individually reconstructed were excluded from the analysis. All calyces that matched the criteria were analyzed from at least one slide for each animal used. If the number of labeled calyces from one slide was too small (<3), an additional slide was used, however, care was taken to avoid examining serial sections (**Table 2**).

Determination of Monoinnervation

At the time of surface rendering, the mono- or polyinnervation status of the postsynaptic neuron was evaluated. Sparse RDA-labeled brainstems were immunolabeled for PV (P8) or VGluT1/2 (P13) and MNTB cells with RDA-labeled input were analyzed. For a monoinnervated MNTB neuron, there would be no PV or VGluT1/2 labeling outside an RDA-labeled calyx (**Figures 1E,E'**). If additional inputs were present, we would see PV or VGluT1/2 along the surface of the MNTB neuron but outside the RDA-labeled calyx (**Figures 1F,F'**). MNTB neurons normally transition through a period in which a single dominant input is seen as early as P6 (Holcomb et al., 2013) and thus smaller calyces may be transiently present. However, small areas of spurious labeling with presynaptic markers outside the RDA label would not necessarily correspond to

additional calyces (Cant and Casseday, 1986; Kuwabara et al., 1991; Smith et al., 1998). We set a threshold such that if PV or VGluT1/2 labeling outside the RDA-labeled calyx covered 25% or more of the MNTB cell surface, we considered that MNTB cell to be polyinnervated. This percent coverage was an estimated subjective score made blind to treatment group, and was roughly the size of the smallest calyces we labeled (see section "Results"). We then determined the percentage of analyzed MNTB neurons that were polyinnervated for each group (**Table 2**).

Neuronal Density

To test for effects of drug treatment on cell number, we determined the cell density in AVCN ($n = 4$ DMSO; $n = 4$ BLZ945) and MNTB ($n = 9$ DMSO; $n = 9$ BLZ945) at P13. Tissue was prepared as described previously and cells were plotted using blue fluorescent Nissl staining. Fiji's "Cell Counter" was used to count neurons within the ROI of each AVCN and MNTB (see above). Principle neurons of the MNTB were identified as homogenous cells that were round or slightly oval in shape and similar in size (Hoffpauir et al., 2006; Weatherstone et al., 2017). Only neuronal bodies with a visible nucleus and nucleolus were counted in every fifth section throughout the MNTB on both sides of the midline, including only intact MNTB sections. Neuronal density was calculated by dividing the sum of neuronal counts from each MNTB by the sum of MNTB area in the slices used for analysis.

TABLE 2 | Numbers of calyces of Held analyzed.

Treatment	Age	Animals used	Animal ID	Calyces measured	N neurons scored for innervation	N polyinnervated neurons
DMSO	P8	6	B143	12	15	13
			B144	8	13	11
			B153	13	11	11
			B156	9	15	10
			B160	11	15	15
			B216	8	9	6
BLZ945	P8	5	B141	13	10	9
			B149	10	22	16
			B154	11	14	11
			B217	12	7	4
			B215	19	23	14
DMSO	P13	6	B432	3	4	1
			B438	10	11	1
			B455	10	12	1
			B459	7	6	0
			B460	8	10	0
			B461	8	10	2
BLZ945	P13	7	B429	5	6	5
			B437	9	11	5
			B439	6	10	5
			B454	9	12	2
			B456	11	12	5
			B457	11	15	4
			B458	11	9	3

VGluT1/2 Co-localization With EGFP-Labeled Microglia

We performed VGluT1/2 immunofluorescence in $CX_3CR1^{+/EGFP}$ reporter mice to investigate a possible internalization of presynaptic proteins in microglia. These mice have enhanced green fluorescent protein (EGFP) inserted into exon 2 of CX_3CR1, resulting in EGFP-positive microglial cells (Jung et al., 2000). We used 3 heterozygous mice at P8 and 3 at P13. Animals were perfused as described above and the same immunolabeling protocol was used to label for VGluT1/2. High resolution confocal imaging was used to build z-stacks through the microglial cell (Leica SP8, 63× oil objective, zoom: 3.5, pinhole: 0.6, resolution of 2048 × 2048 pixels, z-step size of 0.5 μm). Subsequently, z-stacks were deconvolved using Huyens essentials software and examined using Imaris. Due to the nature of on-slide immunolabeling, VGluT1/2 antibody penetration was not always complete, thus, z-stacks were cropped so that EGFP-positive microglia were in the same focal plane as VGluT1/2 labeling. A co-localization function in Imaris was used to build the co-localization channel and thresholds of overlap of EGFP and VGluT1/2 channels were set to 10% and kept the same for all microglial cells analyzed. A total of five microglial cells per animal were imaged from non-consecutive sections, thus, 15 cells per age group in total.

Software Accessibility

FIJI scripts will be made available upon request to Dr. Michael A. Muniak.

Experimental Design and Statistical Analysis

To account for variability among litters, multiple litters were used and each of them was divided into control and experimental animals. Mice for each cohort were allocated randomly. Quantitative results for all cell counts, calyx of Held sizes, neuronal density, and innervation status as well as coverage of positive immunohistochemistry are represented by mean scores ± standard error of mean (SEM) and were analyzed using Prism (v7; GraphPad Software). Comparisons between different treatment and age groups were made using a two-way ANOVA and Sidak's multiple comparisons test unless stated otherwise. Statistical significance was accepted at $p < 0.05$. Details of statistical analysis are presented in **Table 3**.

RESULTS

Microglia Depletion With BLZ945

Two age groups of mice were selected for the experiment: P8 and P13, corresponding to ages before and after hearing onset, respectively. A total of 38 mice were used for the P8 age cohort, of which 20 were injected with DMSO and 18 with BLZ945. A total of 46 animals were included in the P13 age group, of which 26 were injected with DMSO and 20 with BLZ945. Littermate controls were used in each age cohort.

BLZ945-treated mice were smaller and had some craniofacial changes, such as shorter snouts and smaller teeth, than their control littermates. This effect is akin to the phenotype seen in CSF1R KO mice (Dai et al., 2002). Occasionally, BLZ945 treatment resulted in loss of pigment, in particular, in areas around the ears and neck. Overall, survival rates were near 100% for DMSO and 75% for BLZ945. We examined expression patterns of microglial marker IBA1 in response to vehicle or BLZ945 injections. At P8 we found microglia distributed throughout the extent of the AVCN and MNTB in the control group (**Figures 2A,B**). However, three subcutaneous injections (at P2, P4, and P6) of BLZ945 in developing pups, in most cases, completely depleted microglia in the brainstems of the P8 mice (**Figures 2C,D**). At P13, microglia were more numerous in the MNTB of the vehicle treated mice (**Figures 2E,F**), while the experimental group showed very little if any IBA1-positive labeling (**Figures 2G,H**). Thus, BLZ945 injections successfully depleted microglia in the MNTB and its presynaptic nucleus, the AVCN. We did not observe any apparent cellular debris from microglia, likely because the BLZ945 treatment was initiated before microglia normally populate the MNTB (Dinh et al., 2014). Cellular debris was also not present in adult mice after a few days of treatment with CSF1R inhibitors (Elmore et al., 2014).

We quantified IBA1 labeling in the MNTB using the areal coverage ratio of immunolabeling, as described in section "Materials and Methods." BLZ945 treatment resulted in a significant decrease in microglia coverage in both P8 and P13 pups when compared to their age-matched littermate controls (P8: $p = 0.0036$; P13: $p < 0.0001$). Microglial coverage significantly increased with age in the control group ($p < 0.0001$; **Figure 2J**). Consequently, repeated BLZ945 delivery during the first two postnatal weeks depleted microglia and prevented them from reappearing by 3 days after the last injection (at P10). The same IBA1-labeled tissue was used to calculate the average number of microglial cells per MNTB slice (**Figures 2I,K**). At P8, there were significantly more microglia present in control than in BLZ945-treated mice ($p < 0.0001$). The difference remained significant after hearing onset ($p < 0.0001$). Additionally, microglial count increased with age in the control group ($p < 0.0001$) while there was no change observed between the two BLZ945 injected groups ($p = 0.6644$).

Mice treated with BLZ945 appeared smaller than their age-matched littermates, thus, we compared the weights of animals used for the experiment and also measured their brain weight. While there was no significant difference observed at P8 in animal weight ($p = 0.0574$), microglia depleted animals sacrificed at P13 weighed significantly less than DMSO injected controls ($p < 0.0001$). However, both vehicle and CSF1R inhibitor injected mice were significantly heavier after the onset of hearing than before ($p < 0.0001$ and $p < 0.0001$, respectively; **Figure 2L**). Consequently, both experimental groups gained weight with age but animals without microglia gained significantly less. Interestingly, there was no significant difference between the brain weight of control and BLZ945-injected mice at P8 ($p = 0.2951$) and P13 ($p = 0.5375$). Brain weight of mice in

TABLE 3 | Statistical analysis.

Figures	Sample size (*n*)	Mean ± SEM	Treatment effect (DMSO-BLZ945)	Age effect (P8-P13)
Figure 2J	DMSO: P8 = 11, P13 = 11; BLZ945: P8 = 13, P13 = 9	DMSO: P8 0.01 ± 0.001, P13 0.04 ± 0.01; BLZ945: P8 0 ± 0, P13 0.001 ± 0.0002	P8: $p = 0.0036$, DF = 40; P13: $p < 0.0001$, DF = 40	DMSO: $p < 0.0001$, DF = 40; BLZ945: $p = 0.9869$, DF = 40
Figure 2K	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 13; P13 = 9	DMSO: P8 3.41 ± 0.33, P13 5.78 ± 0.30; BLZ945: P8 0 ± 0, P13 0.25 ± 0.06	P8: $p < 0.0001$, DF = 39; P13: $p < 0.0001$, DF = 39	DMSO: $p < 0.0001$, DF = 39; BLZ945: $p = 0.6644$, DF = 39
Figure 2L	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 14; P13 = 9	DMSO: P8 4.28 ± 0.14, P13 7.22 ± 0.19; BLZ945: P8 3.67 ± 0.21, P13 5.36 ± 0.26	P8: $p = 0.0574$, DF = 40; P13: $p < 0.0001$, DF = 40	DMSO: $p < 0.0001$, DF = 40; BLZ945: $p < 0.0001$, DF = 40
Figure 2M	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 14; P13 = 9	DMSO: P8 0.31 ± 0.01, P13 0.41 ± 0.01; BLZ945: P8 0.30 ± 0.01, P13 0.40 ± 0.01	P8: $p = 0.2951$, DF = 39; P13: $p = 0.5375$, DF = 39	DMSO: $p < 0.0001$, DF = 39; BLZ945: $p < 0.0001$, DF = 39
Figure 3M	DMSO: P8 = 8, P13 = 10; BLZ945: P8 = 10; P13 = 8	DMSO: P8 0.24 ± 0.02, P13 0.26 ± 0.01; BLZ945: P8 0.26 ± 0.01, P13 0.26 ± 0.02	P8: $p = 0.2971$, DF = 38; P13: $p = 0.9982$, DF = 38	DMSO: $p = 0.5207$, DF = 38; BLZ945: $p = 0.8819$, DF = 38
Figure 3N	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 12; P13 = 9	DMSO: P8 0.23 ± 0.01, P13 0.27 ± 0.01; BLZ945: P8 0.24 ± 0.01, P13 0.27 ± 0.01	P8: $p = 0.8814$, DF = 32; P13: $p > 0.9999$, DF = 32	DMSO: $p = 0.0667$, DF = 32; BLZ945: $p = 0.1707$, DF = 32
Figure 3O	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 12; P13 = 9	DMSO: P8 0.02 ± 0, P13 0.04 ± 0; BLZ945: P8 0.02 ± 0, P13 0.02 ± 0	P8: $p = 0.9307$, DF = 38; P13: $p = 0.0042$, DF = 38	DMSO: $p < 0.0001$, DF = 38; BLZ945: $p = 0.5687$, DF = 38
Figure 5M	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 13; P13 = 9	DMSO: P8 0.18 ± 0.01, P13 0.17 ± 0.01; BLZ945: P8 0.19 ± 0.01, P13 0.19 ± 0.01	P8: $p = 0.3952$, DF = 39; P13: $p = 0.3401$, DF = 39	DMSO: $p = 0.7678$, DF = 39; BLZ945: $p = 0.9012$, DF = 39
Figure 5N	DMSO: P8 = 11, P13 = 7; BLZ945: P8 = 12; P13 = 9	DMSO: P8 0.16 ± 0.005, P13 0.22 ± 0.02; BLZ945: P8 0.15 ± 0.01, P13 0.20 ± 0.02	P8: $p = 0.8088$, DF = 35; P13: $p = 0.5781$; DF = 35	DMSO: $p = 0.0011$, DF = 35; BLZ945: $p = 0.0018$, DF = 35
Figure 5O	DMSO: P8 = 11, P13 = 10; BLZ945: P8 = 13; P13 = 9	DMSO: P8 0.23 ± 0.005, P13 0.27 ± 0.01; BLZ945: P8 0.24 ± 0.01, P13 0.26 ± 0.02	P8: $p = 0.8992$, DF = 39; P13: $p = 0.9016$, DF = 39	DMSO: $p = 0.0328$, DF = 39; BLZ945: $p = 0.1836$, DF = 39
Figure 7A	DMSO: P8 = 6, P13 = 6; BLZ945: P8 = 5; P13 = 7	DMSO: P8 84.10% ± 6.11%, P13 10.40% ± 4.19%; BLZ945: P8 71.86% ± 5.97%, P13 42.45% ± 8.07%	P8: $p = 0.3914$, DF = 20; P13: $p = 0.0035$, DF = 20	DMSO: $p < 0.0001$, DF = 20; BLZ945: $p = 0.0101$, DF = 20
Figure 7B	AVCN DMO: P13 = 4; AVCN BLZ945: P13 = 4; MNTB DMSO: P13 = 9; MNTB BLZ945: P13 = 9	AVCN DMSO: P13 0.0015 ± 0.0001; AVCN BLZ945:P13 0.0016 ± 0.0001; MNTB DMSO: P13 0.0013 ± 0.00004932; MNTB BLZ945: P13 0.0014 ± 0.00003985	P13 AVCN: $p = 0.8693$, $t = 0.1717$; DF = 6; P13 MNTB: $p = 0.1052$, $t = 1.717$; DF = 16	N/A
Figure 7C	DMSO: P8 = 6, P13 = 6; BLZ945: P8 = 5; P13 = 7	DMSO: P8 654.5 μm^2 ± 70.56 μm^2, P13: 947.7 μm^2 ± 110.6 μm^2; BLZ945: P8 702.6 μm^2 ± 58.57 μm^2, P13 896.6 μm^2 ± 32.52 μm^2	P8: $p = 0.8840$, DF = 20; P13: $p = 0.8480$, DF = 20	DMSO: $p = 0.0191$, DF = 20; BLZ945: $p = 0.1470$, DF = 20
Figure 7D	DMSO: P8 = 6, P13 = 6; BLZ945: P8 = 5; P13 = 7	DMSO: P8 237 μm^3 ± 28.64 μm^3, P13: 358.9 μm^3 ± 53.67 μm^3; BLZ945: P8 242.6 μm^3 ± 22.19 μm^3, P13 318.5 μm^3 ± 19.9 μm^3	P8: $p = 0.9924$, DF = 20; P13: $p = 0.6299$, DF = 20	DMSO: $p = 0.0380$, DF = 20; BLZ945: $p = 0.2487$, DF = 20
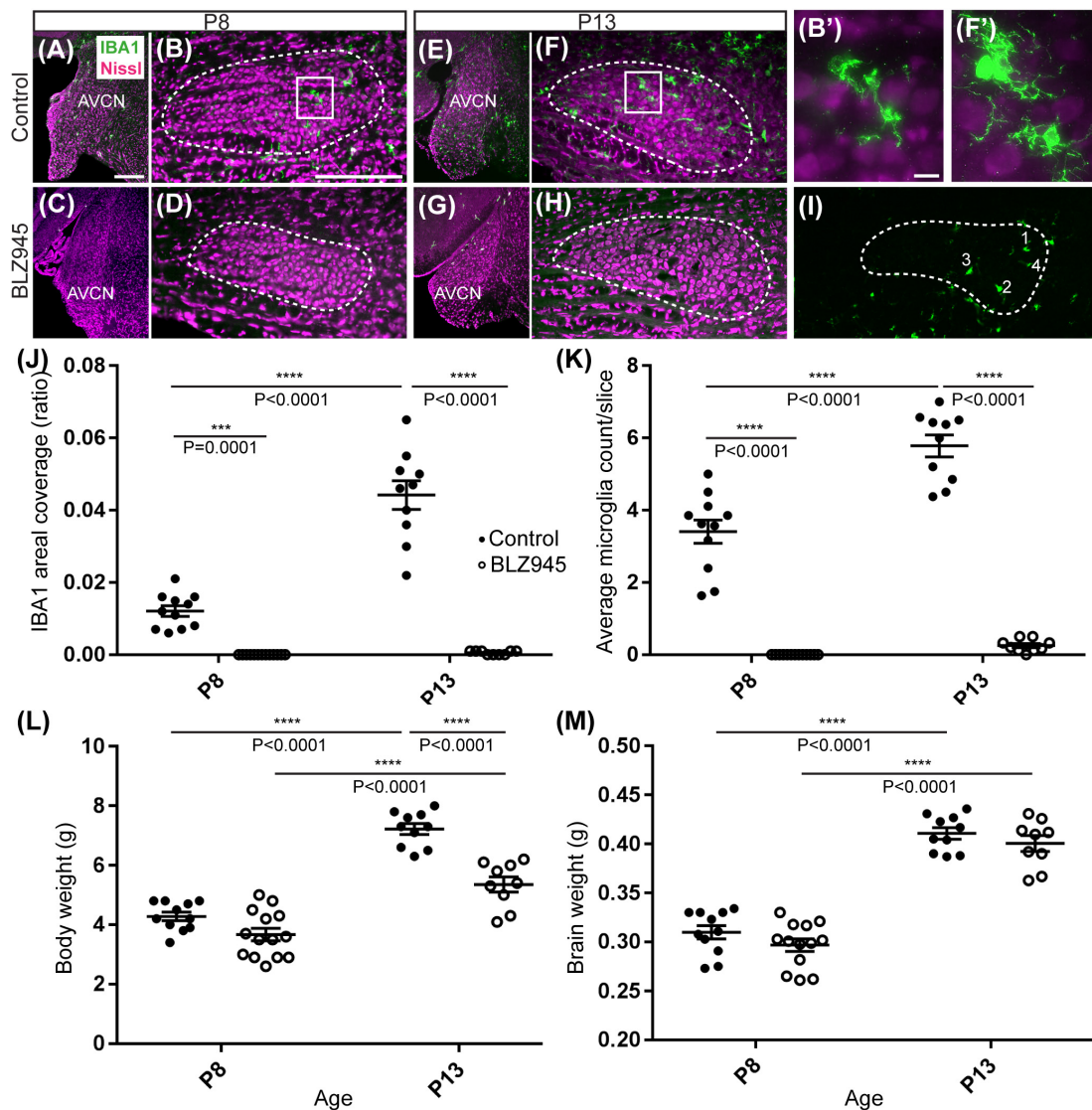
FIGURE 2 | Microglia depletion with BLZ945. **(A)** Microglia (green) are observed within the AVCN and **(B)** MNTB (dashed line) at P8 in control mice. **(B')** Inset from panel **(B)**. A higher magnification of the IBA1-labeled microglia from the MNTB of a control mouse at P8. **(C)** BLZ945 treatment eliminates microglia in the AVCN and **(D)** MNTB at P8. **(E)** At P13 microglia are more abundant than at P8 in control AVCN and **(F)** MNTB. **(F')** Inset from panel **(F)**. A higher magnification of the IBA1-labeled microglia from the MNTB of a control mouse at P13. **(G)** BLZ945 treatment eliminates microglia almost completely in the AVCN and **(H)** MNTB at P13. **(I)** Microglia counts in MNTB. Example of how microglial cell bodies were identified within the MNTB ROI. **(J)** Areal coverage ratio of IBA1 immunolabel. Microglia are significantly reduced at P8 and P13 in BLZ945-injected mice when compared to their age-matched controls. Microglia significantly increase with age in control animals but they remain almost absent in the MNTB of BLZ945-injected mice. **(K)** Microglia numbers per MNTB slice are significantly larger in control than BLZ945-treated mice at P8 and P13. Microglia increase in number in control animals with age but this increase was not observed in the BLZ945 injected group. **(L)** Control and BLZ945 injected animals gain weight with age, but mice without microglia weigh significantly less than their control littermates at P13. **(M)** Brains of control and BLZ945 injected animals significantly increased in weight with age. There was no significant difference between the brain of DMSO- and BLZ945-injected mice at P8 and P13. Scale bar in panel **(A)** = 100 μm, applies to panels **(A,C,E,G)**. Scale bar in panel **(B)** = 200 μm, applies to panels **(B,D,F,H,I)**. Scale bar in panel **(B')** = 10 μm and applies to the panel **(F')**.

both treatment groups significantly increased with age (DMSO: $p < 0.0001$; BLZ945: $p < 0.0001$; **Figure 2M**).

BLZ945 Effect on Astrocytes

Three antibodies for astrocytic markers previously shown to be expressed in the mouse brainstem (Dinh et al., 2014) were used to label astrocytes in the MNTB before and after hearing onset.

S100β and ALDH1L1 are early appearing markers in the MNTB, whereas GFAP appears later (Dinh et al., 2014). Tissue from DMSO- and BLZ945-injected mice was analyzed in the same way as that for IBA1 as described in section "Materials and Methods." Numerous S100β and ALDH1L1 positive astrocytes were present throughout the MNTB of both control and experimental animals at P8 and P13. ALDH1L1 and S100β

immunolabeling appeared to be very similar with both types of astrocytes surrounding neuronal somata (**Figures 3A–H**). After quantifying areal coverage of ALDH1L1 and S100β labeling in the MNTB, no significant difference was observed between the control and treated mice at P8 (ALDH1L1: $p = 0.2971$; S100β: $p = 0.8814$) or at P13 (ALDH1L1: $p = 0.9982$; S100β: $p > 0.9999$). Additionally, the amount of these proteins remained very similar before and after hearing onset (ALDH1L1: DMSO $p = 0.5207$; BLZ945 $p = 0.8819$; S100β: DMSO $p = 0.0667$; BLZ945 $p = 0.1707$; **Figures 3M,N**).

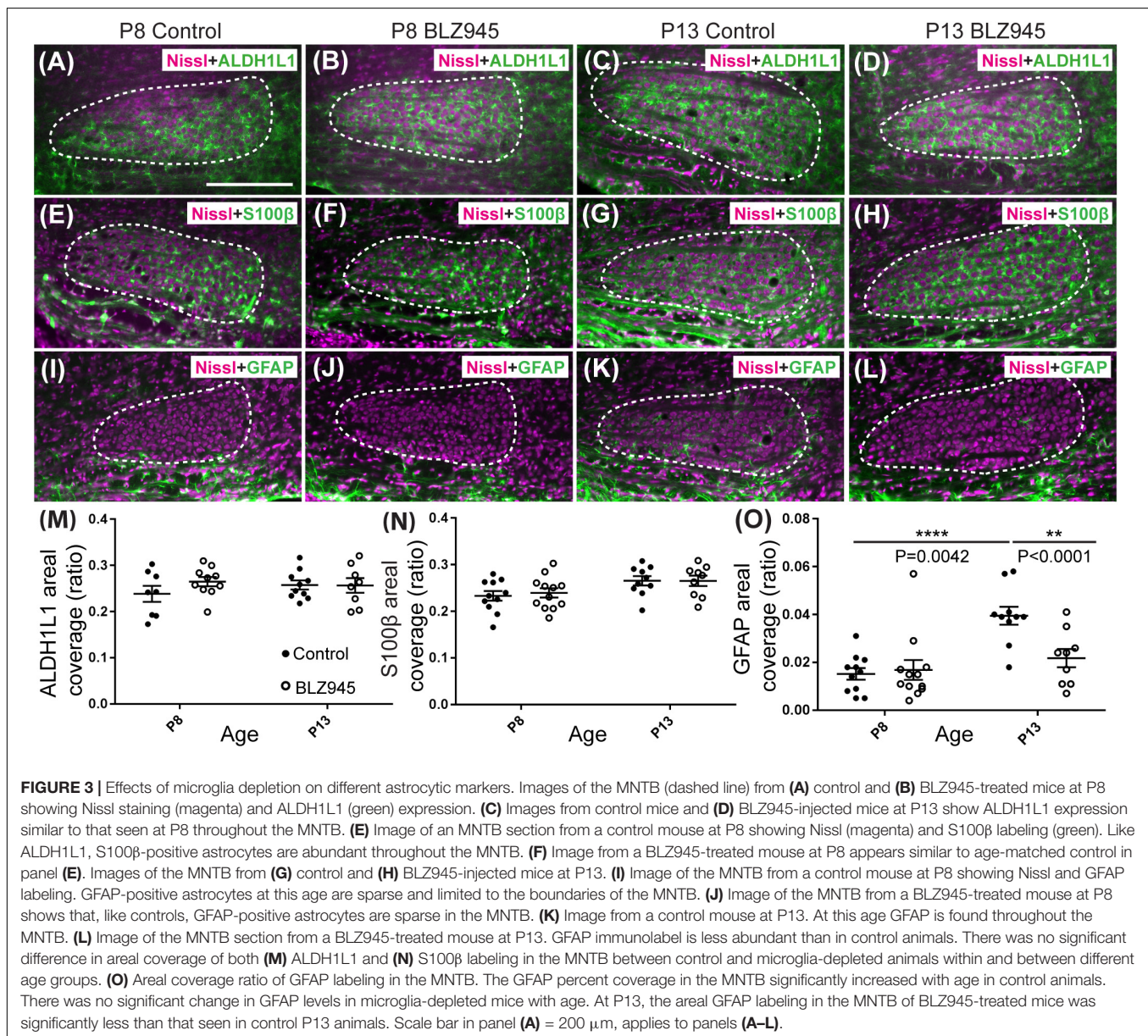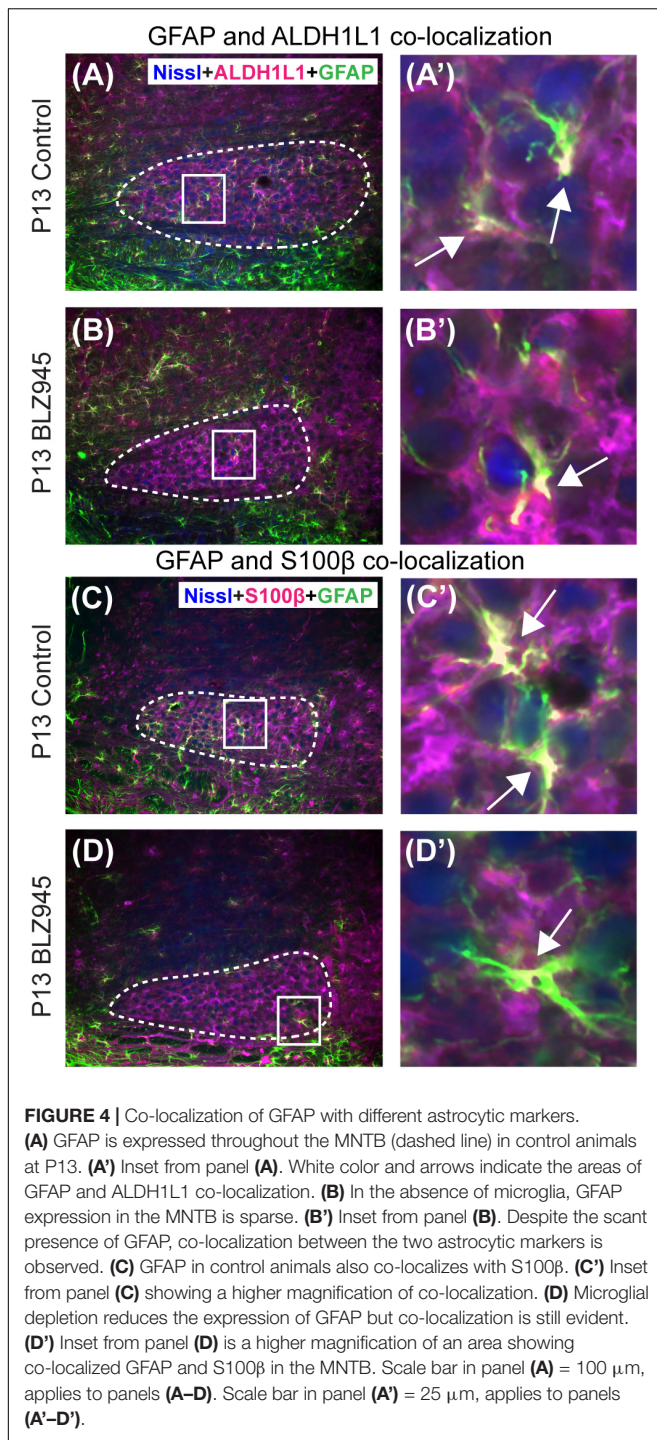As previously shown (Dinh et al., 2014), immunolabeling for the mature astrocyte marker GFAP was minimal at P8 (**Figures 3I,J**). At P13, GFAP immunolabeling could be seen in the MNTB in control mice (**Figure 3K**) but relatively little was seen in the MNTB of experimental animals

(**Figure 3L**). Quantification of GFAP areal coverage revealed no difference between the control and BLZ945-treated groups at P8 ($p = 0.9307$). GFAP-positive MNTB area significantly increased with age in control ($p < 0.0001$) but not experimental animals ($p = 0.5687$). At P13, we found significantly smaller GFAP areal coverage in the MNTB of microglia-depleted mice than in control mice ($p = 0.0042$; **Figure 3O**).

Glial fibrillary acidic protein may be a marker for mature astrocytes (Gomes et al., 1999). We used 5 DMSO and 4 BLZ945-injected mice to label the tissue for GFAP and ALDH1L1 at P13. We examined MNTB area for co-localization between the two astrocytic markers (**Figures 4A,B**). A high degree of co-localization was observed in both control and microglia depleted animals. The same effect was seen when tissue from 10 DMSO and 9 BLZ945-treated mice was labeled for GFAP

FIGURE 3 | Effects of microglia depletion on different astrocytic markers. Images of the MNTB (dashed line) from **(A)** control and **(B)** BLZ945-treated mice at P8 showing Nissl staining (magenta) and ALDH1L1 (green) expression. **(C)** Images from control mice and **(D)** BLZ945-injected mice at P13 show ALDH1L1 expression similar to that seen at P8 throughout the MNTB. **(E)** Image of an MNTB section from a control mouse at P8 showing Nissl (magenta) and S100β labeling (green). Like ALDH1L1, S100β-positive astrocytes are abundant throughout the MNTB. **(F)** Image from a BLZ945-treated mouse at P8 appears similar to age-matched control in panel **(E)**. Images of the MNTB from **(G)** control and **(H)** BLZ945-injected mice at P13. **(I)** Image of the MNTB from a control mouse at P8 showing Nissl and GFAP labeling. GFAP-positive astrocytes at this age are sparse and limited to the boundaries of the MNTB. **(J)** Image of the MNTB from a BLZ945-treated mouse at P8 shows that, like controls, GFAP-positive astrocytes are sparse in the MNTB. **(K)** Image from a control mouse at P13. At this age GFAP is found throughout the MNTB. **(L)** Image of the MNTB section from a BLZ945-treated mouse at P13. GFAP immunolabel is less abundant than in control animals. There was no significant difference in areal coverage of both **(M)** ALDH1L1 and **(N)** S100β labeling in the MNTB between control and microglia-depleted animals within and between different age groups. **(O)** Areal coverage ratio of GFAP labeling in the MNTB. The GFAP percent coverage in the MNTB significantly increased with age in control animals. There was no significant change in GFAP levels in microglia-depleted mice with age. At P13, the areal GFAP labeling in the MNTB of BLZ945-treated mice was significantly less than that seen in control P13 animals. Scale bar in panel **(A)** = 200 µm, applies to panels **(A–L)**.

FIGURE 4 | Co-localization of GFAP with different astrocytic markers. **(A)** GFAP is expressed throughout the MNTB (dashed line) in control animals at P13. **(A')** Inset from panel **(A)**. White color and arrows indicate the areas of GFAP and ALDH1L1 co-localization. **(B)** In the absence of microglia, GFAP expression in the MNTB is sparse. **(B')** Inset from panel **(B)**. Despite the scant presence of GFAP, co-localization between the two astrocytic markers is observed. **(C)** GFAP in control animals also co-localizes with S100β. **(C')** Inset from panel **(C)** showing a higher magnification of co-localization. **(D)** Microglial depletion reduces the expression of GFAP but co-localization is still evident. **(D')** Inset from panel **(D)** is a higher magnification of an area showing co-localized GFAP and S100β in the MNTB. Scale bar in panel **(A)** = 100 μm, applies to panels **(A–D)**. Scale bar in panel **(A')** = 25 μm, applies to panels **(A'–D')**.

and S100β (**Figures 4C,D**). Taken together, the results do not show effects of microglia depletion on astrocytes during early development, but rather that removal of microglia may impede the maturation of astrocytes.

BLZ945 Effect on Synaptic Proteins

The MNTB receives the majority of its excitatory input from the AVCN (Billups, 2005) and inhibitory input from the
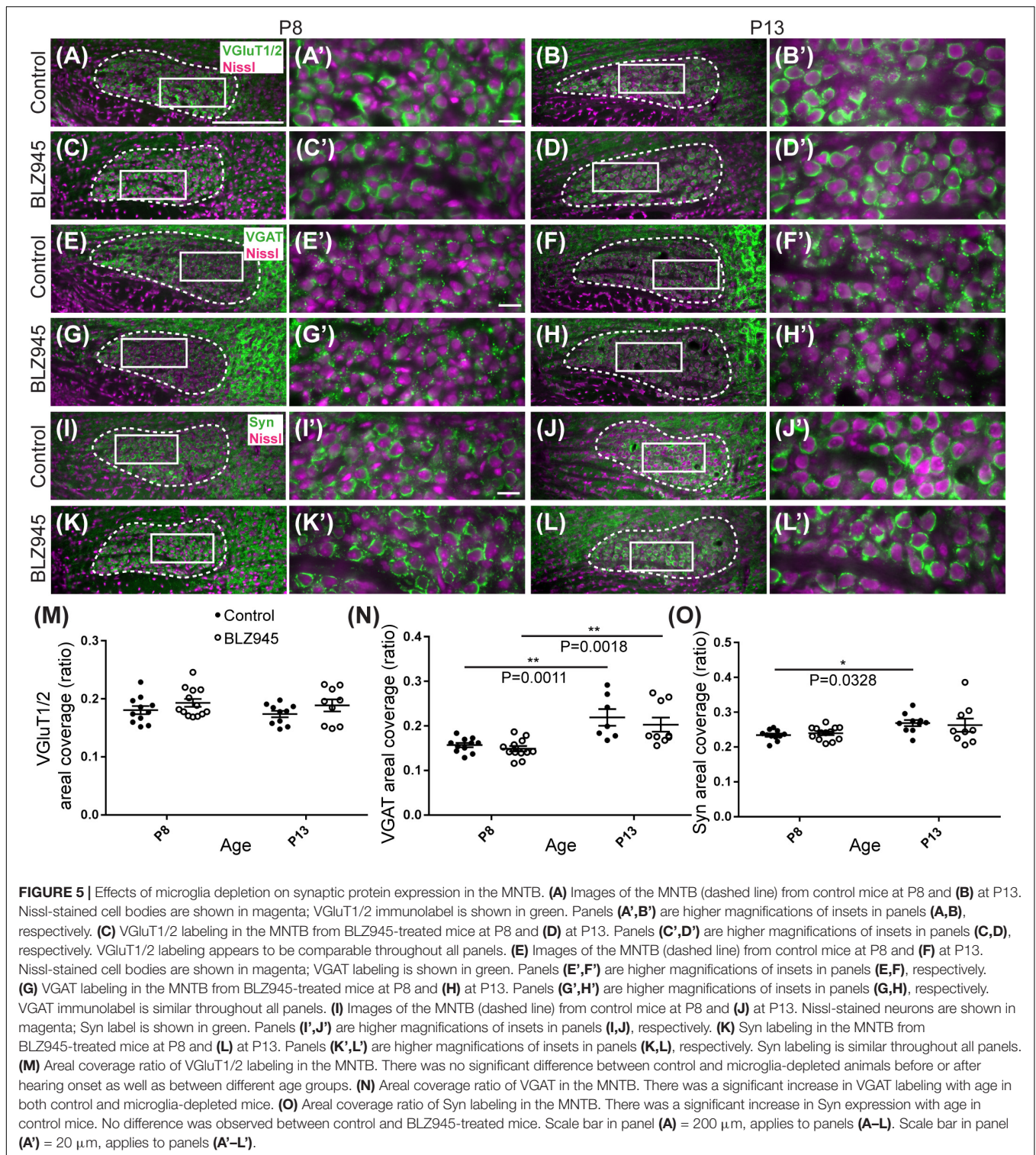
ventral nucleus of the trapezoid body (Albrecht et al., 2014). To evaluate the expression of synaptic inputs in the presence or absence of microglia, we carried out immunofluorescence labeling for VGluT1/2 and VGAT which correspond to excitatory (Fremeau et al., 2001; Herzog et al., 2001; Kaneko et al., 2002) and inhibitory (McIntire et al., 1997; Wang et al., 2009) neurotransmitter transporters, respectively. Additionally, we immunolabeled for Syn, a presynaptic protein associated with small synaptic vesicles (Ngsee et al., 1990; Calakos and Scheller, 1994) to represent combined presynaptic inputs (**Figure 5**).

VGluT1/2 labeling in the MNTB of mature animals produces ring-like structures as calyces of Held nearly completely surround MNTB neurons. We quantified the coverage of VGluT1/2 and found no significant difference when compared to their littermate controls at P8 ($p = 0.3952$) or P13 ($p = 0.3401$). Comparisons between the two age groups did not show any significant change in VGluT1/2 expression either in control ($p = 0.7678$) or in treated animal groups ($p = 0.9012$; **Figure 5M**). Thus, microglia depletion did not significantly impact the expression of VGluT1/2.

While excitatory input dominates MNTB neurons (Billups, 2005), VGAT expression was observed in the MNTB at P8 and P13. Inhibitory inputs contacted mainly neuronal somata (**Figures 5E–H'**). VGAT coverage in BLZ945-treated mice did not differ significantly from control mice at P8 ($p = 0.8088$) or at P13 ($p = 0.5781$). However, in both control and treated animal groups, VGAT coverage significantly increased with age ($p = 0.0011$ and $p = 0.0018$, respectively; **Figure 5N**). Similarly, Syn levels showed no significant difference between control and BLZ945 groups at both P8 ($p = 0.8992$) and P13 ($p = 0.9016$). On the other hand, we found a significant increase in Syn levels in control mice between P8 and P13 ($p = 0.0328$). No age effect was observed in BLZ945-treated animals ($p = 0.1836$; **Figure 5L**). Taken together, these observations suggest that microglia are not needed for normal levels of synaptic protein expression before or after hearing onset.

Microglia Depletion Impairs Pruning of Excess Calyces

We evaluated the innervation status of MNTB neurons in the absence of microglia using sparse labeling of calyces together with broad labeling of presynaptic proteins (see section "Materials and Methods"). For the P8 endpoint, we used PV immunofluorescence to label calyces of Held. Early in development, calyces of Held are PV positive while MNTB neurons are not (Lohmann and Friauf, 1996; Caicedo et al., 1997; Felmy and Schneggenburger, 2004; Kulesza, 2014), allowing us to distinguish inputs from their target somata. Between P8 and P10, MNTB neurons also start expressing PV, making the boundary between the calyx of Held and the cell body less obvious. Therefore, in the P13 age group, we used VGluT1/2 immunofluorescence, which labeled all excitatory terminals surrounding MNTB neurons including calyces of Held. RDA-labeled calyces combined with PV or VGluT1/2

FIGURE 5 | Effects of microglia depletion on synaptic protein expression in the MNTB. **(A)** Images of the MNTB (dashed line) from control mice at P8 and **(B)** at P13. Nissl-stained cell bodies are shown in magenta; VGluT1/2 immunolabel is shown in green. Panels **(A',B')** are higher magnifications of insets in panels **(A,B)**, respectively. **(C)** VGluT1/2 labeling in the MNTB from BLZ945-treated mice at P8 and **(D)** at P13. Panels **(C',D')** are higher magnifications of insets in panels **(C,D)**, respectively. VGluT1/2 labeling appears to be comparable throughout all panels. **(E)** Images of the MNTB (dashed line) from control mice at P8 and **(F)** at P13. Nissl-stained cell bodies are shown in magenta; VGAT labeling is shown in green. Panels **(E',F')** are higher magnifications of insets in panels **(E,F)**, respectively. **(G)** VGAT labeling in the MNTB from BLZ945-treated mice at P8 and **(H)** at P13. Panels **(G',H')** are higher magnifications of insets in panels **(G,H)**, respectively. VGAT immunolabel is similar throughout all panels. **(I)** Images of the MNTB (dashed line) from control mice at P8 and **(J)** at P13. Nissl-stained neurons are shown in magenta; Syn label is shown in green. Panels **(I',J')** are higher magnifications of insets in panels **(I,J)**, respectively. **(K)** Syn labeling in the MNTB from BLZ945-treated mice at P8 and **(L)** at P13. Panels **(K',L')** are higher magnifications of insets in panels **(K,L)**, respectively. Syn labeling is similar throughout all panels. **(M)** Areal coverage ratio of VGluT1/2 labeling in the MNTB. There was no significant difference between control and microglia-depleted animals before or after hearing onset as well as between different age groups. **(N)** Areal coverage ratio of VGAT in the MNTB. There was a significant increase in VGAT labeling with age in both control and microglia-depleted mice. **(O)** Areal coverage ratio of Syn labeling in the MNTB. There was a significant increase in Syn expression with age in control mice. No difference was observed between control and BLZ945-treated mice. Scale bar in panel **(A)** = 200 μm, applies to panels **(A–L)**. Scale bar in panel **(A')** = 20 μm, applies to panels **(A'–L')**.

labeling allowed us to assess whether an MNTB neuron is contacted by multiple excitatory inputs or a single main input. Our RDA labeling method was sparse and extended across the VAS, allowing us to examine RDA-filled endings throughout the medio-lateral extent of the MNTB. A neuron was classified as monoinnervated if the cell was contacted just by an RDA-filled calyx of Held (**Figures 1E,E'**). A cell was classified as polyinnervated if presynaptic proteins on an MNTB neuron were found outside an RDA-labeled calyx (**Figures 1F,F'**, **6**). We found that at P8 in both control
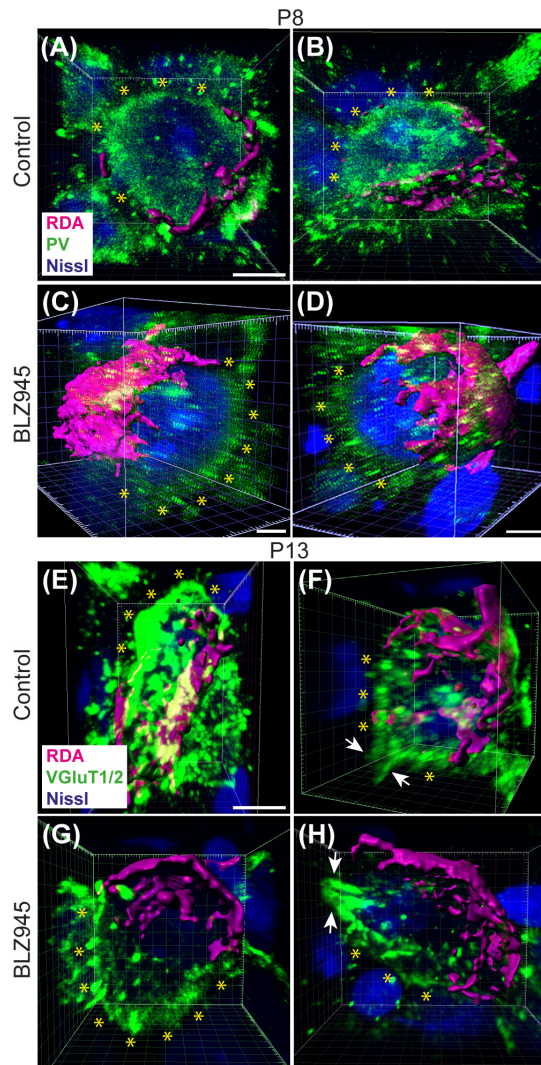
FIGURE 6 | Confocal analysis of polyinnervated neurons in the presence or absence of microglia. **(A,B)** Images showing reconstructed RDA-labeled calyces of Held (magenta) terminating on MNTB principal neurons in control mice at P8. Both are examples of polyinnervated MNTB neurons as evidenced by RDA-labeled calyces and additional PV (green) labeling (asterisks) outside of RDA-labeled calyces. **(C,D)** Examples of polyinnervated neurons in BLZ945-treated mice at P8. **(E,F)** Examples of polyinnervated neurons in control mice at P13. Asterisks indicate VGluT1/2 labeling on neurons in addition to RDA-filled calyces of Held. **(G,H)** Images of polyinnervated neurons in BLZ945-treated mice at P13. Scale bar in panel **(A)** = 3 μm, applies to panels **(A,B)**. Scale bar in panels **(C,D)** = 3 μm. Scale bar in panel **(E)** = 3 μm, applies to panels **(E–H)**.

and BLZ945-injected groups, the majority of neurons in the MNTB were still polyinnervated. Microglial depletion did not affect the number of polyinnervated MNTB neurons at P8 ($p = 0.3914$). As expected, in control animals the percentage of polyinnervated neurons significantly decreased with age ($p < 0.0001$). Polyinnervation also decreased with age for BLZ945-treated mice ($p = 0.0101$). However, microglia-depleted animals contained significantly more polyinnervated neurons
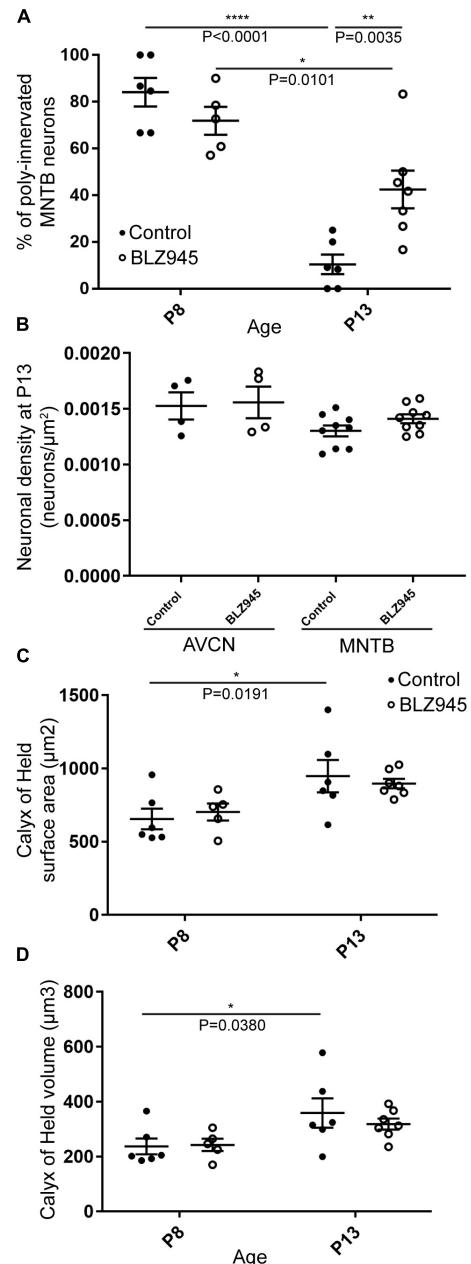

FIGURE 7 | BLZ945 effect on the innervation of MNTB neurons and the size of the calyx of Held. **(A)** Percentage of polyinnervated MNTB neurons was calculated for each group. Before hearing onset, most of neurons in the MNTB were polyinnervated in both control and BLZ945-injected animals. The number of polyinnervated neurons significantly decreased at P13 in both experimental groups, but there were significantly more polyinnervated neurons remaining in microglia-depleted mice. **(B)** Neuronal density in AVCN and MNTB was calculated in control and microglia-depleted animals at P13 and there was no significant difference found between the two cohorts in either of the nuclei analyzed. **(C)** The surface area of calyces of Held was measured after 3D reconstruction of confocal z-stacks. There was no difference in surface area between control and BLZ945-treated mice at P8 or P13. In control animals, calyces grew significantly larger with age. **(D)** The volume of calyces of Held was also measured and was similar in control and treated animals at both ages. The volume of calyces significantly increased with age only in the control group.

than control animals at P13 (p = 0.0035; **Figure 7A**). To test whether BLZ945 treatment had an effect on neuronal counts, we evaluated neuronal density in the AVCN and MNTB at P13. We did not find any difference between control and BLZ945-injected animals, indicating no changes in cell number between the treatment groups in the AVCN (p = 0.8693; unpaired t-test; **Figure 7B**) as well as MNTB (p = 0.1052; unpaired t-test; **Figure 7B**). Thus, the absence of microglia prevented or slowed down the pruning of excitatory inputs from the MNTB neurons resulting in reduced monoinnervation. The observed effect was not due to the increase in neuronal numbers in the AVCN or the decrease in neuronal count in the MNTB.

We did not observe any effect of microglia depletion on the surface area (P8: p = 0.8840; P13: p = 0.8480; **Figure 7C**) or volume (P8: p = 0.9924; P13: p = 0.6299; **Figure 7D**) of calyces of Held. Calyces of Held of control animals significantly increased in both surface area (p = 0.0191) and volume with age (p = 0.0380) while there was no difference observed between calyceal sizes of microglia-depleted animals between P8 and P13 (surface area: p = 0.1470; volume: p = 0.2487; **Figures 7C,D** and **Table 2**).

Multiple Calyces Are Found on Principal Neurons of MNTB in Untreated and DMSO-Injected Mice

Given the unexpectedly high level of polyinnervation in P8 animals in both treatment groups, we used sequential dye labeling (RDA and DA488) in untreated and control animals to verify the presence of multiple inputs. Dextrans tagged with two different fluorophores were electroporated in the VAS of untreated (N = 4) and DMSO-treated animals (N = 3) at P8. In every animal we found examples of polyinnervation (**Figures 8A–F'''**). Both RDA and DA488 labeled calyces were found to converge on the same neuron in the MNTB, often seen with a portion of their preterminal axons. Evaluations were only observational as injections were sparse and differed in size. We used this method to evaluate polyinnervation at P13. Although the percentage of polyinnervated neurons was only ~10% in control animals at P13 (**Figure 7A**), we found examples of polyinnervation in all 3 of the double-labeled brainstems (**Figures 8G–I'''**).

Microglial Association With VGluT1/2 Immunolabel

As microglia depletion was associated with impaired pruning, we next sought to determine whether presynaptic proteins are internalized in microglia during normal development. We collected tissue containing the MNTB from 3 $CX_3CR1^{+/EGFP}$ mice at P8 and P13 and immunolabeled for VGluT1/2 (**Figure 9**). In our analysis of five microglial cells from each animal, we found that every cell made extensive contacts with VGluT1/2-positive terminals. In addition, we consistently found small regions of VGluT1/2-labeled particles inside of the somata of EGFP-positive cells.
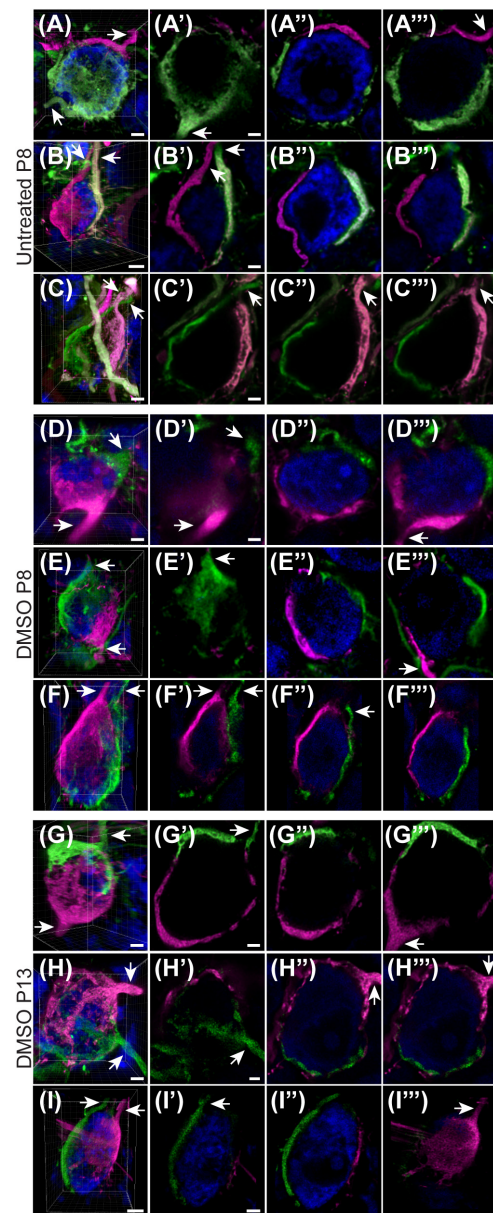

FIGURE 8 | Sequential tracing with RDA and DA488 in the VAS of wild-type and DMSO-injected mice. **(A–C)** Examples of polyinnervated neurons contacted by RDA- and DA488-labeled calyces of Held in wild-type mice at P8. Arrows indicate axons. **(A'–C''')** Individual optical sections through the neurons shown in panels **(A–C)** display polyinnervation on a single MNTB neuron. **(D–F)** 3D reconstructions of two separate calyceal inputs contacting the same neuron in the MNTB of DMSO-treated mice at P8. **(D'–F''')** Optical sections through the neurons shown in panels **(D–F)**. **(G–I)** 3D images of reconstructed RDA- and DA488-filled calyces synapsing on a single MNTB neuron in DMSO-injected mice at P13. **(G'–I''')** Sections through the neurons in panels **(G–I)** revealing multiple calyceal inputs. Scale bars in panels **(A,C,D)** = 3 μm. Scale bars in panels **(A',C')** = 2 μm and apply to panels **(A'–A''',C'–C''')**. Scale bar in panel **(B)** = 4 μm. Scale bar in panel **(B')** = 2 μm and applies to panels **(B'–B''')**. Scale bars in panels **(D,F)** = 3 μm. Scale bar in panel **(D')** = 2 μm and applies to corresponding panels **(D'–F''')**. Scale bars in panels **(G,H)** = 3 μm. Scale bars in panels **(G',H')** = 2 μm and apply to panels **(G'–H''')**. Scale bar in panel **(I)** = 5 μm. Scale bar in panel **(I')** = 3 μm and applies to panels **(I'–I''')**.
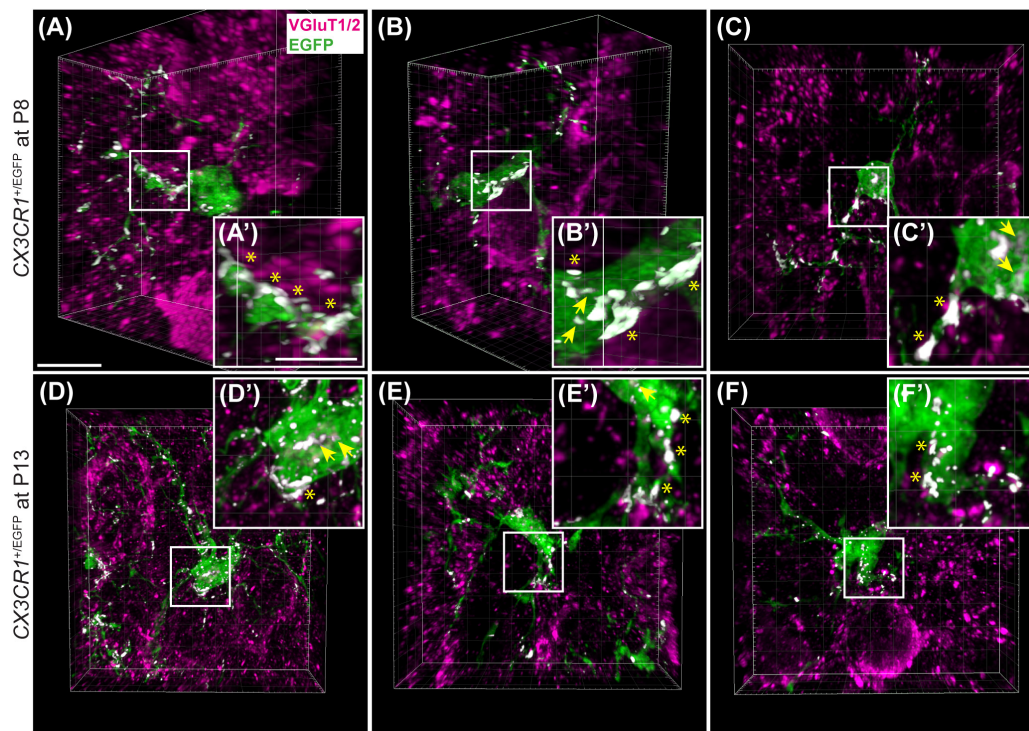
FIGURE 9 | Microglial association with VGluT1/2-positive puncta. **(A–C)** 3D reconstructions of EGFP-positive microglia (green) and VGluT1/2 immunolabeling (magenta) in $CX_3CR1^{+/EGFP}$ mice at P8. White patches denote EGFP and VGluT1/2 co-localization areas. **(A'–C')** Higher magnification images from panels **(A–C)**. Asterisks point to overlapping areas at the surface of microglial processes or cell bodies. Arrows denote internalized VGluT1/2 particles. **(D–F)** 3D confocal z-stack reconstructions of microglial cells and VGluT1/2 labeling in $CX_3CR1^{+/EGFP}$ mice at P13. **(D'–F')** Higher magnification images of areas of co-localization between EGFP and VGluT1/2 (white). At both ages, association between EGFP and VGluT1/2 is clearly visible. Co-localization is more frequently observed at the glial surface than as internalized excitatory puncta. Scale bar in panel **(A)** = 200 μm and applies to panels **(A–F)**. Scale bar in panel **(A')** = 5 μm and applies to panels **(A'–F')**.

DISCUSSION

We have optimized a procedure for near complete elimination of microglia in early postnatal mice. We used this approach to test the contribution of microglia to the development of specialized neural circuits in the brainstem. We found that microglia depletion reduced the expression levels of the mature astrocyte marker GFAP, but did not affect early appearing astrocytic markers or expression levels of excitatory and inhibitory presynaptic proteins. We showed that while microglia depletion did not alter the neuronal numbers or the size of the calyx of Held, it led to impaired pruning of excess synapses terminating in the MNTB (**Figure 10**).
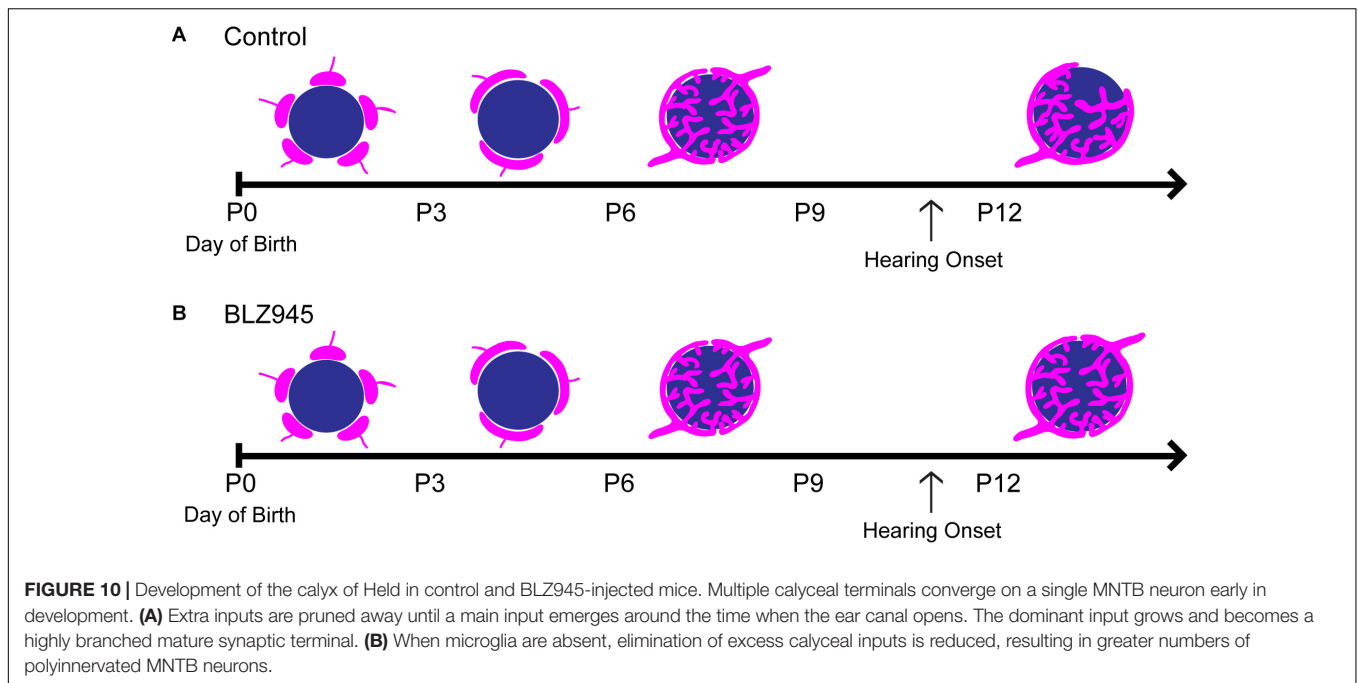
Microglia Function in Calyx of Held Development

The function of microglia in the auditory system has been explored in the context of acoustic trauma (Baizer et al., 2015) and cochlear lesions (Campos Torres et al., 1999; Janz and Illing, 2014) but the role of microglia in developmental pruning at the large and specialized calyx of Held has not been studied. We found that at P8, both control and BLZ945-treated animals contained a rather high percentage of polyinnervated MNTB neurons, and at P13, microglial elimination resulted in

significantly greater numbers of polyinnervated neurons. In the control group, the number of polyinnervated cells in the MNTB significantly decreased with age.

Polyinnervation of MNTB Neurons

Previous studies have described the formation of the calyx from the protocalyx into a cup-shaped structure enveloping ~50% of the postsynaptic neuron (Morest, 1968; Satzler et al., 2002). Rapid growth of calyces during the first postnatal week was determined in the rat using light microscopy (Kandler and Friauf, 1993; Rodriguez-Contreras et al., 2008). In mice, monoinnervation was observed by P4 with the number of polyinnervated neurons decreasing by half in the second postnatal week using electron microscopy and physiological techniques (Hoffpauir et al., 2006). The presence of a single dominant input on the majority of MNTB neurons was shown by P6 and monoinnervation by P9 (Holcomb et al., 2013). In contrast, other studies demonstrated polyinnervation of MNTB neurons after the first postnatal week in rodents using physiological (Bergsman et al., 2004) and anatomical methods (Wimmer et al., 2004), although the incidence was small. Smith et al. (1998) suggested that the variation seen in the excitatory input coverage (in cat, 40–50% of neuronal surface) may be due to some MNTB cells receiving two calyces or non-calyceal excitatory inputs (Smith et al., 1998). We used PV to look for signs of polyinnervation at

FIGURE 10 | Development of the calyx of Held in control and BLZ945-injected mice. Multiple calyceal terminals converge on a single MNTB neuron early in development. **(A)** Extra inputs are pruned away until a main input emerges around the time when the ear canal opens. The dominant input grows and becomes a highly branched mature synaptic terminal. **(B)** When microglia are absent, elimination of excess calyceal inputs is reduced, resulting in greater numbers of polyinnervated MNTB neurons.

P8, as early in development PV is expressed in calyces (Lohmann and Friauf, 1996; Felmy and Schneggenburger, 2004; Kulesza, 2014). Thus, a PV-positive area outside the RDA labeling most likely represents an additional calyx. Moreover, in some cases we observed two calyces along with their preterminal axons converging on a single MNTB neuron. It is also possible that the smallest excitatory inputs observed outside the RDA-labeled calyx are actually non-calyceal collateral inputs from axons forming calyces on the neighboring neurons (Kuwabara et al., 1991; Hamann et al., 2003) or collaterals of axons from SBCs (Cant and Casseday, 1986). However, we occasionally observed small RDA-filled calyces along with their preterminal axons that contacted only about 25% of the neuronal surface, suggesting that small inputs could be actually small calyces rather than excitatory collaterals.

Several factors might account for the discrepancy in levels of polyinnervation using these diverse approaches. Electrophysiological techniques may underestimate the convergence of synaptic inputs due to severing of axons in brain slice preparations or shared stimulation thresholds that are hard to resolve. Additionally, electron microscopy data are challenged by the limited number of cells that can be examined. We used confocal imaging and 3D reconstruction of multiple labeled calyces from multiple animals, thus increasing the number of examples. However, we were restricted by random labeling of calyces throughout the MNTB as well as the number of calyces that fulfilled the selection criteria. It did not appear that DMSO treatment alone impaired the pruning of calyces at P8, as our sequential two-color dye electroporation revealed some examples of polyinnervation in every animal examined, with or without prior DMSO treatment. Our P13 control group showed predominant monoinnervation in the MNTB, in agreement with other studies showing that pruning largely takes place

prior to this age. However, microglial depletion impaired or delayed this developmental process and resulted in significantly more neurons that were still polyinnervated by multiple calyceal synapses. Further studies are needed to determine the physiological effects of increased polyinnervation in MNTB.

Branching of GBC Axons

Some GBC axons can branch and give rise to multiple calyces. The branching site can be distant to (Spirou et al., 1990; Kuwabara et al., 1991; Smith et al., 1991) or near its target (Rodriguez-Contreras et al., 2006). In rodents, branching sites are rarely found outside of the MNTB (Friauf and Ostwald, 1988; Kuwabara et al., 1991; Rodriguez-Contreras et al., 2006). These studies also indicate that multiple calyces formed by the same axon contact different neurons in the MNTB. Our experiments show RDA- and DA488-labeled calyces converging on a single MNTB neuron. We do not know whether we labeled the axon coming from the same GBC and branching before the electroporation site or if the labeled axons belong to two different GBCs in the AVCN. If axon branching occurred close to its target, we would expect to occasionally encounter two RDA- or DA488-labeled calyces contacting the same neuron. However, we observed few such cases, consistent with the interpretation that labeled inputs generally arose from distinct GBCs.

Glial Cell Interactions

Astrocyte-synapse communication plays an important role in synapse development throughout the brain. In rodent cerebral cortex, a significant period of synaptogenesis occurs at postnatal weeks two and three, the time when birth and differentiation of astrocytes is complete (Freeman, 2010; Allen and Eroglu, 2017). Astrocyte-specific transcription factors that drive heterogeneity of astrocytes are still not known (Freeman, 2010). Studies

indicate that the intermediate filament GFAP is a marker for mature astrocytes (Eng et al., 1971; Pixley and de Vellis, 1984; Wofchuk and Rodnight, 1995) as well as for reactive astrocytes, as GFAP immunostaining peaks 1 week after bilateral cochlear ablation in the CN of rats (Fuentes-Santamaria et al., 2013, 2017). In the auditory brainstem, glial cell expansion coincides with neural circuit development (Brandebura et al., 2018) and astrocytic markers appear at distinct times during postnatal development. In the MNTB, ALDH1L1-positive cells are present at P0, while S100β expression is sparse and GFAP expression is completely absent. S100β expression is seen throughout the MNTB at P6, while GFAP is observed at P14 (Dinh et al., 2014; Saliu et al., 2014). Thus, it is possible that expression of specific astrocytic markers signifies distinct processes at different developmental stages.

While ALDH1L1 and S100β expression was not affected by microglia depletion, we showed a decrease in GFAP levels at P13 following BLZ945 injection. Our results differ from some previous microglial depletion studies done in adult mice where a significant increase in GFAP mRNA was found with no change in cell numbers (Elmore et al., 2014; Jin et al., 2017). Our findings also contrast with a study done in 5xfAD Alzheimer's model mice, in which the effect of microglia depletion on GFAP expression depended on the presence of pathology and the brain region examined (Spangenberg et al., 2016). Differences may be attributed to the age of the animals as well as the specific brain region investigated (Grabert et al., 2016; Spangenberg et al., 2016; De Biase and Bonci, 2018). Previous studies demonstrated interactions between microglia and astrocytes (Walton et al., 2006; Antony et al., 2011; Reemst et al., 2016) and an astrocytic role in pruning (Chung et al., 2015; Allen and Eroglu, 2017; Bosworth and Allen, 2017). A study done in rats showed that microglia are necessary for GFAP expression in astrocytes in disease states (Liddelow et al., 2017), a characteristic that may be recapitulated during development in the MNTB. The effects of microglia depletion on GFAP expression suggest a role for microglia in astrocyte maturation. This role occurs after synaptic pruning in the MNTB is largely complete.

Microglial Function in Synaptic Pruning

It is well established that microglia have a fundamental role in effective neural wiring of the brain through regulated apoptosis, neurogenesis (Casano and Peri, 2015; Sato, 2015; Vilalta and Brown, 2018), promotion of synaptic formation and maturation (Hoshiko et al., 2012; Miyamoto et al., 2016), and synaptic pruning in normal and pathological conditions (Paolicelli et al., 2011; Schafer and Stevens, 2015; Neniskyte and Gross, 2017). We observed that microglial processes make substantial contacts with VGluT1/2-positive puncta, and we consistently observed small regions of presynaptic proteins internalized in microglial somata. It is thus possible that microglia may be engulfing some presynaptic materials as well as identifying and tagging synapses for potential elimination by other cells. Moreover, microglia-synapse contacts may reflect trogocytosis (partial phagocytosis, or "nibbling") of presynaptic structures (Schafer et al., 2012; Weinhard et al., 2018). This process was demonstrated in a detailed study carried out in the mouse hippocampus. The

authors showed that boutons descended into the microglial cytoplasm, followed by the closure of the membrane and the subsequent trafficking of engulfed presynaptic material or axonal processes. Interestingly, the study demonstrated that the complement signaling pathway, previously shown to be involved in elimination of apoptotic cells (Iram et al., 2016) and synapses (Stevens et al., 2007; Thielens et al., 2017), is not required for trogocytosis of presynaptic elements (Weinhard et al., 2018). Whether this phenomenon generalizes to auditory system development is not clear. Further exploration of these pathways is needed to understand the mechanisms through which glial cells help to shape neural circuitry in the auditory system.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Animal Subjects: The animal study was reviewed and approved by University of California Irvine Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

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Spontaneous Activity Patterns Are Altered in the Developing Visual Cortex of the *Fmr1* Knockout Mouse

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Fragile X syndrome (FXS) is the most prevalent inherited cause of autism and is accompanied by behavioral and sensory deficits. Errors in the wiring of the brain during early development likely contribute to these deficits, but the underlying mechanisms are unclear. Spontaneous activity patterns, which are required for fine-tuning neuronal networks before the senses become active, are perturbed in rodent models of FXS. Here, we investigated spontaneous network activity patterns in the developing visual cortex of the *Fmr1* knockout mouse using *in vivo* calcium imaging during the second postnatal week, before eye opening. We found that while the frequency, mean amplitude and duration of spontaneous network events were unchanged in the knockout mouse, pair-wise correlations between neurons were increased compared to wild type littermate controls. Further analysis revealed that interneuronal correlations were not generally increased, rather that low-synchronization events occurred relatively less frequently than high-synchronization events. Low-, but not high-, synchronization events have been associated with retinal inputs previously. Since we found that spontaneous retinal waves were normal in the knockout, our results suggest that peripherally driven activity is underrepresented in the *Fmr1* KO visual cortex. Therefore, we propose that central gating of retinal inputs may be affected in FXS and that peripherally and centrally driven activity patterns are already unbalanced before eye opening in this disorder.

Keywords: fragile X mental retardation, *in vivo* calcium imaging, sensory integration, 2-photon microscopy, transgenic mouse

INTRODUCTION

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders that have been characterized traditionally by core features such as weak social communication, restricted interests, and repetitive behaviors. More recently, it has become clear that these disorders are also associated with compromised sensory processing, including vision (Kogan et al., 2004; Knoth et al., 2014; Dickinson et al., 2016; Ethridge et al., 2017; Yamasaki et al., 2017). Changes in sensory perception may underlie the complex behavioral traits described previously (Baum et al., 2015; Yamasaki et al., 2017; Rais et al., 2018). Studies in animal models of ASD found alterations in sensory perception and plasticity as well (Dolen et al., 2007; Berzhanskaya et al., 2016; Orefice et al., 2016; He et al., 2017; Goel et al., 2018; Wen et al., 2019). Miswiring of central sensory pathways may underlie these

symptoms (Yamasaki et al., 2017; Goswami et al., 2019); however, the developmental mechanisms that cause miswiring of sensory pathways in ASD and neurodevelopmental disorders in general are unknown (Di Martino et al., 2014; Park et al., 2016).

The wiring of neuronal networks is driven by molecular cues early on and later refined by activity-dependent mechanisms (Sanes and Yamagata, 2009; Kirkby et al., 2013). Before the onset of sensation, spontaneous activity is required for fine-tuning synaptic connections for sensory processing; thereafter, experience further adapts sensory networks to the prevalent conditions in the environment (Villers-Sidani et al., 2007; Caras and Sanes, 2015; Soutar et al., 2016). Perturbed mechanisms at all these developmental stages may contribute to neurodevelopmental syndromes (Meredith, 2015; Sanders, 2015).

Fragile X syndrome (FXS) is the most common genetic cause of autism and has been investigated extensively in patients and animal models (Hagerman et al., 2017), in particular the *Fmr1* knockout mouse (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006). These studies confirmed sensory phenotypes in both humans and mice (Kogan et al., 2004; Dolen et al., 2007; Knoth et al., 2014; Zhang et al., 2014) and these phenotypes are most likely caused by compromised synaptic connectivity (Bureau et al., 2008; Gibson et al., 2008; La Fata et al., 2014). Importantly, spontaneous activity patterns are perturbed during development, suggesting that errors in early activity-dependent synaptic refinement may impair synaptic connections in the FXS brain. For example, spontaneous network events in the somatosensory cortex of the *Fmr1* KO mouse show increased correlations (Goncalves et al., 2013), which may render these activity patterns less suitable for refining developing networks (Leighton and Lohmann, 2016).

Here, we investigated spontaneous activity patterns in the developing primary visual cortex of the *Fmr1* KO mouse (Mientjes et al., 2006) before eye opening. We find increased inter-neuronal correlations in the developing visual cortex, similar to previous findings in the somatosensory cortex (Goncalves et al., 2013). Further analysis suggested, however, that there is not a general increase in correlations, but rather a relative decrease of low- vs. high-synchronicity network events. Low-synchronicity events (L-events) have previously been associated with inputs from the retina (Siegel et al., 2012). Thus, our results suggest that retinally driven activity is underrepresented in cortical *Fmr1* KO activity patterns. Since we show here that retinal waves are normal in the knockout, we propose that central gating in the ascending visual pathway may be affected in FXS, even before the onset of vision.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences. The *Fmr1* KO mouse line used here was previously backcrossed to C57Bl/6J mice at least seven generations (Mientjes et al., 2006; de Vrij et al., 2008). The mice used in this study were bred from heterozygote *Fmr1*

($Fmr1^{+/-}$) mothers and wild type fathers (WT, C57Bl/6J). Only male KOs ($Fmr1^{y/-}$) were used in experiments, with littermate (wild type, $Fmr1^{y/+}$) males as controls. Experiments and initial analysis were performed blind to the genotype. There were no significant differences in weight (control: 6.79 ± 0.32 g, $n = 20$; FX: 6.51 ± 0.27 g, $n = 18$; $p > 0.05$) or age (control: 10.20 ± 0.29 days, $n = 20$, FX: 10.39 ± 0.26 days, $n = 18$, $p > 0.05$) between KOs and WTs for the cortical experiments. In the retinal experiments the weight (control: 5.58 ± 0.31 g, $n = 15$, FX: 5.34 ± 0.22 g, $n = 17$, $p > 0.05$) and age (control: 9.60 ± 0.31 days, $n = 15$, FX: 9.94 ± 0.20 days, $n = 17$, $p > 0.05$) also did not differ between KOs and WTs. All of the mice had closed eyes at the time of the experiment which fits with previous research showing that C57Bl/6J mice open their eyes at P12–P14 (Rochefort et al., 2009).

Genotyping

Mouse genotypes were determined *post hoc* by polymerase chain reaction (PCR) using the following primers: for KOs (GCCTCACATCCTAGCCCTCTAC and CCCACTGG GAGAGGATTATTTGGG) and for WTs (GCCTCACATCC TAGCCCTCTAC and CCCACAAAGTTGATTCCCCAGA). Tail samples were digested overnight with proteinase K (0.2 μg/μL) in 500 μL tail lysis buffer (in mM: 100 Tris–HCl, 5 EDTA, 200 NaCl, and 0.2% SDS) at 56°C. Proteins were pelleted by centrifugation (14,000 rpm for 3 min); DNA was isolated and precipitated with 500 μL of isopropanol. Following centrifugation (14,000 rpm for 1 min) the DNA pellet was dried and subsequently resuspended in 50 μL Tris–EDTA buffer. The PCR mix was prepared by adding: 2.6 μL of 10× buffer, 0.25 μL dNTPs (20 mM), 0.5 μL primer mix (10 μM), 0.15 μL Taq polymerase, and milliQ water up to a total of 25 μL for each sample. 1 μL of mouse DNA was added and the mixture was kept at 95°C for 5 min followed by 40 cycles (10 s at 95°C, 20 s at 60°C, and 45 s at 72°C) and 72°C for 10 min to finish.

Visual Cortex Imaging

In vivo imaging experiments were performed as described previously (Siegel et al., 2012). Briefly, P8–14 mice were anesthetized with isoflurane (2% in 1.7 L/min O$_2$), attached to a head bar with super glue and stabilized with dental cement (Heraeus Kulzer). Isoflurane was then reduced to 0.7% for the remainder of the experiment during which temperature was maintained at 36–37°C and heart beat was continuously monitored (control: 329.15 ± 16.17 bpm, $n = 20$, FX: 323.89 ± 17.94 bpm, $n = 18$, $p > 0.05$). At this level of isoflurane, animals remained anesthetized as they showed limited movement and a lack of reflexes. However, their breathing was rapid and shallow, and heart rates were high indicating that they were lightly anesthetized, as they lacked low gasping respiration and low heart rates typical in deeply anesthetized mice of this age. A craniotomy was made above the visual cortex without perforating the dura. The exposed cortical surface was kept moist with cortex buffer (in mM: 125 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 MgSO$_4$ and 2 CaCl$_2$, pH 7.4). A large population of layer 2/3 neurons was then labeled by bolus loading with

the calcium-sensitive dye Oregon Green BAPTA-1 AM (OGB1-AM, Life Technologies, O-6807) or Cal-590 AM (AAT Bioquest, 20510) diluted in 4 μL pluronic F-127 (20% solution in DMSO, Life Technologies, P-3000MP), and 36 μL dye buffer (in mM: 150 NaCl, 2.5 KCl, and 10 HEPES, pH 7.4). Dye was injected into layer 2/3 through a glass pipette (3–7 MΩ) using a picospritzer (12 min, 10–12 psi, Toohey). After 1 h the craniotomy was covered with 1.5% agarose for extra stability and imaging was performed to monitor spontaneous network activity in the same set of neurons (Mean cell number: Control: 91.80 $\pm$ 10.03, $n = 20$, FX: 80.17 $\pm$ 6.84, $n = 18$, $p > 0.05$) for approximately 40 min (Mean imaging time: Control: 38.31 $\pm$ 2.69 min, $n = 20$, FX: 43.42 $\pm$ 2.78 min, $n = 18$. Total duration recorded: Control: 766 min, FX: 781 min). Consecutive xyt-stacks (256 $\times$ 256, 600 nm pixel size, 5 Hz) were obtained through a 40$\times$ water-immersion objective (0.8 NA, Olympus) with a two-photon microscope (Movable Objective Microscope, Sutter Instrument or A1R-MP, Nikon) and a mode-locked Ti:Sapphire laser (at $\lambda = 810$ nm; Spectra Physics or Coherent) controlled by custom made Labview (National Instruments) software or ScanImage (Pologruto et al., 2003). 5-min recordings were obtained with only short breaks for re-focusing when necessary.

Visual Cortex Image Analysis

Recordings of spontaneous network activity in the cortex were analyzed with ImageJ (NIH) and custom-written Matlab scripts (MathWorks). To remove movement artifacts and align all recordings we performed image alignment based on the enhanced correlation coefficient algorithm (Evangelidis and Psarakis, 2008). $\Delta F/F_0$ stacks were generated by subtracting and dividing each frame by the baseline fluorescence (F_0). Regions of interest (ROIs) were placed on cells that showed clear activity and were visible in all recordings. Glial cells in the field of view showed elevated basal intensity and were not active. All included ROIs were neuronal. $\Delta F/F_0$ traces were obtained by calculating the mean intensity within the ROI for each frame. Increases in fluorescence intensity, which reflect increases in the intracellular calcium concentration due to action potential firing, were then detected semi-automatically for all ROIs and the maximum amplitudes and timings were determined. The detection threshold was adjusted for each experiment (at least 2$\times$ the noise) but remained the same within an experiment. A network event was defined as activity across multiple neurons during consecutive frames ($<$15 frames separation). The participation rate for each event was determined by summing the number of active cells and dividing by the total number of ROIs. Events with less than 20% participation were not analyzed further. Previously we found that two types of activity occur in the visual cortex at this developmental age: L-events are low participation (20–80%) and low amplitude events that are generated in the retina and transmitted to the cortex, while H-events are high participation ($>$80%) and high amplitude events that are generated within the cortex (Siegel et al., 2012). Here we also divided the data into H-events and L-events based on participation (above or below 80%). We found that varying the cut-off between 70 and 90% did not affect the results presented in this study (not shown).

To analyze synchrony, we calculated Pearson's correlations for each neuron pair in each experiment. Correlations were calculated on binarized activity traces of each neuron where each neuron was given a value of 0 for each inactive frame and a value of 1 for each frame of each burst it was active in. Burst duration was determined as the difference between the peak frame of the first and last cell to become active. To compare the correlation means we averaged across all pairs within 200 μm for each animal. The distance between the two cells was calculated as a straight line connecting the center of each ROI. We excluded animals that had fewer than 10 events in the recorded time (6 mice were excluded, 4 WTs and 2 KOs).

Retinal Imaging

Postnatal day 8–11 (P8–11) mice were anesthetized by inhalation of isoflurane (2% in 1.7 L/min O_2) and killed by decapitation. The eyes were removed and placed into ice cold modified Hank's balanced salt solution (HBSS, Life Technologies, in mM: 3.26 $CaCl_2$, 0.493 $MgCl_2$, 0.406 $MgSO_4$, 5.33 KCl, 0.441 KH_2PO_4, 4.17 $NaHCO_3$, 138 NaCl, 0.336 Na_2HPO_4, and 5.56 D-glucose) and dissected to isolate the retinas. Ganglion cells were labeled by injection of OGB1-AM (prepared as for visual cortex imaging) just below the inner limiting membrane with a glass pipette (1.5–5 MΩ, 3 min, 15 psi). After 1 h cells retinas were placed in a heated chamber (35°C) and imaged to monitor spontaneous network activity for $\sim$ 45 min (Mean imaging time: Control: 47.33 $\pm$ 1.18 min, $n = 15$, FX: 41.76 $\pm$ 1.05 min, $n = 17$. Total duration recorded: Control: 710 min, FX: 710 min). Consecutive xyt-stacks (500 $\times$ 500 pixels, 1.6 μm pixel size, 10 Hz) were obtained through a 20$\times$ water-immersion objective (0.5 NA, Olympus) with a CCD camera (iXon+; Andor Technology) and LED-based excitation illumination (pE-2; CoolLed) controlled by custom-built software (Labview, National Instruments).

Retinal Image Analysis

Recordings of spontaneous network activity in the retina were analyzed in a similar way to the cortical recordings. However, due to the large field of view and resulting small size of neuronal somas we did not analyze retinal activity in individual neurons. Instead, we downsized the images into 10 $\times$ 10 pixel bins and analyzed activity in the resulting 100 pixels. $\Delta F/F_0$ stacks were generated as described above and the $\Delta F/F_0$ traces were obtained for each pixel. Activity was then detected as for the cortical experiments except the same threshold was used for all experiments.

Retinal wave front velocities were quantified using custom python code implemented as part of the python-microscopy project[1]. Full resolution $\Delta F/F_0$ stacks were smoothed with a Gaussian (radius of 10 pixels) to reduce noise. The positions of wave front peaks in each frame were estimated as the zero-crossings of the time derivative of intensity as follows: (1) A low threshold was used to establish a mask of areas where calcium was elevated and to eliminate the inactive areas whose intensity was roughly constant over time, (2) in pixels above this threshold, an approximate temporal derivative of the intensity ($\Delta_t I$) was

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[1]www.python-microscopy.org

calculated by taking the difference between consecutive frames, (3) zero-crossings were detected by finding all pixels where $|\Delta_t I|$ was less than a threshold (chosen to give a gap-free wave front ~4–5 pixels wide) and skeletonizing the resulting masks. The direction of propagation of these wave fronts was estimated using a regularized version of optical flow algorithm described previously (Fleet and Weiss, 2006) applied to the filtered intensity data. Velocities were then estimated at each point on the wave front by looking along the optical flow direction, extracting the closest position of the wave front along this vector in each of the 5 frames before and after the current frame, and performing a linear least-squares fit to these positions as a function of time.
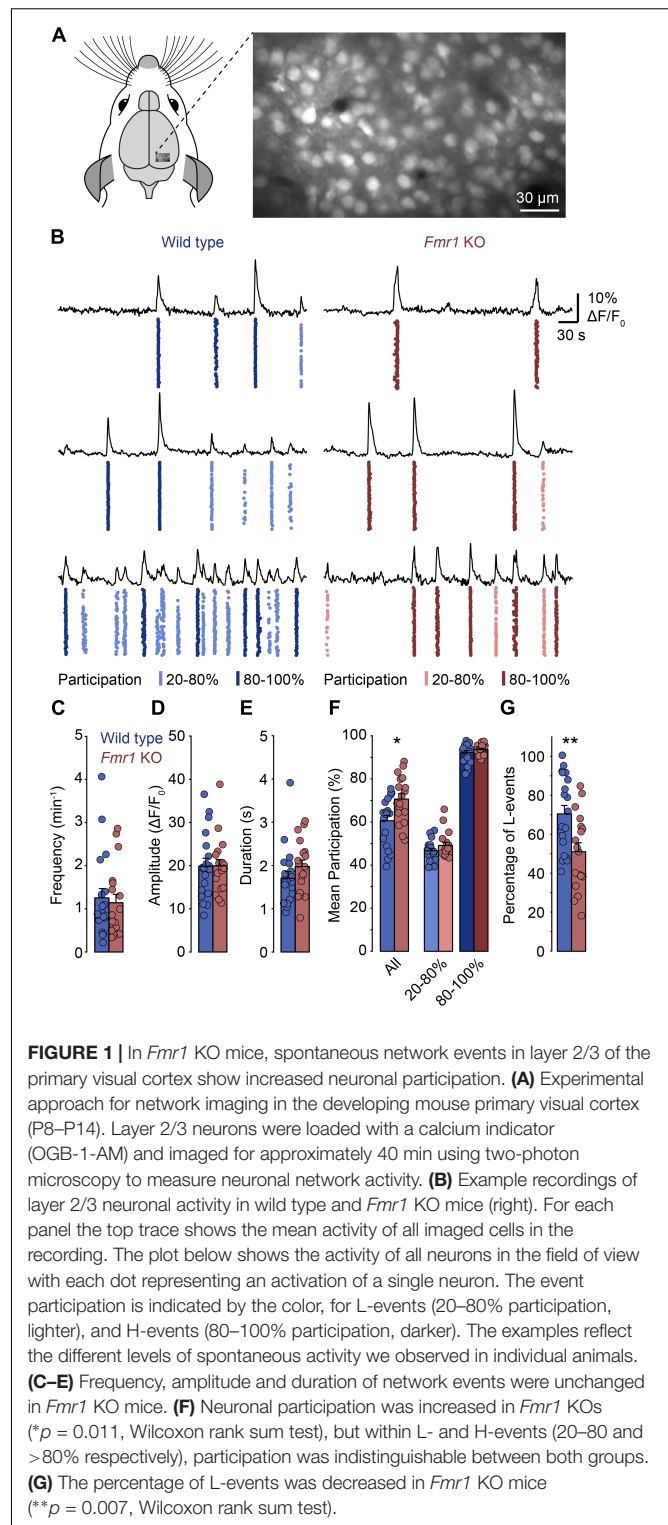
Statistics

To test for statistical differences between groups we used the Wilcoxon rank sum test. Data are presented as means ± SEM with n as the number of animals.
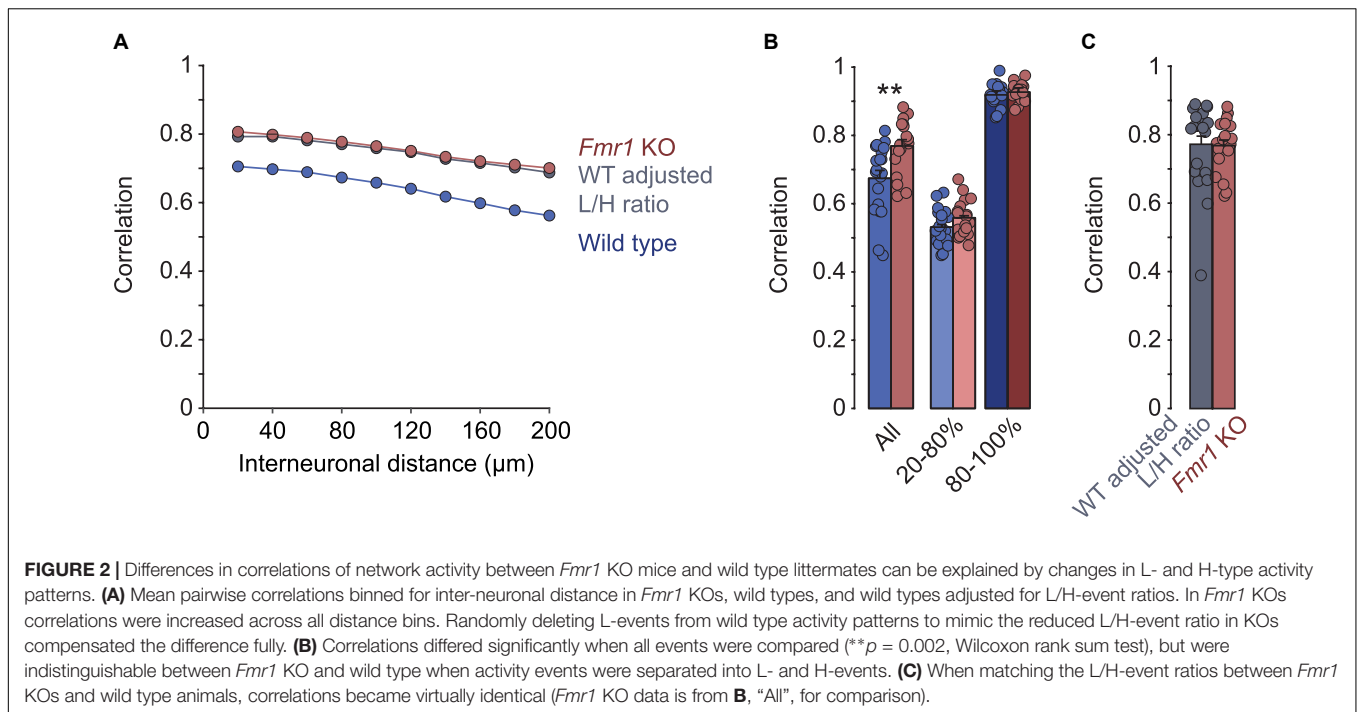
RESULTS

To determine whether spontaneous network activity patterns are altered in the developing visual cortex of FXS mice we performed *in vivo* calcium imaging in the primary visual cortex of lightly anesthetized *Fmr1* knockout mice (*Fmr1*$^{-/y}$) and wild type littermate controls (*Fmr1*$^{+/y}$) at postnatal day (P) 8–14 (**Figure 1**). Previously, we found that low levels of anesthesia (0.7 – 1% isoflurane) reduce the frequency of spontaneous network events, but do not change their basic properties (Siegel et al., 2012). We labeled layer 2/3 neurons with a fluorescent calcium indicator (Oregon Green BAPTA-1 or Cal-590) by bolus injection (**Figure 1A**) and recorded neuronal activity using two-photon imaging. Increases in somatic calcium, which reflect action potential firing, were monitored continuously in large populations of neurons for approximately 40 min (**Figure 1B**).

In both wild type and *Fmr1* KO mice, we observed repetitive events of synchronized network activity as described previously (**Figure 1B**; Hanganu et al., 2006; Golshani et al., 2009; Rochefort et al., 2009; Colonnese et al., 2010; Siegel et al., 2012). In most animals, there was no or little activity in between these network events, as is typically observed during this developmental stage. We found that the frequency, the mean amplitude and the duration of network events were indistinguishable between *Fmr1* KOs and controls (**Figures 1C–E**). In contrast, the mean participation was significantly higher in *Fmr1* KOs than in wild type animals (**Figure 1F**). Previously, we had identified two activity patterns in the developing visual cortex before eye-opening: L-events with low participation rates that are dependent on retinal wave activity and H-events where almost all neurons participate, which are independent of retinal inputs (Siegel et al., 2012). When we separated network events in the present data sets and compared participation rates within L-events (20–80% participation) and H-events (80–100% participation), we found that within these groups there was no change in participation (**Figure 1F**). This suggested that in *Fmr1* KO mice overall participation was not generally increased, but that the relative contribution of L- and H-events differed in the knockout mouse.

FIGURE 1 | In *Fmr1* KO mice, spontaneous network events in layer 2/3 of the primary visual cortex show increased neuronal participation. **(A)** Experimental approach for network imaging in the developing mouse primary visual cortex (P8–P14). Layer 2/3 neurons were loaded with a calcium indicator (OGB-1-AM) and imaged for approximately 40 min using two-photon microscopy to measure neuronal network activity. **(B)** Example recordings of layer 2/3 neuronal activity in wild type and *Fmr1* KO mice (right). For each panel the top trace shows the mean activity of all imaged cells in the recording. The plot below shows the activity of all neurons in the field of view with each dot representing an activation of a single neuron. The event participation is indicated by the color, for L-events (20–80% participation, lighter), and H-events (80–100% participation, darker). The examples reflect the different levels of spontaneous activity we observed in individual animals. **(C–E)** Frequency, amplitude and duration of network events were unchanged in *Fmr1* KO mice. **(F)** Neuronal participation was increased in *Fmr1* KOs (*p = 0.011, Wilcoxon rank sum test), but within L- and H-events (20–80 and >80% respectively), participation was indistinguishable between both groups. **(G)** The percentage of L-events was decreased in *Fmr1* KO mice (**p = 0.007, Wilcoxon rank sum test).

Indeed, we found that the proportion of L-events was decreased in the *Fmr1* KO mouse (**Figure 1G**).

A previous study found that in the somatosensory cortex of *Fmr1* KO mice, neuronal activity was more correlated than in wild type controls (Goncalves et al., 2013). To compare these

FIGURE 2 | Differences in correlations of network activity between *Fmr1* KO mice and wild type littermates can be explained by changes in L- and H-type activity patterns. **(A)** Mean pairwise correlations binned for inter-neuronal distance in *Fmr1* KOs, wild types, and wild types adjusted for L/H-event ratios. In *Fmr1* KOs correlations were increased across all distance bins. Randomly deleting L-events from wild type activity patterns to mimic the reduced L/H-event ratio in KOs compensated the difference fully. **(B)** Correlations differed significantly when all events were compared (*******p* = 0.002, Wilcoxon rank sum test), but were indistinguishable between *Fmr1* KO and wild type when activity events were separated into L- and H-events. **(C)** When matching the L/H-event ratios between *Fmr1* KOs and wild type animals, correlations became virtually identical (*Fmr1* KO data is from **B**, "All", for comparison).

results with the primary visual cortex, we analyzed pair-wise correlations of the calcium traces of layer 2/3 neurons in our recordings. Like in the somatosensory cortex, we observed a decrease in correlations with increasing distance between them, in both *Fmr1* KO ($R^2 = 0.99$, $p < 0.001$) and control ($R^2 = 0.98$, $p < 0.001$) mice (**Figure 2A**). In addition, correlations were higher in the *Fmr1* KO mouse compared to controls across all distance bins (**Figures 2A,B**). We did not observe significant age related changes of these correlations or their differences between genotypes (**Supplementary Figure 1**). Surprisingly, we found that correlation coefficients when determined for L- and H-events separately were virtually identical in *Fmr1* KO and control mice (**Figure 2B**). Again, this observation suggested that we did not observe a global increase in correlations, but rather a shift toward a higher fraction of high-correlation network events (H-events). To test the plausibility of this idea we randomly deleted L-events from the control dataset to match the percentage of L-events of *Fmr1* KO and control recordings (WT adjusted L/H ratio). This adjustment increased the overall correlations to the same level as those seen in *Fmr1* KO mice (**Figures 2A,C**), confirming that a shift in L-/H-event ratio could explain the increased correlations in the *Fmr1* KO visual cortex.
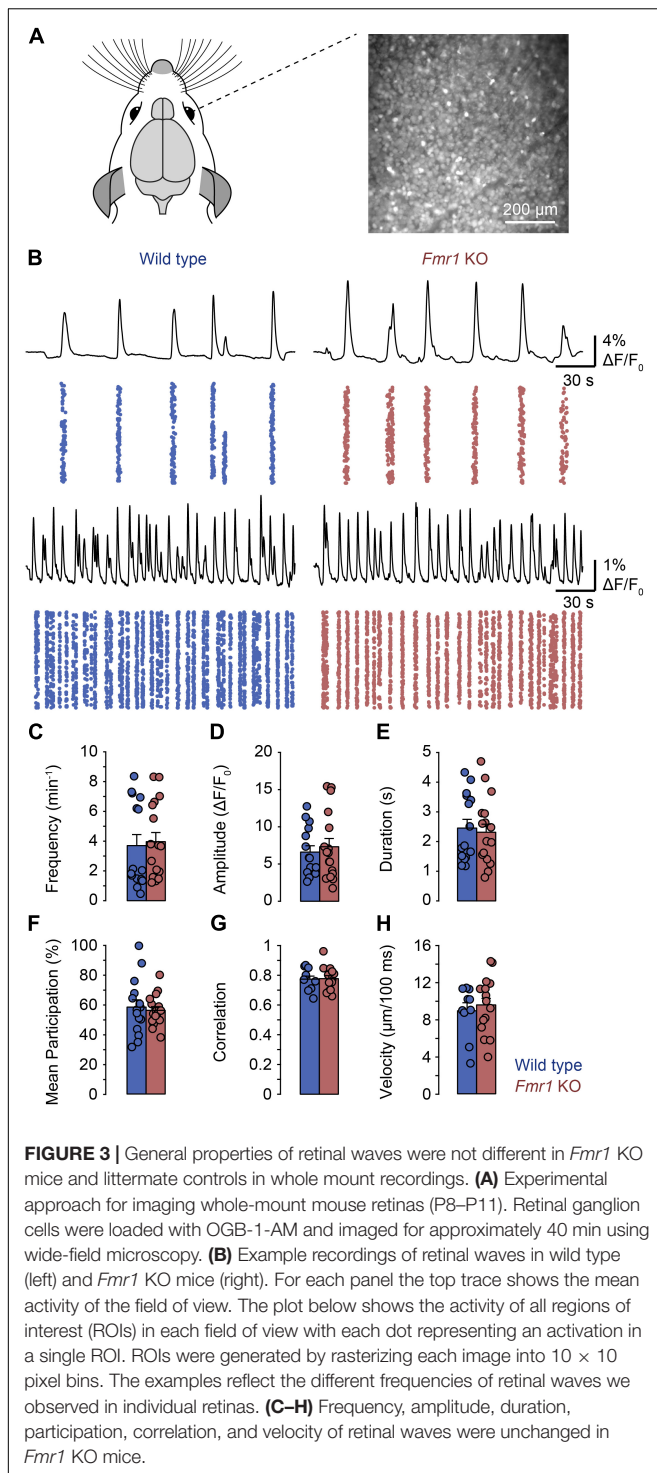
Since we previously found that L-, but not H-events are dependent on retinal wave activity, the present observations suggested that the representation of inputs from the sensory periphery is reduced in comparison to intrinsically generated activity patterns. Therefore, we asked whether retinal waves were affected in the *Fmr1* KO mouse. We performed calcium imaging in retinal whole mounts from P8–P11 *Fmr1* KO and control mice (**Figure 3A**). We observed retinal waves in both preparations (**Figure 3B**) and found no differences in frequency, amplitude, duration, participation or correlation

between them (**Figures 3C–G**). In addition, we analyzed the velocity of retinal waves but found no difference between *Fmr1* KO and control mice (**Figure 3H**). Thus, peripheral activity is underrepresented in the *Fmr1* KO visual cortex, despite normal retinal waves, suggesting differences in central processing of peripheral activity in FXS.

DISCUSSION

Neurodevelopmental disorders are associated with aberrant spontaneous activity patterns during early stages of brain development. Here, we show that in the visual cortex of the *Fmr1* KO mouse, neuronal correlations are increased compared to wild type littermates. The distribution of correlations suggest that this is not a consequence of generally increased correlations, but rather a redistribution of the contribution of two distinct, previously described activity patterns. Specifically, we see a relative decrease in those activity patterns that are dependent on retinal inputs even though retinal waves are unchanged in this FXS mouse model.

Like in the somatosensory cortex (Goncalves et al., 2013), we observe here an increase in the mean correlation coefficient when we compare activity patterns between pairs of neurons in *Fmr1* KO mice and wild type littermates. This increase is quantitatively and qualitatively very similar to the increase in the somatosensory cortex, as it affects pairs of neurons of all distances and translates to a change of approximately 10–20%. While in the somatosensory cortex differences become most pronounced by P14, we see differences in the visual cortex already at P8–P14. These differences are apparent in lightly anesthetized animals, whereas a significant difference between KO and wild

FIGURE 3 | General properties of retinal waves were not different in *Fmr1* KO mice and littermate controls in whole mount recordings. **(A)** Experimental approach for imaging whole-mount mouse retinas (P8–P11). Retinal ganglion cells were loaded with OGB-1-AM and imaged for approximately 40 min using wide-field microscopy. **(B)** Example recordings of retinal waves in wild type (left) and *Fmr1* KO mice (right). For each panel the top trace shows the mean activity of the field of view. The plot below shows the activity of all regions of interest (ROIs) in each field of view with each dot representing an activation in a single ROI. ROIs were generated by rasterizing each image into 10 × 10 pixel bins. The examples reflect the different frequencies of retinal waves we observed in individual retinas. **(C–H)** Frequency, amplitude, duration, participation, correlation, and velocity of retinal waves were unchanged in *Fmr1* KO mice.

type has been described in the somatosensory cortex only in awake animals (Goncalves et al., 2013). Despite the differences in detail between the primary visual and somatosensory cortices, these studies show that cortical activity patterns in *Fmr1* KO mice are associated with increased neuronal correlations. Similar findings have been observed at P0–P7 (La Fata et al., 2014).

Surprisingly, we find that increases in correlations do not distribute evenly across all types of events. When splitting events into high and low participation activity as described previously (Siegel et al., 2012) participation rates and correlations are unchanged in either group. We conclude therefore, that correlations are not generally increased, but rather that a change in contribution of these two types of activity patterns causes the increased mean correlations in the *Fmr1* KO visual cortex. This conclusion is supported by (1) the overall reduction in the L-/H-event ratio and (2) the fact that matching the L/H-event ratio between *Fmr1* KO and control activity fully equalizes the correlations between both conditions. Since L-events, but not H-events, have been associated with retinal inputs (Siegel et al., 2012), these observations suggest that peripheral activity is underrepresented in the *Fmr1* KO visual cortex before eye opening. Peripheral and central inputs may have complementary functions in cortical wiring (Siegel et al., 2012; Leighton and Lohmann, 2016), consequently, changes in their relative frequencies are likely to perturb synapse development.

Deficits in photoreceptor function have been described in the *Fmr1* KO mouse (Rossignol et al., 2014) therefore, we tested whether changes in early retinal activity could explain this difference. However, retinal waves are not affected in the *Fmr1* KO mouse, since their frequency, amplitude, and correlation are indistinguishable between KOs and controls.

What could explain the reduced representation of retinal inputs in the *Fmr1* KO visual cortex? A good candidate might be alterations in inhibitory neuron function. Early differences in inhibitory function have been implicated in neurodevelopmental disorders in general and FXS in particular (Marin, 2012; Le Magueresse and Monyer, 2013; Cellot and Cherubini, 2014; Tyzio et al., 2014; Goel et al., 2018). Furthermore, different types of interneurons are at pivotal positions within cortical networks to gate specifically bottom-up or horizontal activity. While PV neurons selectively target bottom-up connections, other interneurons, like the somatostatin expressing interneuron type, regulate horizontal activity spread (Tremblay et al., 2016; van Versendaal and Levelt, 2016; Wood et al., 2017). In addition, during development, cortical interneurons control spontaneous and early sensory evoked activity in a cell-type specific manner (Marques-Smith et al., 2016; Tuncdemir et al., 2016; Che et al., 2018). Therefore, selective alterations e.g., in PV neuron function could specifically decrease the effectiveness of ascending sensory pathways and thereby dampen cortical inputs from the periphery (Khazipov et al., 2013; Goel et al., 2018). Alternatively or in addition, differences in the excitability or synaptic function of excitatory connections at the level of the cortex or the lateral geniculate nucleus (Murata and Colonnese, 2016) might be involved as well. For example, a delay in the maturation of thalamo-cortical synapses has been described in the somatosensory cortex of *Fmr1* KO mice (Harlow et al., 2010). A similar delay of synapse development in the ascending visual pathways could explain the reduced transmission of retinal activity into the cortex during the second postnatal week, too.

Imbalanced contribution of external vs. internal activity streams have been observed in neurodevelopmental disorders (Courchesne and Pierce, 2005; Geschwind and Levitt, 2007).

Thus, the findings described here, suggest that network imbalances already manifest early in development, before the onset of sensation. If this can be generalized beyond FXS, diagnosis of neurodevelopmental disorders may be possible earlier than previously thought and thus facilitate earlier and more promising treatments in the future.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences.

AUTHOR CONTRIBUTIONS

JC and CL conceived and designed the study, and wrote the manuscript. JC and NZ collected the data. JC and DB contributed to the data and analysis of the tools. JC performed the analysis,

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncir.2019.00057/full#supplementary-material

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Multiplex Neural Circuit Tracing With G-Deleted Rabies Viral Vectors

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Neural circuits interconnect to organize large-scale networks that generate perception, cognition, memory, and behavior. Information in the nervous system is processed both through parallel, independent circuits and through intermixing circuits. Analyzing the interaction between circuits is particularly indispensable for elucidating how the brain functions. Monosynaptic circuit tracing with glycoprotein (G) gene-deleted rabies viral vectors (RVΔG) comprises a powerful approach for studying the structure and function of neural circuits. Pseudotyping of RVΔG with the foreign envelope EnvA permits expression of transgenes such as fluorescent proteins, genetically-encoded sensors, or optogenetic tools in cells expressing TVA, a cognate receptor for EnvA. Trans-complementation with rabies virus glycoproteins (RV-G) enables trans-synaptic labeling of input neurons directly connected to the starter neurons expressing both TVA and RV-G. However, it remains challenging to simultaneously map neuronal connections from multiple cell populations and their interactions between intermixing circuits solely with the EnvA/TVA-mediated RV tracing system in a single animal. To overcome this limitation, here, we multiplexed RVΔG circuit tracing by optimizing distinct viral envelopes (oEnvX) and their corresponding receptors (oTVX). Based on the EnvB/TVB and EnvE/DR46-TVB systems derived from the avian sarcoma leukosis virus (ASLV), we developed optimized TVB receptors with lower or higher affinity (oTVB-L or oTVB-H) and the chimeric envelope oEnvB, as well as an optimized TVE receptor with higher affinity (oTVE-H) and its chimeric envelope oEnvE. We demonstrated independence of RVΔG infection between the oEnvA/oTVA, oEnvB/oTVB, and oEnvE/oTVE systems and *in vivo* proof-of-concept for multiplex circuit tracing from two distinct classes of layer 5 neurons targeting either other cortical or subcortical areas. We also successfully labeled common input of the lateral geniculate nucleus to both cortico-cortical layer 5 neurons and inhibitory neurons of the mouse V1 with multiplex RVΔG tracing. These oEnvA/oTVA, oEnvB/oTVB, and oEnvE/oTVE systems allow for differential labeling of distinct circuits to uncover the mechanisms underlying parallel processing through independent circuits and integrated processing through interaction between circuits in the brain.

Keywords: rabies virus, transsynaptic targeting, neural circuit, multiplex, anatomy

INTRODUCTION

The function of the nervous system arises from complex interactions between networks of neurons composed of multiple cell types. Each cell type has its own molecular, morphological, neurophysiological, and anatomical properties and organizes unique neural circuits. The wiring patterns of individual cell types underlie how neural circuits process and represent information. Detailed information on the cell types and their connectivity, in addition to the spatiotemporal patterns of activity in neural circuits, is essential for understanding how the brain functions. Parallel information processing through independent circuits is a commonly-used strategy in the brain (Nassi and Callaway, 2009), but processed information is combined by integration of neural circuits. Interaction between circuits underlies diverse and complex computations that generate perception, cognition, memory, and behavior. Thus, the combination of parallel processing through independent circuits and integrated processing through interaction between circuits is the fundamental principle of neural circuits and computations in the brain. Despite the advances in methods for linking cell types to neural circuits (Arenkiel and Ehlers, 2009; Luo et al., 2018), a major impediment in elucidating how information processing is integrated in the nervous system is lack of means for the dissection of the complex interactions between neural circuits.

The development of viral vectors has transformed the field of neuroanatomy. In particular, G-deleted rabies viral vectors (RVΔG) have been used to reveal neuronal connectivity in the mammalian nervous system (Wickersham et al., 2007; Marshel et al., 2010; Wall et al., 2010; Osakada et al., 2011; Rancz et al., 2011; Watabe-Uchida et al., 2012; Miyamichi et al., 2013; Osakada and Callaway, 2013; Zhu et al., 2014; Bourane et al., 2015; Wertz et al., 2015; Vaziri and Connor, 2016; François et al., 2017; Sun et al., 2017; Kaelberer et al., 2018). Rabies viruses infect neurons through axon terminals and spread across the synapses between neurons in a retrograde direction (Ugolini, 1995, 2010). To take advantage of their trans-synaptic spread property, cell-type-specific infection of rabies viral vectors is required for mapping the neuronal connectivity of target cell types in the brain. Rabies viruses are enveloped viruses with the rabies virus glycoprotein (RV-G) gene as a native envelope. As the glycoprotein gene is deleted from the viral genome, the envelope of G-deleted rabies viruses can be replaced with a foreign one to change their tropism, so-called pseudotyping (Mebatsion et al., 1996; Etessami et al., 2000; Wickersham et al., 2007). Pseudotyping of RVΔG with a foreign glycoprotein allows viral targeting to specific cell populations that express its receptor. The EnvA/TVA is an avian sarcoma leukosis virus (ASLV) infection system that is not recognized by mammalian cells (Bates et al., 1993; Young et al., 1993; Wickersham et al., 2007). The basic approach for targeting specific cell types is that the initial RV infection is targeted to cells of interest by selectively expressing TVA, a specific receptor for EnvA, in those cells *via* viral vectors, transgenic mice, or *in vivo* electroporation (Wickersham et al., 2007; Marshel et al., 2010; Wall et al., 2010,

2016; Rancz et al., 2011; Watabe-Uchida et al., 2012; Miyamichi et al., 2013; Osakada and Callaway, 2013; Zampieri et al., 2014; Zhu et al., 2014; Kim et al., 2015; Wertz et al., 2015; Faget et al., 2016; Beier et al., 2017; Kaelberer et al., 2018). Co-expression of TVA and rabies glycoprotein RV-G in target neurons referred to as "starter cells," allows RVΔG to retrogradely spread to presynaptic neurons directly connected to the starter cells.

This RVΔG monosynaptic tracing represents a powerful approach in mice, zebrafish, cats, and non-human primates (Liu et al., 2013; Dohaku et al., 2019). However, several challenges regarding the development of rabies viral vectors still remain. Especially, analyzing the relationships between neural circuits currently presents a substantial bottleneck to revealing the segregation and integration of complex neural circuits. The EnvA/TVA system for rabies viral tracing allows labeling of only single cell types and their presynaptic neurons. Thus, it has been impossible to reveal the structure, function, and interaction of complicated intermixing circuits organized by multiple cell types (Glickfeld et al., 2013; Yamashita et al., 2013; Lur et al., 2016; Kim et al., 2018). In addition, development of multi-colored, genetically-encoded fluorescent proteins, biosensors, and actuators have allowed us to simultaneously observe different populations in real-time (Chen et al., 2013; Marvin et al., 2013, 2019; Dana et al., 2016; Jing et al., 2018; Luo et al., 2018; Patriarchi et al., 2018; Sun et al., 2018; Abdelfattah et al., 2019; Inoue et al., 2019). By incorporating these tools into the RVΔG tracing system, it is possible to directly link circuits and function (Osakada et al., 2011; Wertz et al., 2015; Tian et al., 2016). Visualizing distinct neural populations with their corresponding fluorescent reporters in real-time is a powerful approach for physiological and behavioral studies. To distinguish starter neurons and presynaptic neurons, it is also required to label starter cells with a reporter that differs in color and is included in the expression cassette of TVA and RV-G. In the present study, we aimed to develop new infection systems of RVΔG to reveal circuit structure, function, and interaction of multiple cell types in the brain.

Here, we first introduced new infection systems using EnvB, EnvC, and EnvE that can be used for pseudotyping RVΔG to restrict viral infection to a specific population in the mouse brain. To eliminate pseudo-negative populations as well as cross-infectivity, we further optimized receptors of EnvB, EnvC, and EnvE to simultaneously dissect multiple circuits. We named these optimized infection systems "oEnvX/oTVX systems" (optimized EnvX/TVX). In conjunction with different colors of reporter fluorescence such as GFP, RFP, BFP, and iRFP, and functional fluorescent probes including genetically encoded sensors for Ca^{2+}, voltage, and neurotransmitters (Chen et al., 2013; Marvin et al., 2013, 2019; Dana et al., 2016; Jing et al., 2018; Patriarchi et al., 2018; Sun et al., 2018; Abdelfattah et al., 2019; Inoue et al., 2019), the multiplexable virus infection systems will allow for *in vivo* interrogation of complex overlapping neural circuits in a single animal. The present study expands the utility of the RVΔG tracing system to simultaneously label and characterize multiple circuits in single animals. The multiplex RVΔG tracing system promises to advance our understanding of the circuit-level

mechanisms underlying information processing in the central nervous system.

MATERIALS AND METHODS

Plasmid Construction

All plasmid construction using polymerase chain reaction (PCR) was performed with PrimeSTAR Max DNA Polymerase (TaKaRa, Tokyo, Japan) on a PCR thermal cycler (Dice Touch, TaKaRa). Plasmids and PCR products were digested with appropriate restriction enzymes (New England BioLabs, Ipswich, MA, USA) at optimal conditions. Plasmid and PCR fragments were assembled with either NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs, Ipswich, MA, USA) or DNA Ligation Kit Mighty Mix (TaKaRa). Chemically-competent Stbl3 *E.coli* (Thermo Fisher Scientific, Waltham, MA, USA) was transformed with AAV plasmids, and chemically-competent XL10-Gold *E. coli* (Agilent Technology, Santa Clara, CA, USA) was used for the other plasmids. The *E. coli* was grown in LB medium (Kanto chemical, Tokyo, Japan) on an LB plate containing ampicillin (100 μg/ml). Plasmids were inspected by diagnostic digestions with appropriate restriction enzymes and sequenced before virus production.

Cell Culture

HEK293t cells were obtained from RIKEN Cell Bank (Saitama, Japan). HEK-TVA, HEK-TVB, BHK-T7, BHK-EnvA, BHK-EnvB, and B7GG cells were gifted by Dr. Callaway (Salk Institute for Biological Studies, La Jolla, CA, USA; Wickersham et al., 2007; Choi et al., 2010; Osakada et al., 2011; Osakada and Callaway, 2013). These cells were maintained in DMEM (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MI, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Wako). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Generation of Cell Lines

HEK-DR46TVB and B7-oEnvE cells were established by the piggyBac system (Ding et al., 2005). pPB-CAG-DR46TVB-IRES-BSD or pPB-CAG-oEnvE-IRES-BSD with pCAG-PBase were co-transfected in HEK293t cells or BHK-T7 cells using Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) and Polyethylenimine "Max" (Polyscience Inc., Warrington, PA, USA). Transfected cells were selected in the presence of blasticidin at a concentration of 100 μg/ml (InvivoGen, San Diego, CA, USA). HEK-DR46TVB and B7-oEnvE cells were maintained in DMEM (Wako), supplemented with 10% FBS (Sigma, St. Louis, MI, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

RVΔG Production

RVΔG was produced as described previously (Osakada et al., 2011; Osakada and Callaway, 2013). Briefly, RVΔG-GFP, RVΔG-DsRedexpress, and RVΔG-tagBFP were recovered in B7GG cells by transfection with pcDNA-B19G, pcDNA-B19N, pcDNA-B19P, pcDNA-B19L, and corresponding

genomic plasmid (pSADΔG-GFP, pSAD-ΔG-DsRedexpress, or pSAD-ΔG-tagBFP) by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The B7GG cells during virus production were cultured in DMEM and supplemented with 10% FBS (Sigma, St. Louis, MI, USA) in a humidified atmosphere of 3% CO_2 at 35°C. BHK-oEnvA, BHK-oEnvB, or B7-oEnvE cells were infected with recovered viruses for pseudotyping RVΔG with oEnvA, oEnvB, or oEnvE (see also **Figure 1B**). The virus-containing medium was concentrated by two rounds of ultra-centrifugation (Beckman Coulter, Brea, CA, USA). The infectious titers were determined on HEK-TVA, HEK-TVB, and HEK-DR46TVB cells. HEK293t cells were used to inspect for contamination with unpseudotyped rabies viruses. Virus aliquots were stored at −80°C until use. The titers of the rabies viral vectors used in the present study were 7.0×10^5–2.9×10^9 infectious units/ml.
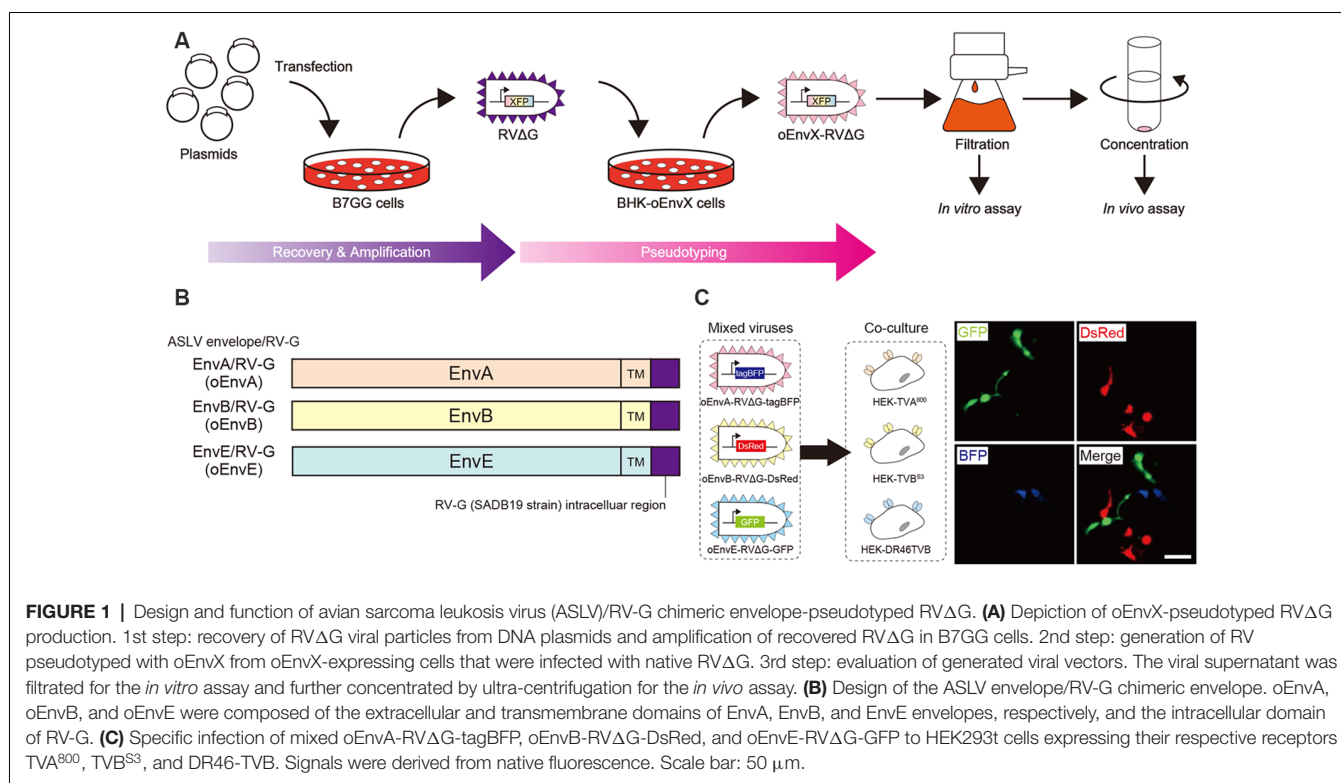
AAV Production

AAVs were generated in HEK293t cells with triple-transfection methods with some modifications (Xiao et al., 1998). Briefly, AAV2/2, AAV2/9, and AAV2retro were generated by transfection of HEK293t cells with pHelper (Cell BioLabs, San Diego, CA, USA), the rep/cap vector (pXR2 for AAV2/2, pAAV2.9 for AAV2/9, or rAAV2-retro helper for AAV2retro) and pAAV genomic vector. Three days post-transfection, virus-producing cells were collected and lysed by freeze and thaw for purification. After centrifugation, the supernatant was loaded on gradients (15%, 25%, 40%, and 58%) of iodexanol OptiPrep (Alere Technologies AS, Stirling, Scotland). After centrifugation at 16000 *g* at 15°C for 3 h, 100–150 μl upper from 58% iodexanol layer was collected. Virus aliquots were stored at −80°C until use. The titers of AAV used in the present study were 5.0×10^2 infectious units/ml (HEK293t cells) or 1.0×10^{11}–1.8×10^{14} viral genome/ml quantitative PCR (qPCR; Aurnhammer et al., 2012).

Animals

Animals were treated in accordance with the Guidelines of Animal Experiments of Nagoya University. All animal experiments in this study were approved by the Animal Care and Use Committee of Nagoya University. All efforts to reduce the number of animals used and minimize the suffering and pain of animals were made. C57BL/6J mice were purchased from Nihon SLC (Shizuoka, Japan). Tlx3-Cre mice (PL56) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Genotyping for Tlx3-Cre mice was performed according to the protocol of Mutant Mouse Resource and Research Center: UC Davis. These animals were maintained in a temperature-controlled room (24 ± 1°C) under a 12 h light/dark cycle with *ad libitum* access to food and water.

Viral Injection

Viral injections were conducted according to the procedures described previously (Osakada et al., 2011) with slight modifications. Briefly, 8-week-old male mice were anesthetized with mixed anesthetic agents (1 ml/kg, i.p.) composed of

FIGURE 1 | Design and function of avian sarcoma leukosis virus (ASLV)/RV-G chimeric envelope-pseudotyped RVΔG. **(A)** Depiction of oEnvX-pseudotyped RVΔG production. 1st step: recovery of RVΔG viral particles from DNA plasmids and amplification of recovered RVΔG in B7GG cells. 2nd step: generation of RV pseudotyped with oEnvX from oEnvX-expressing cells that were infected with native RVΔG. 3rd step: evaluation of generated viral vectors. The viral supernatant was filtrated for the *in vitro* assay and further concentrated by ultra-centrifugation for the *in vivo* assay. **(B)** Design of the ASLV envelope/RV-G chimeric envelope. oEnvA, oEnvB, and oEnvE were composed of the extracellular and transmembrane domains of EnvA, EnvB, and EnvE envelopes, respectively, and the intracellular domain of RV-G. **(C)** Specific infection of mixed oEnvA-RVΔG-tagBFP, oEnvB-RVΔG-DsRed, and oEnvE-RVΔG-GFP to HEK293t cells expressing their respective receptors TVA^{800}, TVB^{S3}, and DR46-TVB. Signals were derived from native fluorescence. Scale bar: 50 μm.

75 μg/ml medetomidine hydrochloride (Domitol; Meiji Seika Pharma Company Limited, Tokyo, Japan), 400 μg/ml midazolam (Dormicum; Astellas Pharma Inc., Tokyo, Japan), and 500 μg/ml butorphanol (Vetorphale; Meiji Seika Pharma Company Limited). Anesthetized mice were mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skull was exposed *via* a small incision, and a small hole was drilled for injection. The virus was loaded into glass pipettes (tip diameter, ≈30 μm; bevel, 45°), pulled on a micropipette puller (P-1000, Sutter Instrument, Novato, CA, USA) and grinded on a microgrinder (EG-401, Narishige, Tokyo, Japan). Before injection of the virus, the pipette was retained in the brain for 5 min after penetration. Three hundred to five hundred nanoliter of virus was injected into the following locations at a rate of 100 nl/min: the V1 (following coordinates: 3.0–3.4 mm rostral, 2.4 mm lateral relative to the bregma and 0.4–0.5 mm ventral to the pia), V2L (3.7 mm rostral, 3.5 mm lateral relative to the bregma and 0.4–0.5 mm ventral to the pia), and SC (3.2 mm rostral, 0.3–0.4 mm lateral relative to the bregma and 2.0 mm ventral to the pia) using a picospritzer (General Valve Corp. Fairfield, NJ, USA). After completion of the injection, the pipette was retained in the brain for another 5 min. No mouse that received viral injection in the brain showed abnormal behavior.

Histological Analysis

Animals were deeply anesthetized by pentobarbital (Somnopentyl, Kyoritsu Seiyaku Company Limited, Tokyo, Japan) and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were post-fixed with 4% paraformaldehyde in PBS and cryopreserved overnight in 2% paraformaldehyde/15% sucrose in PBS and then maintained in 30% sucrose in PBS. The brains were sectioned on a freezing microtome (REM-710, Yamato, Saitama, Japan) at 50 μm thickness. For enhancement of native signals, some brain sections were processed for immunostaining as described previously (Osakada et al., 2008, 2011). The primary antibodies and their working dilutions were as follows: rabbit anti-GFP polyclonal (1:2,000, Abcam, UK) and rabbit anti-DsRed polyclonal (1:1,000, Clontech-Takara, Japan). Labeled cells were visualized with the following fluorescent secondary antibodies: anti-mouse IgG and anti-rabbit IgG conjugated with Alexa Flour 488 or Alexa Flour 594 (1:1,000, Jackson Immunoresearch, West Grove, PA, USA). The sections were mounted on slide glasses with anti-fade solution (Nakalai, Kyoto, Japan). Labeled cells were imaged with a confocal laser-scanning microscope with GaAsP detectors (LSM800, Zeiss, Jena, Germany) using a 10× (NA 0.45, Zeiss, Jena, Germany), 20× (NA 0.75, Zeiss, Jena, Germany), or 40× objective lens (NA 1.2, Zeiss, Jena, Germany).

Statistical Analysis

Data are expressed as means ± SEM. All sets of experiments were performed at least three times. The statistical significance of the difference between groups was determined by one-way ANOVA, followed by Tukey's or Dunnett's test using R (R Foundation, Vienna, Austria[1]). Probability values lower than 5% were considered statistically significant.

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[1]https://www.r-project.org/

RESULTS

Development of a Chimeric ASLV/RV-G Envelope-Pseudotyped RVΔG

Glycoproteins of enveloped viruses such as rabies viruses recognize and bind to their cell receptors, leading to viral entry. Rabies viruses infect neurons through axon terminals (Ugolini, 1995, 2010). Through replication and transcription in the infected cells, rabies viruses spread across the synapses located between the neurons in the nervous system. Deleting native envelope glycoprotein genes from the rabies genome and pseudotyping the RVΔG with a foreign glycoprotein, such as the EnvA derived from ASLV, allows targeting of viral vectors to specific cell populations that express the receptors of foreign glycoproteins such as TVA (Mebatsion et al., 1996; Etessami et al., 2000; Wickersham et al., 2007; Osakada and Callaway, 2013; **Figure 1A**). Notably, ASLV uses various other viral envelopes such as EnvB, C, D, E, and J, in addition to EnvA (Barnard and Young, 2003; Barnard et al., 2006). The cloning of ASLV envelopes and their receptors has revealed their specificity (Barnard et al., 2006; Chai and Bates, 2006): TVB[S1] for EnvB and EnvE, TVB[S3] for EnvB, TVC for EnvC, and TVJ for EnvJ (Smith et al., 1998; Adkins et al., 2000; Elleder et al., 2005; Chai and Bates, 2006). By using these systems, lentiviral vectors pseudotyped with EnvB, EnvC, and EnvE have been reported to infect cells expressing their corresponding receptors (Matsuyama et al., 2015).

To take advantage of this system to target rabies viral vectors to a particular cell population, RVΔG needs to be pseudotyped with the foreign ASLV envelopes. However, ASLV native envelope proteins are optimized to ASLV but not to rabies viruses. Thus, engineering envelope proteins is required for packaging the rabies viral core with the foreign envelopes. First, we designed chimeric glycoproteins because the native EnvXs envelopes are not compatible with rabies viral cores. Previous reports have used the EnvA/RV-G chimeric protein consisting of an extracellular domain of EnvA and an intracellular domain of rabies viral glycoproteins for RVΔG pseudotyping (Wickersham et al., 2007; Osakada and Callaway, 2013). According to the EnvA/RV-G design, we replaced the intracellular domain of EnvB, EnvC, or EnvE with that of the rabies viral glycoprotein RV-G to generate EnvB/RV-G, EnvC/RV-G, or EnvE/RV-G, respectively. To differentiate the chimeric glycoproteins (EnvX/RV-G) for RVΔG from the native glycoproteins (EnvX) for ASLV, we termed the optimized chimeric glycoproteins EnvX/RV-G "oEnvX" (i.e., EnvA/RV-G for oEnvA, EnvB/RV-G for oEnvB, EnvC/RV-G for oEnvC, and EnvE/RV-G for oEnvE; **Figure 1B**). EnvD was excluded from this procedure because the EnvD-packaged virus may infect mammalian cells that lack foreign receptors (Bova et al., 1988). EnvJ was also omitted because TVJ, an EnvJ receptor, is a sodium-proton exchanger that may potentially affect neuronal activity (Chai and Bates, 2006; Matsuyama et al., 2015).

Using these chimeric envelopes, we next produced pseudotyped RVΔG with oEnvB, oEnvC, or oEnvE, by transiently expressing them in BHK cells by plasmid transfection.

First, we determined whether these pseudotyped RVΔG can infect the mammalian HEK293t cells without introducing the ASLV receptors. No infection with oEnvB- or oEnvE-pseudotyped RVΔG was observed in HEK293t cells (**Table 1**). However, we found a degree of infection with oEnvC-pseudotyped RVΔG in HEK293t cells (data not shown), suggesting non-specific infection in mammalian cells.

Thus, we excluded the oEnvC/TVC system from the following studies. To test for the specificity of the pseudotyped RVΔG, we determined whether a mixture of oEnvA-, oEnvB-, and oEnvE-pseudotyped RVΔGs could cause cross-infection to a coculture of TVA[800]-, TVB[S3]-, and DR46-TVB-expressing cells. To distinguish viral infection in three independent cell groups in a mixed population, we prepared pseudotyped RVΔG with distinct envelopes that encode different fluorescent proteins: oEnvA-RVΔG-tagBFP (blue), oEnvB-RVΔG-DsRed (red), and oEnvE-RVΔG-GFP (green; **Figure 1C**). We applied the mixture of the viruses to the mixed culture of those TVX-expressing cells. Three days after viral infection, the infected cells expressed only one fluorescent protein. No cells expressed multiple colored fluorescent proteins. Therefore, we conclude that oEnvA-, oEnvB-, and oEnvE-RVΔG specifically infect cells expressing TVA[800], TVB[S3], and DR46-TVB, respectively. These results imply that oEnvA/TVA, oEnvB/TVB, and oEnvE/DR46-TVB can be applied to multiplexing RVΔG infection.

To apply the multiplex infection system *in vivo*, we need to obtain the pseudotyped RVΔG at high titers. We next attempted to establish an efficient virus-production system. Since cell lines expressing EnvA/RV-G or EnvB/RV-G generated high titers of the pseudotyped RVΔG (Wickersham et al., 2007; Choi et al., 2010; Osakada et al., 2011), we also generated an oEnvE-expressing cell line. Generally, the efficiency of RVΔG pseudotyping depends on the expression level of the envelope of the host cell. Therefore, to establish a cell line expressing an envelope protein at a higher expression level, we used the piggyBac transposon system that enables integrating multiple copies of exogenous genes into the chromosomal DNA of the host cells (Ding et al., 2005). We successfully generated BHK-T7 cells stably expressing oEnvE and named them B7-oEnvE cells. Using these oEnvX-cell lines, we successfully produced pseudotyped RVΔG at a high titer: oEnvA-RVΔG at 5.3×10^7, oEnvB-RVΔG at 4.2×10^6, and oEnvE-RVΔG at 7.0×10^5 (**Table 1**). These results suggest that the stable cell lines expressing chimeric envelopes can efficiently generate high titers of pseudotyped RVΔG that can be used for *in vivo* experiments.

Generation of the Optimized oEnvA/oTVA System

For multiplex RVΔG tracing, we need to reliably visualize starter neurons expressing TVX receptors. For this purpose, we also envisioned to functionally characterize the connectionally defined neurons by combining RVΔG trans-synaptic tracing with *in vivo* functional imaging. To reveal the relationship between neuronal connectivity and function, we used fluorescent indicators for calcium, voltage, or neurotransmitters together with the RVΔG system for *in vivo* functional imaging. The hurdle

Virus Name	Infectious Units (/ml)			
	HEK-TVA[800]	HEK-TVB[S3]	HEK-DR46-TVB	HEK293t
oEnvA-RVΔG-DsRed	5.3×10^7	n.d.	n.d.	n.d.
oEnvB-RVΔG-GFP	n.d.	4.2×10^6	n.d.	n.d.
oEnvE-RVΔG-DsRed	n.d.	n.d.	7.0×10^5	n.d.

HEK cells expressing either TVA, TVB, or DR46TVB were respectively infected with oEnvA-RVΔG, oEnvB-RVΔG, or oEnvE-RVΔG. No infections with oEnvA-RVΔG, oEnvB-RVΔG, and oEnvE-RVΔG were observed in HEK293t cells. Data represent the infectious units. n.d.: not detected.

of such an experiment is that co-labeling of the connectionally defined neurons with fluorescent reporters (green or red) and indicators is not useful, as many fluorescent indicators such as GCaMP, XCaMPs, jRGECO, iGluRSnFr, and dLight1 are of the same color (Chen et al., 2013; Marvin et al., 2013, 2019; Dana et al., 2016; Jing et al., 2018; Patriarchi et al., 2018; Sun et al., 2018; Inoue et al., 2019). Thus, infrared fluorescent reporters that do not interfere with green and red fluorescence are required for co-labeling of the connectionally defined neurons (Shcherbakova and Verkhusha, 2013). Therefore, to simultaneously visualize multiple circuits with different colors, we generated an infrared-tagged TVA receptor for starter neuron labeling.

TVA has variants with different affinities. Among them, the original type TVA[800] displays the highest affinity to EnvA; however, TVA[800] is a glycophosphatidylinositol-anchored receptor without a transmembrane region (Bates et al., 1993; Gray et al., 2011). Thus, its N-terminal cannot be fused to a fluorescent protein for its visualization. Conversely, TVA[950] is a single transmembrane receptor and can be fused to a fluorescent protein for the visualization of TVA-expressing starter cells: TVA[950]-EYFP (Faget et al., 2016), TVA[950]-mCherry (Watabe-Uchida et al., 2012), and low-affinity mutant TVA[950E66T]-mCherry (Miyamichi et al., 2013). The high sensitivity of TVA[950]-mCherry causes oEnvA-RVΔG-GFP infection to cells expressing TVA[950]-mCherry at a level where mCherry signals are not detected. Using TVA[950]-mCherry for rabies trans-complementation experiments, the starter cells appear mCherry-negative and GFP-positive with subsequent pseudo-negative results. To overcome this issue, we utilized TVA[950E66T], a mutant TVA at E66T, with a lower affinity to oEnvA-RVΔG viruses (Rong et al., 1998; Miyamichi et al., 2013). Accordingly, the cells expressing TVA[950E66T]-mCherry at higher levels can be infected with oEnvA-RVΔG viruses allowing elimination of pseudo-negative cell populations because starter cells expressing high levels of mCherry can be unambiguously observed. Cells expressing lower levels of TVA[950E66T] with undetectable mCherry cannot be infected with oEnvA-RVΔG viruses. The strength of TVA[950E66T]-mCherry is the clear and reliable visualization of starter neurons during rabies tracing experiments because a high TVA[950E66T]-mCherry expression is required for oEnvA-RVΔG infection.

To develop a new TVA that could be used together with green and red fluorescence, we generated TVA[950E66T] fused with iRFP670 (termed "oTVA-L-iRFP") as a reporter, the brightest infrared fluorescent protein at present (Shcherbakova and Verkhusha, 2013; Shcherbakova et al., 2018; **Figure 2A**).

First, to examine the subcellular localization of oTVA-L-iRFP, the encoding plasmid was transfected into HEK293t cells (**Figure 2B**). Confocal microscopy revealed that the iRFP670 signals derived from oTVA-L were localized on the cell membrane, consistent with TVA[950E66T]-mCherry membrane localization. We next investigated whether oEnvA-RVΔG could infect the oTVA-L-iRFP-expressing cells (**Figure 2C**). oEnvA-RVΔG-GFP was applied to the oTVA-L-iRFP-transfected HEK293t cells. Most iRFP^{+} cells were also GFP-positive. While the TVA[950]-expressing cells were efficiently infected with oEnvA-RVΔG, infection of oTVA-L-iRFP-expressing cells was comparable to that of oTVA-L-mCherry-expressing cells. These results indicate that oTVA-L-iRFP can be used as a receptor for oEnvA-RVΔG *in vitro*, similar to oTVA-L-mCherry.

We next evaluated the *in vivo* function of oTVA-L-mCherry and oTVA-L-iRFP to determine whether they could be used for rabies tracing experiments in the mouse brain. To introduce oTVA-L to a specific population in the brain, we used Tlx3-Cre mice, in which layer 5 cortical neurons specifically express Cre recombinase (Kim et al., 2015; **Figure 2D**). AAV2/9-CAG-DIO-oTVA-mCherry or AAV2/9-CAG-DIO-oTVA-L-iRFP that expresses oTVA-L-mCherry or oTVA-L-iRFP in a Cre-dependent manner, respectively was used as control. We injected AAV2/9-CAG-DIO-oTVA-L-mCherry or AAV2/9-CAG-DIO-oTVA-L-iRFP into the primary visual cortex (V1) of Tlx3-Cre mice and then oEnvA-RVΔG-GFP in the AAV-injection location (**Figure 2D**). Seven days after oEnvA-RVΔG injection, we fixed and sectioned the brain of the virus-injected mice. The signal of mCherry was specifically observed in layer 5 neurons around the V1 injection site (**Figure 2E**), indicating that oTVA-L was expressed in layer 5 neurons in a Cre-dependent manner. Part of the mCherry^{+} cells expressed GFP in layer 5. All GFP^{+} cells were also mCherry-positive in layer 5, and none of the GFP^{+} cells were distributed in the other V1 layers (total 444 mCherry-expressing neurons/444 GFP-expressing neurons from three mice). These results indicate that oEnvA-RVΔG-GFP specifically infected the oTVA-L-mCherry-expressing neurons in layer 5 of the mouse cortex.

Similar results were obtained for oTVA-L-iRFP (**Figure 2F**). iRFP-positive cells were localized in layer 5 of the V1. Part of the iRFP^{+} cells expressed GFP in layer 5. All GFP^{+} cells were positive for iRFP670 in layer 5 (total 261 iRFP-expressing neurons/261 GFP-expressing neurons from three mice). These results indicate that oEnvA-RVΔG-GFP specifically infected the oTVA-L-iRFP-expressing neurons *in vivo*. No infection with

FIGURE 2 | Generation of an optimized oEnvA/oTVA system for unambiguously marking starter cells. **(A)** Design of TVA variants fused to a fluorescent protein. TVA^{950} and its lower-affinity version TVA^{950E66T} (oTVA-L) were fused to mCherry or iRFP670. **(B)** Membrane localization of

(Continued)

FIGURE 2 | Continued

oTVA-L-mCherry (left) and oTVA-L-iRFP (right) in transfected HEK293t cells. Scale bar: 20 μm. **(C)** Infection efficiency of oEnvA-RVΔG to cells expressing TVA^{950}-mCherry, oTVA-L-mCherry, or oTVA-L-iRFP. Each column represents the mean ± SEM ($n = 3$). **$P < 0.01$ vs. TVA^{950}-mCherry (Tukey's multiple tests). n.s.: not significant. n.d.: not detected. **(D)** *In vivo* evaluation of oTVA-L-iRFP function in the mouse brain. AAV2/9-DIO-oTVA-L-mCherry or AAV2/9-DIO-oTVA-L-iRFP was injected to the V1 of layer 5-specific Tlx3-Cre mice to introduce oTVA-L-mCherry or oTVA-L-iRFP in layer 5 neurons. oEnvA-RVΔG-GFP was injected at the location of the AAV injection. **(E)** Reliable targeting of oEnvA-RVΔG-GFP to oTVA-L-mCherry-expressing cells. oTVA-L-mCherry was expressed in layer 5 neurons in the V1 of Tlx3-Cre mice. All oEnvA-RVΔG-GFP-infected neurons expressed oTVA-L-mCherry. Signals were derived from native fluorescence. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Scale bar: 20 μm. **(F)** Reliable targeting of oEnvA-RVΔG-GFP to oTVA-L-iRFP670-expressing cells. oTVA-L-iRFP was expressed in layer 5 neurons in the V1 of Tlx3-Cre mice. All oEnvA-RVΔG-GFP-infected neurons expressed oTVA-L-iRFP. Signals were derived from native fluorescence. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Scale bar: 20 μm.

oEnvA-RVΔG-DsRed was observed in the absence of oTVA-L in mice (**Supplementary Figure S1A**). Thus, the initially-infected cells can be reliably visualized with oTVA-L-iRFP *in vivo*, similar to oTVA-L-mCherry (**Figures 2E,F**).

To determine the specificity and cross-infectivity of oTVA-L to EnvX-RVΔG, we applied each oEnvX-pseudotyped RVΔG to oTVA-L-expressing HEK293t cells and quantified the number of oEnvX-RVΔG-infected cells (**Table 2**). oEnvA-RVΔG infected the oTVA-L-expressing cells, whereas oEnvB-RVΔG and oEnvE-RVΔG did not. Taking together, we conclude that oTVA-L is specific to oEnvA-RVΔG and that its fusion reporter allows reliable visualization of the oTVA-L-expressing starter cells *in vivo*.

Generation of the Optimized oEnvB/oTVB System

EnvA/TVA allows viral targeting to a particular cell population. To target two distinct populations with two different viruses, we next designed an additional infection system based on the EnvB/TVB system. oEnvB-pseudotyped rabies viral vectors have the potential to infect *via* TVB *in vivo* (Choi et al., 2010). We first attempted to develop TVB, a receptor for oEnvB-enveloped viruses. As the original TVB has several use issues, we optimized the TVB receptors for the rabies tracing experiments focusing on envelope affinity and fluorescence reporter detectivity to utilize the EnvB/TVB system for restricted viral transduction in a specific neuronal population.

TVB reportedly has the following variants with different affinities for various ASLVs: TVB^{S1}, TVB^{r2}, TVB^{S3}, and TVB^{T} (Brojatsch et al., 1996; Adkins et al., 1997, 2000; Smith et al., 1998). Among the several subtypes, we selected TVB^{S3} because it does not recognize and bind to EnvE, whereas the other TVB variants bind to both EnvB and EnvE (Adkins et al., 2000; Klucking and Young, 2004; Reinisová et al., 2008; Matsuyama et al., 2015). However, TVB is a homolog of the mammalian TNF-related apoptosis-inducing ligand death receptor 4/5; when ASLV-B and ASLV-E infected cells *via* TVB, TVB induced

TABLE 2 | Specificity of oTVA-L, oTVB-L, and oTVE-H.

Chimeric Envelope	Number of Infected Cells			
	oTVA-L (TVA^{950E66T})	oTVB-H (TVB^{S3}-TVA)	oTVE-H (DR46-TVB-TVA)	(−)
oEnvA-RVΔG	119 ± 8.5	n.d.	n.d.	n.d.
oEnvB-RVΔG	n.d.	112 ± 11	n.d.	n.d.
oEnvE-RVΔG	n.d.	n.d.	>200	n.d.

HEK cells expressing either oTVA-L, oTVB-L, and oTVE-H were specifically infected with oEnvA-RVΔG, oEnvB-RVΔG, or oEnvE-RVΔG. oTVA-L, oTVB-L, and oTVE-H were specific to oEnvA-RVΔG, oEnvB-RVΔG, and oEnvE-RVΔG, respectively. No infections with oEnvA-RVΔG, oEnvB-RVΔG, and oEnvE-RVΔG were observed in HEK293t cells. Data represent the infectious units. n.d.: not detected.

apoptosis (Brojatsch et al., 1996, 2000; Smith et al., 1998; Klucking et al., 2005). The death domain of TVB has been reported to be responsible for inducing apoptosis (Adkins et al., 2000). To maintain the infected neurons healthy during the viral tracing experiments, we deleted the death domain from the full-length of the TVB receptor. Additionally, to visualize the TVB-expressing cells, we replaced the TVB^{S3} death domain with tagBFP, the brightest fluorescent blue protein (**Figure 3A**). The tagBFP signal was weakly observed at the cell membrane and strongly at the cytoplasm of HEK293t cells transfected with a TVB^{S3}-tagBFP plasmid (**Figure 3B**). Cytoplasm aggregation could cause cell cytotoxicity (Eisele et al., 2015; Boland et al., 2018). Thus, to reduce TVB^{S3}-tagBFP aggregation, we designed a new TVB receptor. First, we attempted to insert a GGG peptide linker as a fusion protein, such as TVA^{950}-mCherry, into the TVB^{S3}-tagBFP segment. However, no change was shown (data not shown). As TVA^{950} was distributed on the membrane (**Figure 2B**), we replaced the TVB cytoplasmic tail with TVA^{950} to generate a TVB^{S3}-TVA chimera receptor (**Figure 3A**, **Supplementary Figure S2**). The TVB^{S3}-TVA chimera receptor was fused to tagBFP and expressed in the cell membrane without aggregating inside the cell (**Figure 3B**). To determine whether the cells expressing these TVB variants could be infected with oEnvB-RVΔG, we transfected HEK293t cells with TVB^{S3}-TVA-tagBFP and then we applied oEnvB-RVΔG-DsRed to the transfected cells (**Figure 3C**). Three days after the viral infection, TVB^{S3}-TVA-tagBFP-expressing cells were positive for DsRed. TVB^{S3}-TVA-tagBFP had higher infection efficiency by oEnvB-RVΔG compared to TVB^{S3}-tagBFP. We termed the TVB^{S3}-TVA receptor "oTVB-H" (optimized TVB with the highest affinity). We further assessed the cross-infectivity of oTVB-H to oEnvX-pseudotyped RVΔG. Each oEnvX-pseudotyped RVΔG was applied to oTVB-H-expressing HEK293t cells (**Table 2**). The oEnvB-RVΔG infected the oTVB-H-expressing cells whereas oEnvA-RVΔG and oEnvE-RVΔG did not. Taken together, these results indicate that oTVB-H is a high-affinity receptor with a specificity to oEnvB-RVΔG.

We next investigated whether oTVB-H can be used for viral targeting of a specific population in the brain. To restrict the viral infection to a particular cell population, we again used Tlx3-Cre mice, a layer 5-specific Cre-line; we injected Cre-dependent AAV expressing oTVB-H-tagBFP (AAV2/9-CAG-DIO-oTVB-H-tagBFP) to the V1 of Tlx3-Cre mice (**Figure 3D**). oEnvB-RVΔG-DsRed was injected at the same location in the V1 2–3 weeks after the AAV injection. The DsRed signal was observed in layer 5 neurons in Tlx3-Cre mice, but not in the

neurons or glia of other layers; however, the DsRed$^+$ cells in layer 5 were tagBFP-negative (**Figure 3E**). Tlx3-Cre mice that received injections of oEnvB-RVΔG-DsRed but not of AAV2/9-CAG-DIO-oTVB-H-tagBFP were used as controls, and as expected, no DsRed$^+$ cells were observed in the absence of oTVB-H. Our results suggest that the oEnvB-RVΔG-DsRed-infected layer 5 neurons express oTVB-H in a Cre-dependent manner, probably because oTVB-H is markedly sensitive to oEnvB-RVΔG infection; even when the BFP reporter signal cannot be detected due to the low expression level of oTVB-H-BFP, oEnvB-RVΔG can still infect these cells.

To overcome this issue, we attempted to clearly visualize the starter cells with fluorescent reporters by developing a low-affinity type TVB receptor, as shown in the cases of TVA^{950} and TVA^{950E66T} (Watabe-Uchida et al., 2012; Miyamichi et al., 2013; **Figure 2**). To obtain an ideal receptor, we compared it with other TVB subtypes (**Figure 3F**). TVB has four subtypes (TVB^{S1}, TVB^{r2}, TVB^{S3}, and TVB^T) that can recognize and bind to ASLV-B (Brojatsch et al., 1996; Reinisová et al., 2008). The extracellular domain of TVB is composed of three cysteine-rich domains (CRDs; Hymowitz et al., 1999; Adkins et al., 2000; Reinisová et al., 2008). In general, CRD1 is essential for ASLV-B recognition, but CRD2 and CRD3 are not necessary for ASLV-B interaction. Importantly, when these CRD2 and CRD3 domains were mutated or deleted in TVB, ASLV-B infection efficiency tended to decrease (Adkins et al., 2001; Klucking and Young, 2004). Thus, we hypothesized that we can design TVB receptors with lower affinity and higher specificity to EnvB by mutations or deletions of CRD2 and CRD3 from the TVB extracellular domain. Consequently, we designed four TVB mutants that contained a TVA intracellular domain: TVB^{S1}-TVA-tagBFP, TVB^{r2}-TVA-tagBFP, TVB^{S3ΔCRD2}-TVA-tagBFP, and TVB^{S3ΔCRD3}-TVA-tagBFP (**Figure 3F**). Compared to TVB^{S3}, TVB^{S1} has only one mutation (S62C) at CRD2, and TVB^{r2} has two mutations at CRD2 (S62C) and CRD3 (C125S; Reinisová et al., 2008). Previous reports have revealed that TVB^{S1} whose CRD2 or CRD3 was deleted showed reduced ASLV-E and stable or slightly reduced ASLV-B infectivity (Adkins et al., 2001; Klucking and Young, 2004). Thus, we expected that chimeric TVB^{r2} or TVB^{S3} without CRD2 or CRD3 would have a lower affinity and higher specificity to EnvB. Accordingly, we generated a CRD2-deleted mutant TVB^{S3ΔCRD2} and a CRD3-deleted mutant TVB^{S3ΔCRD3} (**Supplementary Figure S2**). To evaluate the *in vitro* localization and function of these TVB mutant receptors, we transfected HEK293t cells with plasmids coding for those mutant receptors and then applied oEnvB-RVΔG-DsRed or oEnvE-RVΔG-GFP to those cells (**Figures 3G,H**)
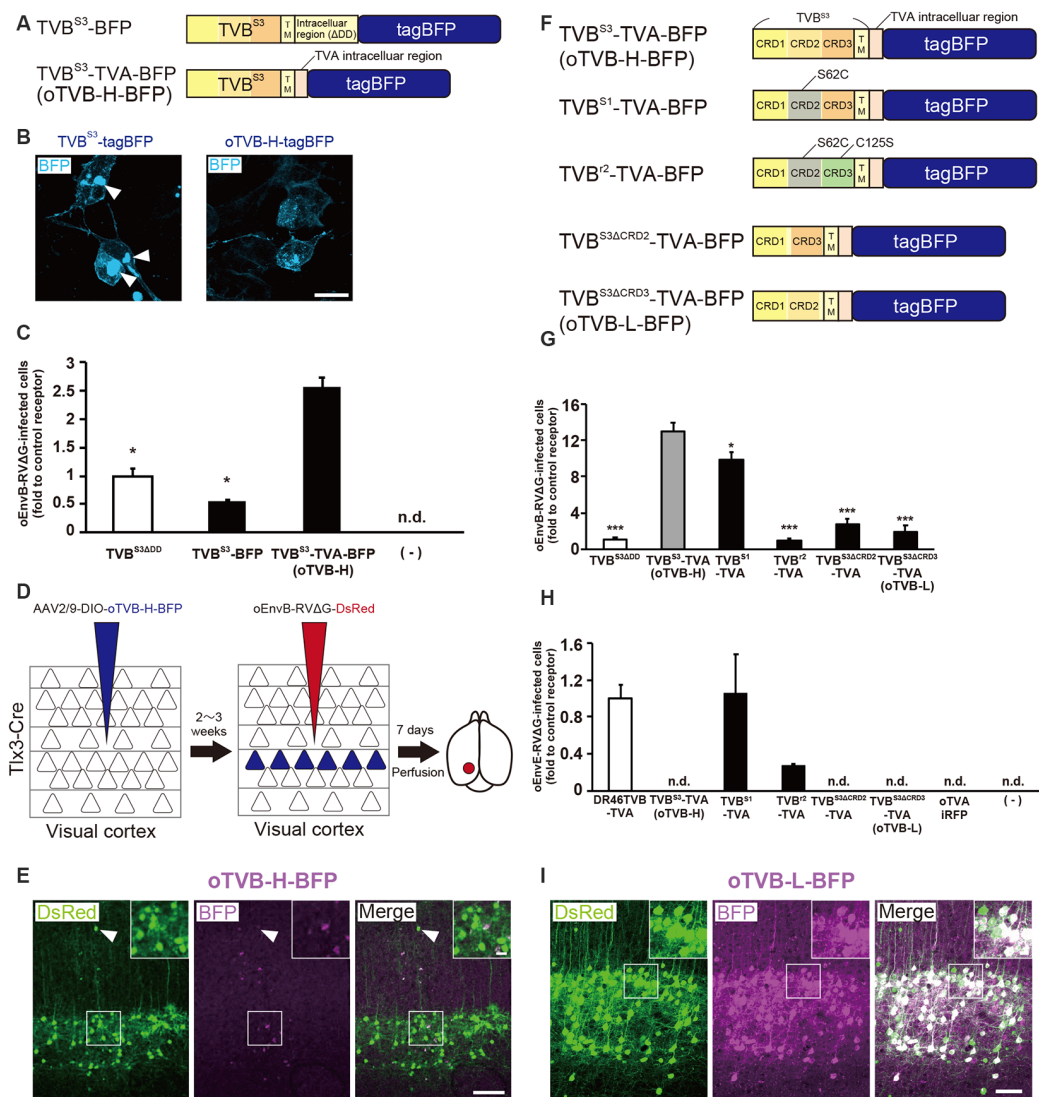
FIGURE 3 | Generation of an optimized oEnvB/oTVB system for viral targeting. **(A)** Design of TVB variants fused to a blue fluorescent protein. TVB$^{S3\Delta DD}$-BFP was deficient in the death domain from TVB^{S3} and fused to tagBFP. TVB$^{S3\Delta DD}$-TVA-BFP (oTVB-H) has a TVA^{950} intracellular region instead of the TVB^{S3} intracellular region and fused-tagBFP. **(B)** Subcellular localization of TVB^{S3}-BFP (left) and oTVB-H-BFP (right) in transfected-HEK293t cells. Abnormal localization of TVB^{S3}-BFP in the cell membrane and cytoplasmic region. Arrowhead: aggregation of TVB^{S3}-BFP. Membrane localization of oTVB-H-BFP. Scale bar: 20 μm. **(C)** Infectious efficiency of oEnvB-RVΔG to cells expressing TVB$^{S3\Delta DD}$, TVB^{S3}-BFP, or oTVB-H-BFP. Each column represents the mean ± SEM ($n = 3$). *$P < 0.05$ vs. oTVB-H (Tukey's multiple tests). n.d.; not detected. **(D)** *In vivo* evaluation of oTVB-H function in the mouse brain. AAV2/9-DIO-oTVB-H-BFP was injected to the V1 of Tlx3-Cre mice to introduce oTVB-H in layer 5 neurons. oEnvA-RVΔG-DsRed was injected at the location of the AAV injection. **(E)** Evaluation of *in vivo* function of TVB^{S3}-TVA-BFP (oTVB-H) in Tlx3-Cre mice. oEnvB-RVΔG-DsRed infected layer 5 neurons without visible oTVB-H-tagBFP signals, suggesting that oTVB-H was sufficiently sensitive for oEnvB-RVΔG-DsRed to infect cells expressing oTVB-H at a low expression level. The arrowhead indicates non-specific infection to neurons in layer 2/3. Signals were derived from native fluorescence. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Scale bar: 20 μm. **(F)** Generation of TVB mutants fused with a blue fluorescent protein. TVB^{S1} has one mutation (S62C). TVB^{r2} has two mutations (S62C and C125S). TVB$^{S3\Delta CRD2}$ is devoid of CRD2 from TVB^{S3}. TVB$^{S3\Delta CRD3}$ is devoid of CRD3 from TVB^{S3}. **(G,H)** Infection efficiency of **(G)** oEnvB-RVΔG and **(H)** oEnvE-RVΔG to cells expressing one of the tagBFP-fused TVB mutants. Each column represents the mean ± SEM ($n = 3$). *$P < 0.05$, ***$P < 0.001$ vs. TVB^{S3}-TVA-BFP (oTVB-H) (Dunnett's multiple test). **(I)** Evaluation of *in vivo* function of TVB$^{S3\Delta CRD3}$-TVA-BFP (oTVB-L). oEnvB-RVΔG-DsRed infected oTVB-L-BFP-expressing neurons of layer 5-specific Tlx3-Cre mice. Signals were derived from native fluorescence. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Scale bar: 20 μm.

because several lines of evidence have indicated that TVB^{S1} and TVB^{T} have affinities to ASLV-B and ASLV-E (Brojatsch et al., 2000; Klucking and Young, 2004; Matsuyama et al., 2015). We counted the number of infected cells 3 days after the infection.

The cells expressing each TVB mutant receptor were infected with oEnvB-RVΔG-DsRed at a different efficiency (**Figure 3G**). The oTVB-H-expressing cells were the most efficiently infected with oEnvB-RVΔG-DsRed but did not show infectivity with

oEnvE-RVΔG-GFP (**Figures 3G,H**). TVB^{r2}-TVA showed the lowest affinity to oEnvB-RVΔG and limited affinity to oEnvE-RVΔG. Notably, HEK293t cells expressing DR46TVB-TVA, TVB^{S1}-TVA, and TVB^{r2}-TVA were infected with oEnvE-RVΔG, but no infection with oEnvE-RVΔG was observed in HEK293t cells expressing TVB$^{S3\Delta CRD2}$-TVA, TVB$^{S3\Delta CRD3}$-TVA, or oTVA-iRFP (**Figure 3H**). TVB$^{S3\Delta CRD2}$-TVA-tagBFP and TVB$^{S3\Delta CRD3}$-TVA-tagBFP had the lowest affinity and highest specificity to oEnvB-RVΔG but not to oEnvE-RVΔG. These results suggest that TVB$^{S3\Delta CRD3}$-TVA can be used as a specific TVB receptor with the lowest affinity to oEnvB-RVΔG. Hereafter, we refer to TVB$^{S3\Delta CRD3}$-TVA as "oTVB-L" (optimized TVB with the lowest affinity).

Then, we tested whether oTVB-L-tagBFP can be used for *in vivo* viral targeting to specific cell types and unambiguous visualization of the infected cells. We injected Cre-dependent AAV expressing oTVB-L-tagBFP (AAV2/9-CAG-DIO-oTVB-L-tagBFP) into the V1 of Tlx3-Cre mice to restrict oTVB-L-tagBFP expression to layer 5 neurons (**Figure 3I**). Three weeks after injection, the tagBFP signal was distributed in layer 5 neurons of the V1. We subsequently examined whether oEnvB-RVΔG could infect oTVB-L-expressing neurons *in vivo* and whether the oTVB-L-expressing cells could be visible and unambiguously identified as starter neurons for rabies tracing experiments. We injected AAV2/9-CAG-DIO-oTVB-L-tagBFP into the V1 of Tlx3-Cre mice, and then oEnvB-RVΔG-DsRed into the same location. BFP signals were observed in layer 5 neurons of the mouse V1. A total of 84.8 $\pm$ 4.2% of DsRed^{+} cells co-expressed tagBFP (total 325/347 neurons from four mice; **Figure 3I**). Conversely, no infection with oEnvB-RVΔG-DsRed was observed in the absence of oTVB-L in mice that were not subjected to injection of oTVB-L-tagBFP-expressing AAV (**Supplementary Figure S1B**). These results indicate that oEnvB-RVΔG infected the oTVB-L-expressing neurons and that oTVB-L-tagBFP reliably visualized the oEnvB-RVΔG-infected neurons. Moreover, oTVB-H is too sensitive for use in *in vivo* viral targeting because of its low expression level that can lead to viral infection, thereby generating pseudo-negative populations even when its intrinsic reporter signal is invisible (**Figure 3F**). Taken together, we conclude that oTVB-L is suitable for oEnvB-RVΔG-targeting to particular populations *in vivo*.

Generation of the Optimized oEnvE/oTVE System

We next aimed to develop optimized DR46-TVB receptors that would allow specific targeting of EnvE viruses in the mammalian brain (Klucking and Young, 2004; Matsuyama et al., 2015). Intriguingly, DR46-TVB has been developed as an EnvE-specific receptor by replacing the TVB^{S1} CRD1 region with a corresponding region of death receptor 5 (Klucking and Young, 2004). Thus, we further optimized DR46-TVB as a tumor virus subgroup E (TVE)-specific viral receptor suitable for the rabies tracing experiments (**Figure 4A**). Interestingly, DR46-TVB shares the same transmembrane and cytoplasmic domains with the original TVB. As TVB did not show clear membrane localization in the mammalian cells, we endowed the clear membrane localization and the

fluorescent-protein-tagging of DR46-TVB to efficiently and unambiguously visualize the starter neurons. For that purpose, we followed the same strategy as that used for oTVB. We swapped the intracellular domain of DR46-TVB with the intracellular domain of TVA to generate DR46-TVB-TVA and then we fused the C-terminus of the DR46-TVB-TVA to tagBFP or to mCherry to use it along with TVA^{950E66T}-iRFP670 (**Figure 4A**, **Supplementary Figure S2**). We termed DR46-TVB-TVA "oTVE-H" (optimized DR46-TVB with a high affinity for oEnvE-pseudotyped viruses). Both oTVE-H-tagBFP and oTVE-H-mCherry were localized on the cell membrane of the transfected HEK293t cells (**Figure 4B**).

Next, we determined whether the cells expressing oTVE-H can be infected with oEnvE-RVΔG *in vitro* (**Figure 4C**). We transfected HEK293t cells with the oTVE-H-coding plasmid and subsequently applied oEnvE-RVΔG. oEnvE-RVΔG significantly infected the oTVE-H-expressing HEK293t cells. The infection efficiency of oTVE-H was higher than that of DR46-TVB. The higher infection efficiency of oTVE-H can be attributed to its higher membrane localization compared with that of DR46-TVB. We further investigated the specificity and cross-infectivity of oTVE-H to oEnvX-RVΔG *in vitro* (**Table 2**). We applied oEnvX-RVΔG to oTVE-H-expressing HEK cells and we found that oEnvE-RVΔG infected them, whereas oEnvA-RVΔG and oEnvB-RVΔG did not. Consequently, these results suggest that oTVE-H can be used as a specific receptor for oEnvE-RVΔG targeting.

To evaluate whether oTVE-H allows viral targeting to a specific population *in vivo*, we injected Cre-dependent AAV expressing oTVE-H-mCherry (AAV2/9-CAG-DIO-oTVE-H-mCherry) in the V1 of layer 5-specific Tlx3-Cre mice, as previously performed with oTVA-L and oTVB, and then injected oEnvE-RVΔG-GFP into the same location (**Figure 4D**). We observed some GFP-expressing neurons in layer 5 around the injection site, but not any mCherry-expressing neurons. No infection with oEnvE-RVΔG-GFP was observed in mice lacking oTVE-H expression (**Supplementary Figure S1C**). It is plausible that although a small amount of oTVE-H-mCherry was expressed in layer 5 neurons at the level of an invisible fluorescent reporter signal, its affinity to oEnvE-RVΔG was too high; thus, oTVE-H recognized oEnvE-RVΔG and led to viral infection.

As previously performed with oTVA-L or oTVB-L for unambiguous labeling of target cells *in vivo* (**Figures 2**, **3**), we sought to reduce oTVE-H affinity. Previous reports have indicated that the CRD2 region of TVB^{S1} is important for ASLV-E infection, and the recognition site is composed of three residues in CRD2 (Klucking and Young, 2004). Thus, according to the TVB^{S1} mutation (Klucking and Young, 2004), we generated CRD region-deleted oTVE-H (DR46-TVB$^{\Delta CRD2}$-TVA and DR46-TVB$^{\Delta CRD3}$-TVA) and single mutated oTVE-H receptors (DR46-TVB^{Y102A}-TVA, DR46-TVB^{N107A}-TVA, and DR46-TVB^{N108A}-TVA) fused to tagBFP (**Supplementary Figure S3**). The tagBFP signals of every receptor mutant were observed on the membrane of the transfected-HEK293t cells but none of them recognized oEnvE-RVΔG (data not shown).
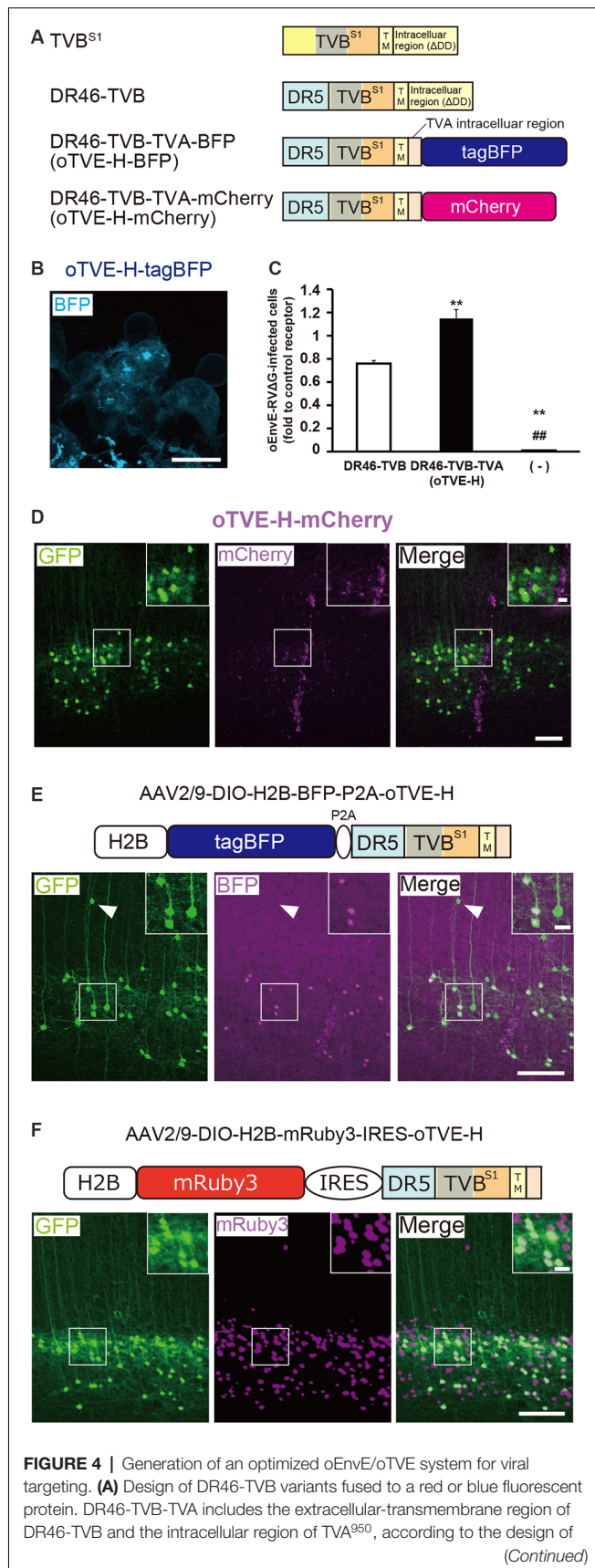
FIGURE 4 | Continued

oTVB (see **Figures 3A,B**). **(B)** Membrane localization of DR46-TVB-TVA (oTVE-H)-BFP in HEK293t cells. The TagBFP signal was observed in the cell membrane. Signals were derived from native fluorescence. Scale bar: 20 μm. **(C)** Infection efficiency of oEnvE-RVΔG to cells expressing DR46-TVB or oTVE-H. Each column represents the mean ± SEM ($n = 3$). **$P < 0.01$ vs. DR46-TVB, ##$P < 0.01$ vs. oTVE-H (Tukey's multiple tests). **(D)** Evaluation of *in vivo* function of oTVE-H-mCherry in Tlx3-Cre mice. Typical images of oEnvE-RVΔG-GFP-infected layer 5 neurons although the layer 5 neurons do not have visible expression of oTVE-H-mCherry. These data suggest that oTVE-H was sufficiently sensitive for oEnvE-RVΔG-GFP to infect layer 5 neurons expressing oTVE-H at a low expression level. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Signals were derived from native fluorescence. Scale bar: 20 μm. **(E)** The 2A-mediated oTVE-H expression system. oTVE-H was introduced by the H2B-tagBFP-P2A-oTVE-H cassette. oEnvE-RVΔG-GFP infected neurons expressing both nuclear H2B-tagBFP and oTVE-H in Tlx3-Cre mice. The arrowhead indicates non-specific infection to neurons in layers 2/3. Signals were derived from native fluorescence. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Scale bar: 20 μm. **(F)** The IRES-mediated the oTVE expression system for reliable detection of oTVE-H-expressing cells. oTVE-H was expressed downstream of the IRES sequence. oEnvE-RVΔG-GFP infected neurons expressing visible H2B-mRuby3 as well as oTVE-H in Tlx3-Cre mice. oEnvE-RVΔG-GFP-infected neurons expressed nuclear mRuby3. Signals were derived from native fluorescence. Scale bar: 100 μm. The inset shows high magnifications of areas indicated by the white square. Scale bar: 20 μm.

FIGURE 4 | Generation of an optimized oEnvE/oTVE system for viral targeting. **(A)** Design of DR46-TVB variants fused to a red or blue fluorescent protein. DR46-TVB-TVA includes the extracellular-transmembrane region of DR46-TVB and the intracellular region of TVA[950], according to the design of
(Continued)

Clear labeling of starter neurons is essential to determine the post-synaptic neurons in rabies tracing experiments, although the cells expressing oTVE-H-mCherry can be infected with oEnvE-RVΔG even when its expression is too low to be detected. It is conceivable that oTVE-H is inefficiently expressed in the mouse brain and its fused-reporter signal is barely detected. Thus, to reliably visualize target cells expressing oTVE-H, we first used a 2A-mediated bicistronic expression system (Szymczak et al., 2004; Chng et al., 2015) with a fluorescence reporter but without immunostaining (**Figure 4E**). We designed a bicistronic viral vector that expressed the two nuclear-localized transgenes tagBFP and oTVE-H (AAV2/9-CAG-DIO-H2B-tagBFP-p2A-oTVE-H). Then, we injected this AAV into the V1 of Tlx3-Cre mice and 2–3 weeks later oEnvE-RVΔG-GFP was injected into the same site. Seven days after RV injection, we counted the number of GFP/tagBFP-double-positive neurons in the V1. The results showed that 84.4 ± 4.4% of the GFP+ neurons also expressed H2B-tagBFP (total 547/659 neurons from three mice). GFP-positive and H2B-tagBFP-negative neurons were observed outside layer 5 of the cortex. This is because leak expression of oTVE-H derived from AAV-DIO vectors induced oEnvE-RVΔG-GFP infection. These results suggest that the 2A-system permits labeling and detection of the starter neurons with a fluorescence reporter and that only a small amount of oTVE-H is needed for the infection of oEnvE-RVΔG even at a barely visible expression level of its reporter fluorescence.

To reliably label and detect oTVE-H-expression cells for circuit mapping, we hypothesized that if oTVE-H expression could be reduced and its reporter fluorescence could be increased, we would be able to unambiguously detect oEnvE-RVΔG-infected neurons without immunostaining. Thus, we used another bicistronic system, the internal ribosome entry
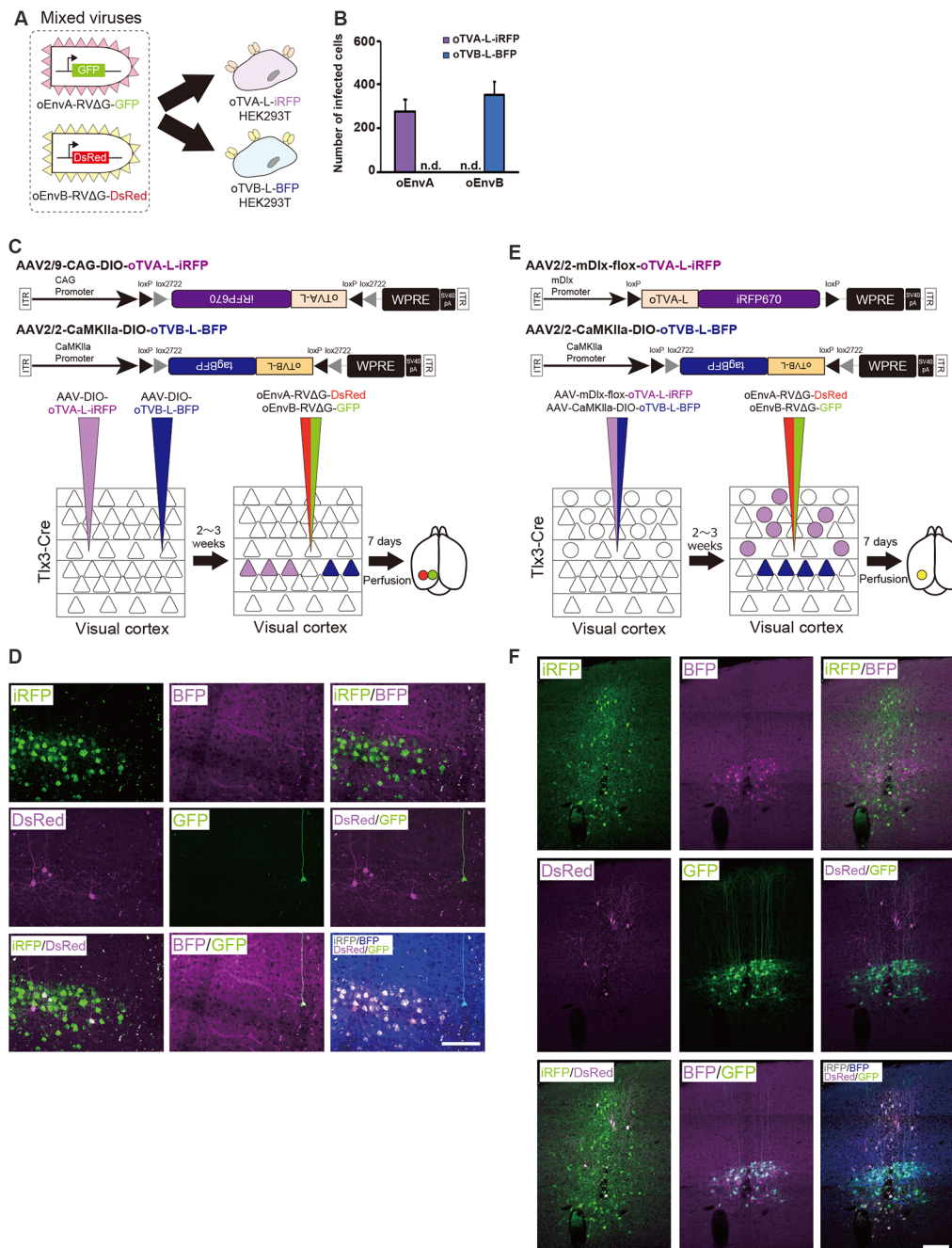
FIGURE 5 | Independence of the oEnvA/oTVA and oEnvB/oTVB systems. **(A)** *In vitro* evaluation of specificity of oEnvA-RVΔG-GFP and oEnvB-RVΔG-DsRed to cells expressing either oTVA-L-iRFP or oTVB-L-BFP. oEnvA-RV and oEnvB-RV mixed viruses applied to HEK293t cells expressing oTVA-L-iRFP or oTVB-L-BFP transitorily. **(B)** No cross-infectivity of oEnvA-RVΔG-GFP and oEnvB-RVΔG-DsRed to cells expressing either oTVA-L-iRFP or oTVB-L-BFP. Evaluation of *in vitro* infectious specificity. Each column represents the mean ± SEM (*n* = 3). n.d.: not detected. **(C)** *In vivo* infectious specificity of oTVA-L-iRFP and oTVB-L-BFP that are expressed in two different locations of the brain. AAV-CAG-DIO-oTVA-L-iRFP and AAV-CaMKIIa-DIO-oTVB-L-BFP were injected in two different locations of Tlx3-Cre mice, 800 μm apart. A mixture of oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP was injected between the two injection sites of AAV. **(D)** Non-co-infection with oEnvA-RVΔG and oEnvB-RVΔG. Signals were derived from native fluorescence. Scale bar: 100 μm. **(E)** *In vivo* infection specificity of oTVA-L-iRFP and oTVB-L-BFP that are expressed in two different cell types. oTVA-L-iRFP and oTVB-L-BFP are exclusively expressed by a distinct promotor and a transgenic Cre line. Inhibitory neurons were targeted by the inhibitory neuron-specific mDlx promoter and layer 5 neurons recombined the AAV genome to excise the flox cassette and eliminate gene expressions. Excitatory neurons in layer 5 of the cortex were targeted by a combination of the excitatory neuron-specific CaMKIIa promoter and the layer 5-specific Tlx3-Cre line. AAV-mDlx-oTVA-L-iRFP and AAV-CaMKIIa-DIO-oTVB-L-BFP were co-injected into the V1 of Tlx3-Cre mice. oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP were co-injected into the location of the AAV injection. **(F)** No cross-infectivity between oEnvA-RVΔG and oEnvB-RVΔG. Signals were derived from native fluorescence. Scale bar: 100 μm.

site (IRES) system, which can adjust oTVE-H expression to a lower level (**Figure 4F**). We generated AAV vectors that allow for lower expression of oTVE-H (AAV2/9-CAG-DIO-H2B-mRuby3-IRES-oTVE-H). This system enables bicistronic expression but the expression level of the second gene (oTVE-H) after the IRES sequence is lower than that of the first gene (H2B-mRuby3; Mizuguchi et al., 2000; Mardinly et al., 2018). We injected AAV2/9-CAG-DIO-H2B-mRuby3-IRES-oTVE-H into the V1 and then applied oEnvE-RVΔG-GFP into the same injection site (**Figure 4F**); 99.0 ± 0.4% of GFP$^+$ cells also expressed H2B-mRuby3 (total 590/595 neurons from three mice). Compared to the 2A system that enables the bicistronic expression of two genes at the same expression level (Chng et al., 2015), the IRES-mediated oTVE expression system facilitates the unambiguous detection of oEnvE-RVΔG-infected neurons without the need of immunostaining signal enhancement (**Figures 4E,F**).

Next, we examined whether the oEnvE/oTVE system can be used for circuit tracing. We injected AAV expressing both oTVE and oG in a Cre-dependent manner into the mouse V1 of Tlx3-Cre mice. Three weeks after the AAV injection, oEnvE-RVΔG-GFP was injected into the same injection site. Ten days after the rabies injection, we found GFP$^+$ presynaptic cells in the V1 and dorsal lateral geniculate nucleus (dLGN; **Supplementary Figure S4**), which is consistent with the previous report (Kim et al., 2015). These results suggest that the oEnvE/oTVE system could be used for trans-synaptic circuit tracing.

In the present study, we developed the chimeric envelope protein, oEnvE, and its cognate high-affinity receptor, oTVE. AAV vectors carrying the IRES-mediated bicistronic system could introduce oTVE-H in starter cells for oEnvE-RVΔG targeting. The oEnvE/oTVE system can be used for circuit mapping for particular cell populations in the mammalian nervous system.

Independence Between the oEnvA/oTVA and oEnvB/oTVB Systems *in vivo*

To differentially dissect and visualize two independent cell populations with fluorescent proteins, we next applied both the oEnvA/oTVA and oEnvB/oTVB systems to the mouse brain. To trace circuits with RVΔG, we needed to reliably label starter cells and introduce two genes RV-G and oTVX in the starter cells. For that purpose, we used a 2A element to express both genes from the AAV genome. To label two different populations, we selected oTVA-L and oTVB-L, as both are low-affinity receptors to the corresponding envelopes (**Figures 2E,F, 3I**). Initially, to assess the specificity and cross-infectivity of oTVA-L-iRFP and oTVB-L-tagBFP, we performed an *in vitro* assay by preparing the oTVA-L-iRFP- and oTVB-L-tagBFP-expressing HEK293t cells individually and then applied a mixture of oEnvA-RVΔG-GFP and oEnvB-RVΔG-DsRed into each culture (**Figure 5A**). Three days after the viral infection, 100% of the GFP$^+$ cells expressed iRFP670 and 0% of the GFP$^+$ cells expressed BFP (**Figure 5B**). Similarly, 100% of the DsRed$^+$ cells expressed BFP and 0% of the DsRed$^+$ cells expressed iRFP670. These results indicate that

oTVA-L and oTVB-L, respectively recognized oEnvA-RVΔG and oEnvB-RVΔG without cross-reaction.

Next, we investigated the *in vivo* specificity and cross-infectivity of oEnvA-RVΔG and oEnvB-RVΔG. We introduced oTVA-L and oTVB-L separately in two different locations of the mouse brain (**Figure 5C**). We injected oTVA-L-iRFP-expressing AAV and oTVB-L-tagBFP-expressing AAV at the medial and lateral V1 (0.8 mm apart) of Tlx3-Cre mice, respectively, and subsequently injected a mixture of oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP between the AAV-injection sites. Seven days after the RV injection, we sacrificed the mice and observed the injection sites of the sectioned brain. Indeed, iRFP$^+$ and tagBFP$^+$ cells were exclusively distributed in the V1 (from three mice). No co-infection was observed between the oTVA-L-iRFP- and oTVB-L-tagBFP-expressing AAV (**Figure 5D**). All oEnvA-RVΔG-derived DsRed$^+$ neurons also expressed iRFP670. Moreover, most oEnvB-RVΔG-derived GFP$^+$ neurons also expressed BFP; the rest of the GFP$^+$ neurons there was no BFP signal observed, but all GFP$^+$ neurons were observed in the medial V1 injection site of the oTVB-L-tagBFP-expressing virus (**Supplementary Table S1**). Thus, the receptors did not recognize the non-corresponding envelopes.

To further demonstrate the specificity and independence of the oEnvA/oTVA and oEnvB/oTVB systems, we constructed two helper viruses: the first one carried an inhibitory-neuron-specific mDlx promotor (Dimidschstein et al., 2016) that allowed the inhibitory neurons to express oTVA-L-iRFP, and the other expressed oTVB-L-tagBFP in Tlx3-Cre mice, a layer 5 excitatory neuron-specific line, in a Cre-dependent manner (**Figure 5E**). We injected the mixture of these two AAVs to the V1 of Tlx3-Cre mice and then the mixture of oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP to the location of the AAV injection. Most DsRed-expressing neurons were iRFP-positive while majority of GFP-expressing neurons were tagBFP-positive (**Figure 5F**, **Supplementary Table S1**). No GFP/DsRed-double-positive cells were observed in the injected mice. These results indicate that the oEnvA/oTVA-L and oEnvB/oTVB-L systems targeted inhibitory and layer 5 excitatory neurons in the mouse cortex, respectively, in a mutually exclusive manner.

In conclusion, we demonstrated that the oEnvA/oTVA-L and oEnvB/oTVB-L systems can be used in simultaneous *in vivo* targeting of RVΔG in single animals. Using the oEnvA/oTVA-L and oEnvB/oTVB-L systems for RVΔG enables differentially dissecting and visualizing two independent cell populations with fluorescent proteins.

Simultaneous Trans-synaptic Tracing Using the oEnvA/oTVA and oEnvB/oTVB Systems

To perform a simple proof-of-concept experiment for simultaneous multiplex circuit tracing, we aimed to dissect multiple neural circuits using the oEnvA/oTVA and oEnvB/oTVB systems that we developed for the purposes of the present study. V1 neurons are heterogeneous; excitatory neurons even on the same layer have different connection patterns, and inhibitory neurons have different biochemical markers,

morphological features, electrophysiological properties, and connection patterns. Layer 5 neurons in the V1 comprise at least two different cell types depending on their projection targets (Brown and Hestrin, 2009; Lur et al., 2016). The first one projects to the cortical areas, such as the higher visual areas, and the other to subcortical areas, such as the superior colliculus (SC; Brown and Hestrin, 2009; Lur et al., 2016). These two types of layer 5 neurons that project to different target regions are locally intermingled.

To distinguish these two different layer-5 cell types and their corresponding presynaptic networks, we introduced the oEnvA/oTVA and oEnvB/oTVB systems to each population (**Figure 6A**). The AAV2retro capsid allows AAV to retrogradely infect projection neurons through axon terminals (Tervo et al., 2016). We first planned to develop an AAV2retro-CAG-oTVA-L-iRFP-P2A-oG (ITR-ITR: >4.9 kbps) that would simultaneously express both oTVA-L-iRFP and the optimized rabies glycoprotein oG. However, the AAV genome is limited (<4.7 kbps; Dong et al., 1996; Allocca et al., 2008), so we could not obtain sufficient AAV to be usable *in vivo*. Then, to maximize space for transgenes within the AAV packaging capacity without compromising transgene expression, we used a shorter universal promotor and a shorter polyA sequence to accommodate TVX, oG, and a fluorescent reporter such as, pAAV-CBh-oTVA-L-iRFP-P2A-oG-WPRE3-SV40 late polyA (ITR-ITR: approximately 4.3 kbps; Choi et al., 2014).

We administered two injections of retrograde AAVs to Tlx3-Cre mice. First, Cre- and projection-dependent, retrograde AAV expressing oTVA-L-iRFP and oG (AAV2retro-CBh-DIO-oTVA-L-iRFP-P2A-oG-WPRE3-SV40 late polyA) were injected into the lateral higher visual area (V2L: the putative area LM) to introduce both TVA and oG to V2L-projecting layer 5 neurons (Kim et al., 2016; **Figures 6A,B**). Second, projection-dependent, retrograde AAV expressing oTVB-L-tagBFP and oG (AAV2retro-CBh-oTVB-L-BFP-P2A-oG-WPRE3-SV40 late polyA) was injected in the SC to introduce both oTVB and oG to SC-projecting neurons (Lur et al., 2016; **Figures 6A,B**). iRFP+ cells were observed in layer 5 of the mouse V1, suggesting that V2L-projecting layer 5 neurons of V1 were labeled in a Cre-dependent and retrograde manner (**Figure 6C**). BFP+ neurons were also observed in layer 5, suggesting that the SC-projecting V1 neurons were retrogradely labeled. Using the conditional AAVs in Tlx3-Cre mice, we succeeded in specific labeling of layer 5 neurons projecting to either the V2L or SC area with oTVA-L or oTVB-L, respectively.

To identify their presynaptic inputs, we next injected oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP into the V1 of the AAV-injected Tlx3-Cre mice 2–3 weeks after the AAV injections. Ten days after the rabies viral injection, we sacrificed the animals for histological analysis. Most DsRed+ cells in upper layer 5 were positive for iRFP670, indicating that oEnvA-RVΔG-DsRed infected oTVA-L-iRFP+ cells in upper layer 5 of the V1 and that the DsRed/iRFP-double-positive cells were starter cells for trans-synaptic tracing of RVΔG-DsRed. RVΔG-DsRed spread to the presynaptic cells following trans-complementation with oG (**Figures 6B–I**). DsRed-positive and

iRFP670-negative cells were mainly observed in layer 2/3 and 4 of the V1 (**Figure 6C**), V2L (**Figures 6D,E**), and dLGN (**Figures 6F,H**). Likewise, most GFP+ cells in lower layer 5 were also tagBFP-positive, indicating that oEnvB-RVΔG-GFP infected the oTVB-L-tagBFP+ cells in lower layer 5 of the V1, while the GFP/BFP-double-positive cells were the starter cells for trans-synaptic tracing of RVΔG-GFP. The GFP+ cells were more widely spread in V1, the higher visual areas V2ML and V2MM (**Figure 6E**), association cortical areas (medial parietal association; **Figure 6G**), and various thalamic areas including the dLGN, LP (**Figures 6F,H**), and anterior thalamic nuclei (**Figure 6I**). Additionally, GFP+ and DsRed+ neurons were not observed in any of the brain regions. These results suggest that V2L-projecting V1 neurons on layer 5 received inputs from V1 local circuits, feedback from higher visual areas, and visual-related thalamic areas, whereas SC-projecting V1 neurons received inputs from V1 local circuits, feedback from higher visual and association cortical areas, and visual and non-visual thalamic nuclei.

Therefore, we conclude that the multiplexed neural circuit tracing with RVΔG enabled the simultaneous dissection of multiple neural circuits in the brain.

Labeling of Common Input to Different Cell Populations With Multiplex Circuit Tracing

In the next set of experiments, we examined whether common input to two distinct cell populations can be detected by multiplex circuit tracing *in vivo*, which can be detected as co-infected cells with two viral vectors in multiplex circuit tracing. Many lines of evidence have shown that dLGN neurons send projections to neurons in layers 4, 5, and 6 of the V1 (Blasdel and Lund, 1983; Antonini et al., 1999; Constantinople and Bruno, 2013; Kim et al., 2015). A recent study revealed that parvalbumin (PV)-expressing inhibitory neurons in the V1 receives input from dLGN neurons (Kim et al., 2016).

Based on these reports, we performed multiplex RV tracing from the inhibitory neurons and layer 5 neurons of the V1 in single animals to determine whether inhibitory neurons and layer 5 excitatory neurons of the V1 share common input (**Figure 7A**). Cortico-coritcal layer 5 neurons of the V1 were labeled with Cre-dependent AAV expressing oTVB under the excitatory-neuron-specific CaMKIIa promoter in layer 5-specific Tlx3-Cre mice. Inhibitory neurons of the V1 were labeled with AAV expressing oTVA under the inhibitory-neuron-specific mDlx promoter using the flox cassette that is excised by recombination in the Cre-expressing cells to eliminate oTVA expression in layer 5 excitatory neurons of Tlx3-Cre mice. Using this viral system, oTVA and oTVB can be exclusively introduced to layer 5 excitatory neurons and inhibitory neurons, respectively. Additionally, to complement RVΔG *in trans*, we co-injected AAV expressing the rabies glycoprotein oG under the strong ubiquitous CAG promotor, as the level of gene expressions driven by the CaMKIIa and mDlx promotors differs. Three weeks after the AAV injection, oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP were co-injected in the same location of the V1 injection of AAVs. We observed oTVA-L-iRFP-positive cells in inhibitory
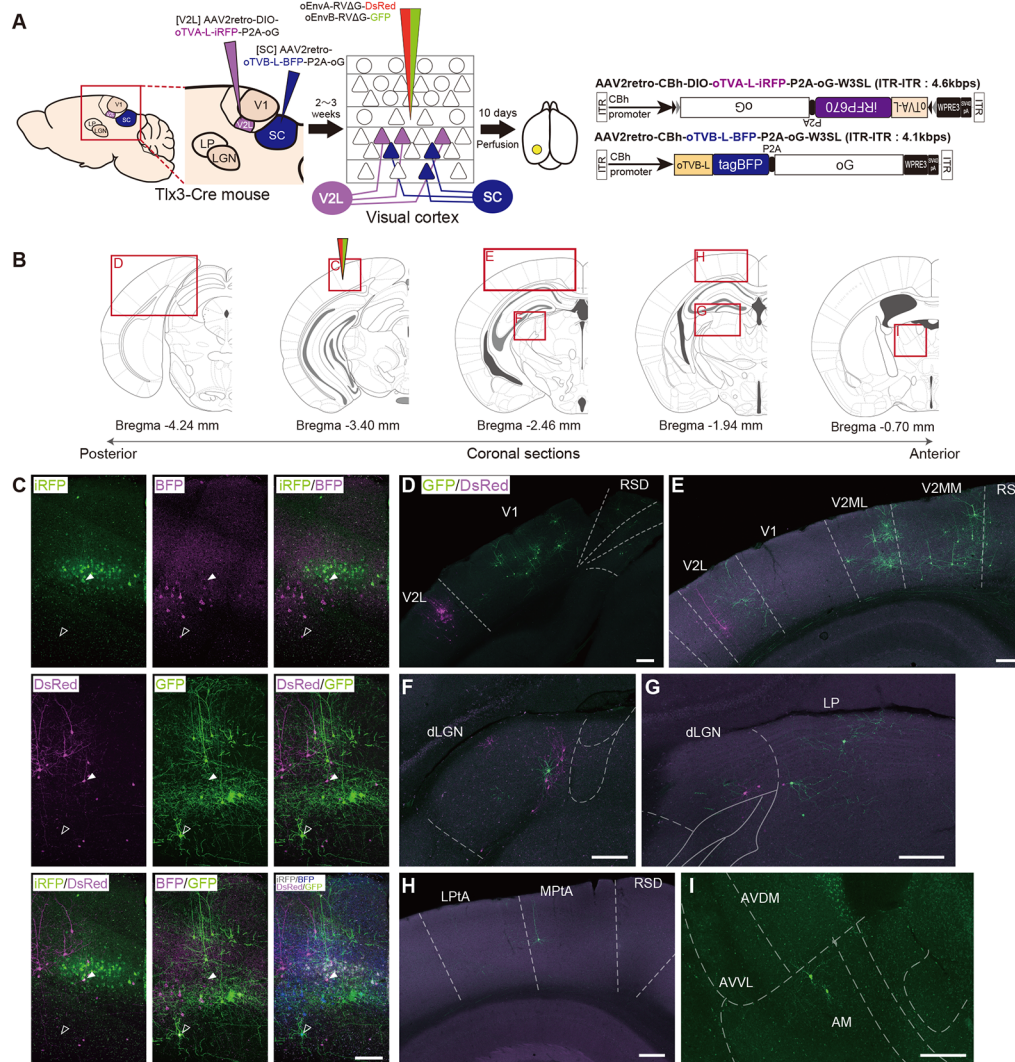
FIGURE 6 | Multiplex circuit tracing with the oEnvA/oTVA and oEnvB/oTVB systems. **(A)** Multiplex monosynaptic tracing of two distinct classes of layer 5 neurons targeting either other cortical or subcortical areas. Retrograde AAVs were injected into two different locations of layer 5-specifc Tlx3-Cre mice: AAV2retro-CBh-DIO-oTVA-L-iRFP-P2A-oG and AAV2retro-CBh-L-oTVB-L-BFP-P2A-oG in the lateral area of the higher visual cortex (V2L) and superior colliculus (SC), respectively. V2L-projecting V1 neurons in layer 5 expressed both oTVA-L-iRFP and oG while SC-projecting V1 neurons expressed both oTVB-L-BFP and oG. After the AAV injections, oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP were co-injected into the V1. **(B)** Coronal brain sections illustrating anatomical regions shown in (**C–I**; adapted from The Mouse Brain in Stereotaxic Coordinates, 3rd edition, Paxinos and Franklin, 2008; **G**). **(C)** Viral targeting to two distinct classes of layer 5 neurons depending on projection targets. oTVA-L-iRFP-expressing starter neurons were specifically infected with oEnvA-RVΔG-DsRed, whereas oTVB-L-BFP-expressing starter neurons were infected with oEnvB-RVΔG-GFP. DsRed and GFP signals were enhanced by immunostaining. Closed arrowheads indicate oTVA-L-iRFP+/oEnvA- RVΔG-DsRed+ starter neurons. Opened arrowheads indicate oTVB-L-BFP+/oEnvB-RVΔG-GFP+ starter neurons. Scale bar: 100 μm. **(D–I)** Distributions of presynaptic neurons connected to two distinct classes of layer 5 neurons. Neurons trans-synaptically infected with RVΔG-DsRed and RVΔG-GFP were distributed in the V1, V2L, and RSD **(D)**, V1, V2L, V2ML, V2MM, and RSD **(E)**, dLGN **(F)**, dLGN and LP **(G)**, LPtA, MPtA, and RSD **(H)**, AM, AVDM, and AVVL **(I)**. DsRed and GFP signals were enhanced by immunostaining. Scale bar: 200 μm. Abbreviations: AM, anteromedial thalamic nucleus; AVDM, anterovent thalamic nucleus, dorsomedial part; AVVL, anteroventral thalamic nucleus, ventrolateral part; RSD, retrosplenial dysgranular cortex; dLGN, dorsal lateral geniculate nucleus; LP, lateral parietal association cortex; LPtA, medial parietal association cortex; MPtA, lateral posterior thalamic nucleus; V2L, secondary visual cortex, lateral area; V2ML, secondary visual cortex, mediolateral area; V2MM, secondary visual cortex, mediomedial area.

neurons across layers and oTVB-L-BFP-positive cells (cell membrane-localized BFP) in layer 5 of the V1. We also found GFP+ and DsRed+ cells, indicating that common input was detected as co-infection with both oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP. Interestingly, GFP+/DsRed+ neurons were distributed in the V1 (**Figure 7B**) and dLGN

(**Figures 7C–E**). These results indicate that inhibitory neurons and layer 5 excitatory neurons of the V1 share common input from V1 local circuits and from dLGN neurons.

We found that dLGN neurons provide divergent input to both layer 5 excitatory and inhibitory neurons of the V1 in the mouse visual system. The common input to both layer
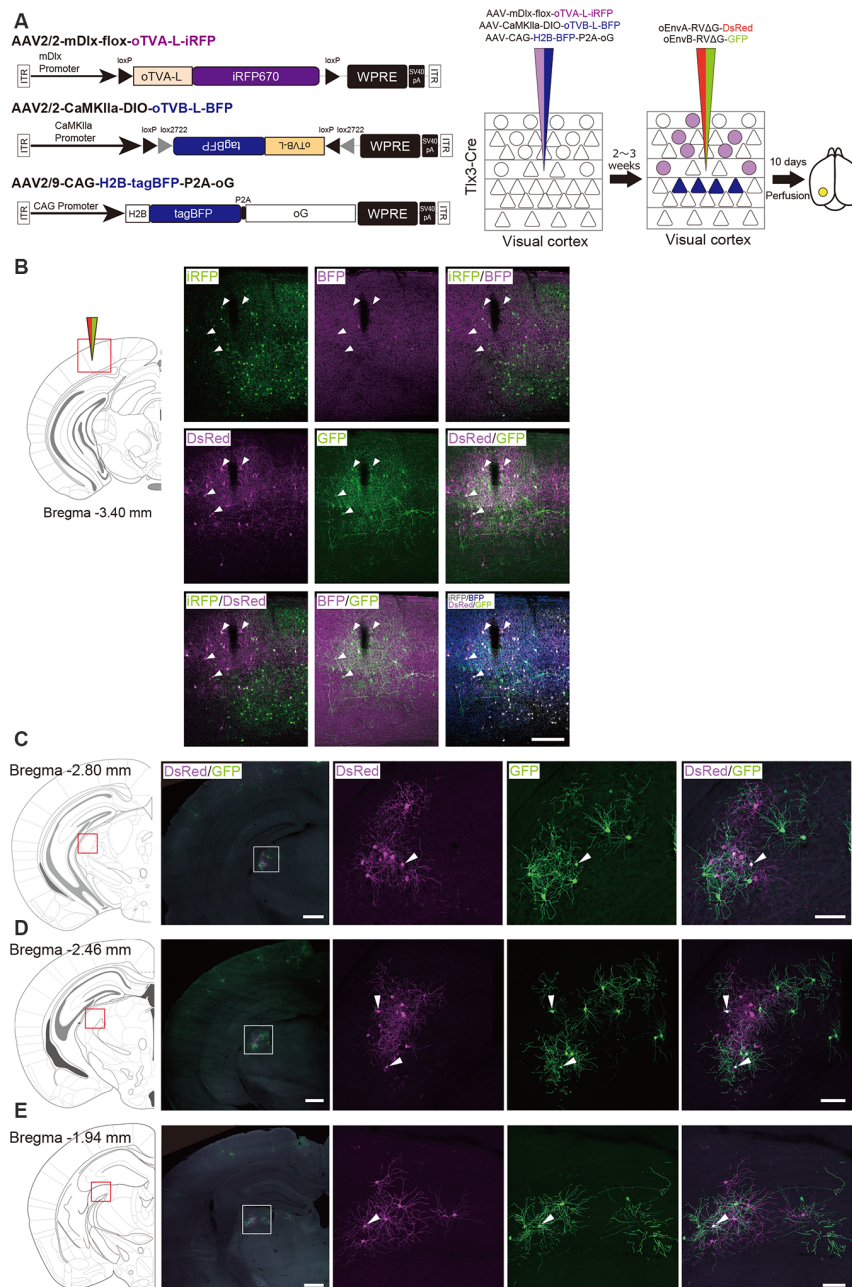
FIGURE 7 | Labeling of common input with multiplex circuit tracing. **(A)** Multiplex monosynaptic tracing of two distinct classes of layer 5 excitatory or inhibitory neurons. Mixed AAVs were injected into the V1 of layer 5-specifc Tlx3-Cre mice: AAV2/2-mDlx-flox-oTVA-L-iRFP, AAV2/2-CaMKIIa-DIO-oTVB-L-BFP, and AAV2/9-CAG-H2B-tagBFP-P2A-oG. After the AAV injection, oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP were co-injected into the same location of the V1. **(B)** Viral targeting to two distinct classes of V1 neurons depending on cell types. oTVA-L-iRFP-expressing inhibitory neurons were specifically infected with oEnvA-RVΔG-DsRed, whereas oTVB-L-BFP-expressing excitatory neurons in layer 5 were infected with oEnvB-RVΔG-GFP. The rabies glycoprotein oG was expressed in cells positive for nuclear tagBFP. Closed arrowheads indicate presynaptic neurons co-infected with both oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP. DsRed and GFP signals were enhanced by immunostaining. Scale bar: 200 μm. **(C–E)** Low and high magnification images of dLGN neurons monosynaptically connected to two distinct classes of V1 neurons. DsRed and GFP signals were enhanced by immunostaining. Closed arrowheads indicate presynaptic neurons co-infected with both oEnvA-RVΔG-DsRed and oEnvB-RVΔG-GFP. Scale bar of overview: 500 μm. Scale bar of dLGN view: 100 μm. Coronal brain sections illustrating anatomical regions shown in (**B–E**; adapted from The Mouse Brain in Stereotaxic Coordinates, 3rd edition, Paxinos and Franklin, 2008).

5 excitatory and inhibitory neurons could provide a sub-network structure crucial for shaping the receptive field properties of V1 neurons. Taking together, we conclude that multiplex neural

circuit tracing with RVΔG allows detecting common input to different cell populations and analyzing complex computations between circuits.

DISCUSSION

Multiplex Neural Circuit Tracing With G-Deleted Rabies Viral Vectors

The brain is a complex and dynamic structure composed of heterogeneous neuronal populations that are organized in distinct neural circuits to generate perception and behavior (Harris and Mrsic-Flogel, 2013; Tasic et al., 2016, 2018). Information is processed not only through paralleled, independent circuits but also by integration between circuits in the nervous system including the retina and brain (Nassi and Callaway, 2009). Much progress has been made toward elucidating cell-type-specific connectivity at the whole-brain level. However, information integration in the brain remains elusive. Thus, dissection of the interaction between intermixing circuits will provide better opportunities to reveal the neural circuits and computations underlying integration of information processing. Here, we multiplexed the infection systems of RVΔG and demonstrated their implementation in the mouse brain. We introduced oEnvA/oTVA, oEnvB/oTVB, and oEnvE/oTVE systems to simultaneously target distinct cell populations with RVΔG. Our work provided proof-of-concept for multiplex circuit tracing with RVΔG in a single animal.

Our *in vivo* proof-of-concept experiment showed the simultaneous labeling of the two distinct classes of layer 5 neurons targeting either other cortical or subcortical areas. Using both the oEnvA/oTVA and oEnvB/oTVB systems, we differentially labeled two distinct populations with different colors in single animals. Their presynaptic inputs were also labeled with trans-synaptic spread of different-colored RVΔG. We found that SC-projecting layer 5 neurons of the V1 received input from local circuits and motor-related areas, while LM-projecting layer 5 neurons of the V1 received input from local circuits and visual-related areas including higher visual areas. We did not find any connections between SC-projecting layer 5 neurons and LM-projecting layer 5 neurons in V1 as co-infection. This result is consistent with the recent study by Maruoka et al. (2017). Maruoka et al. (2017) revealed lattice organization composed of cell type-specific microcolumns in layer 5; subcortical projection neurons formed radial clusters called microcolumns, while cortical projection neurons were also organized into microcolumns that were aligned radially in an orientation parallel to the microcolumns composed of subcortical projection neurons. Maruoka et al. (2017) also demonstrated that subcortical projection neurons were rarely connected with cortical projection neurons whereas subcortical projection neurons had reciprocal connections with each other and cortical projection neurons were also interconnected. In addition, we did not observe any co-infected cells with two rabies viral vectors outside V1, suggesting that the presynaptic network organization of layer 5 neurons differs depending on the output target; layer 5 neurons in V1 pool distinct information depending on their projection target. However, it is difficult to conclude that there is no common input between SC-projecting and LM-projecting layer 5 neurons in the V1 in this set of experiments. Although the SC-projecting layer 5 starter neurons

were distributed in the vicinity of the LM-projecting starter neurons in the V1, the retinotopic location of SC-projecting layer 5 starter cells can be mismatched with that of LM-projecting layer 5 starter cells. It is possible that the cells responsible for the same location of the visual space can interact with each other and their presynaptic networks may overlap, whereas the cells responsible for different locations of the visual space may not share common input.

In another set of experiments, the multiplex circuit tracing labeled the common input to different cell populations; we found that dLGN neurons were directly connected to both cortico-cortical layer 5 neurons and inhibitory neurons of the mouse V1. It is known that the most canonical thalamocortical connection is between dLGN neurons and layer 4 neurons of the V1. However, Kim et al. (2016) demonstrated using rabies tracing that PV-expressing inhibitory neurons in the V1 receive input from dLGN neurons. Additionally, they revealed that layer 5 excitatory neurons in the V1 also receive input from dLGN neurons (Kim et al., 2015). Several lines of evidence have indicated that dLGN neurons provide collateral connections to layer 5 and 6 neurons of the V1, in addition to layer 4 neurons (Blasdel and Lund, 1983; Antonini et al., 1999). To our knowledge, no study has revealed that layer 5 and inhibitory neurons of the V1 share common excitatory input from dLGN neurons. This divergent thalamocortical input could play unique roles in shaping the receptive field properties of V1 neurons. Further physiological studies are warranted to determine how the common input participates in circuit computation and visual information processing in the V1.

Viral Receptors With Higher or Lower Affinity

TVA fused to a fluorescent protein has been used to visualize TVA-expressing starter cells: TVA^{950}-EYFP (Faget et al., 2016), TVA^{950}-mCherry (Watabe-Uchida et al., 2012), and the low-affinity mutant TVA^{950E66T}-mCherry (Miyamichi et al., 2013). TVA^{950}-EYFP and TVA^{950}-mCherry are highly sensitive to viral infection despite low expression of TVA^{950} caused by leak expression even though their fluorescent reporters (EYFP and mCherry) fused to TVA^{950} were invisible. For example, in transgenic mice expressing Cre in a specific cell population, Cre-negative cells can express a small amount of TVA^{950} due to leak expression, which leads to non-specific infection with EnvA-RVΔG. However, when using TVA^{950E66T}-mCherry for rabies viral targeting in Cre-expressing transgenic mice, oEnvA-RVΔG cannot infect the cells expressing a small amount of TVA^{950E66T}-mCherry, which are invisible under a fluorescent microscope because of the low affinity of TVA^{950E66T} to oEnvA (Rong et al., 1998; Miyamichi et al., 2013). The strength of this TVA^{950E66T}-mCherry constitutes clear and reliable visualization of starter neurons in rabies tracing experiments because high expression of TVA^{950E66T}-mCherry is required for oEnvA-RVΔG infection. Thus, a lower-affinity version of TVA (oTVA-L) is ideal for rabies viral tracing.

oEnvA-RVΔG, oEnvB-RVΔG, and oEnvE-RVΔG showed specific infectivity to cells expressing their corresponding receptors, TVA^{800}, TVB^{S3}, and DR46-TVB (**Figure 1C** and
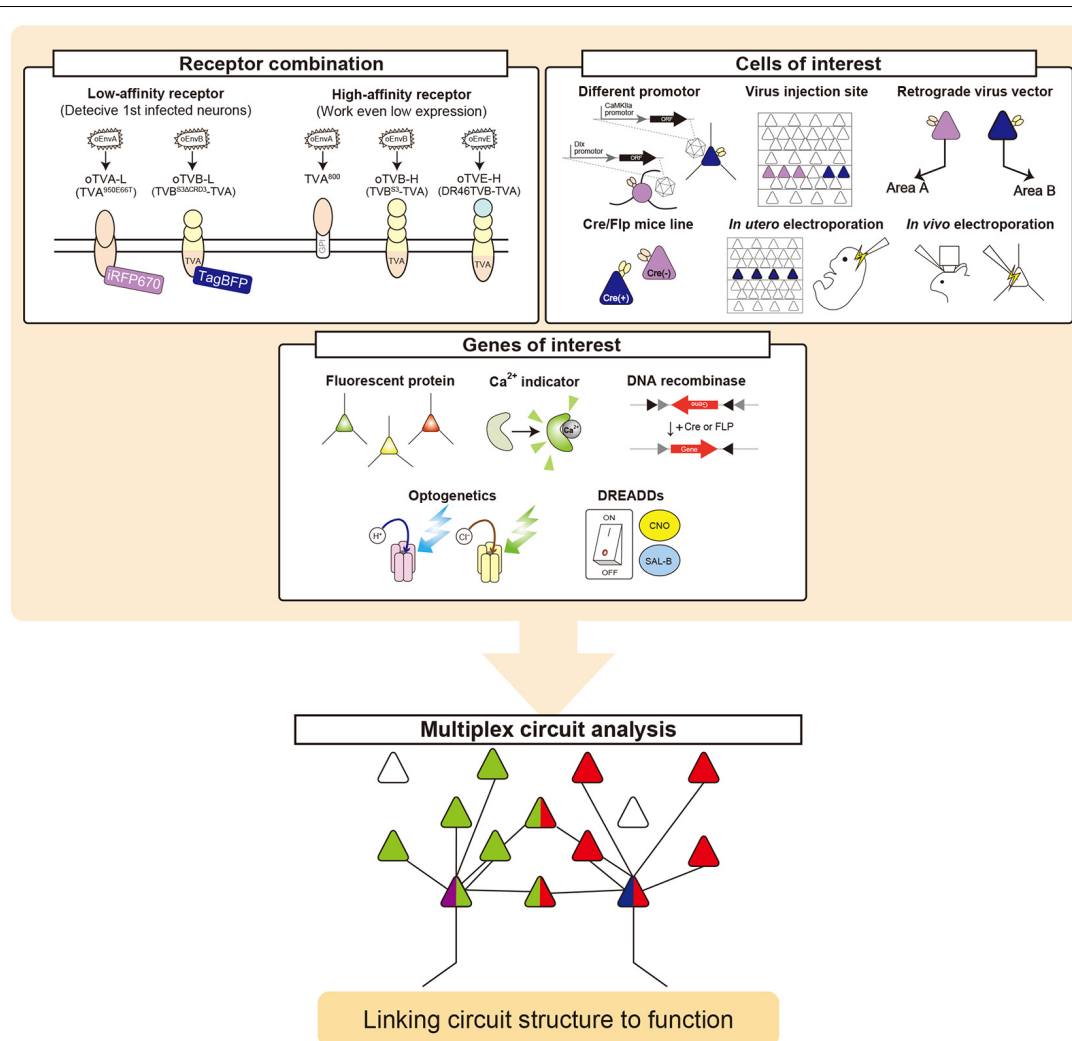
FIGURE 8 | Multiplex circuit analysis with rabies viral vectors. Simultaneous RV trans-synaptic tracing can be achieved by designing three steps. (1) Select viral envelopes and their receptors. oEnvX/oTVX systems developed in the present study can be used for rabies viral targeting. (2) Select cells of interest. Cell-type-specific promoters, connection-mediated gene transfer, electroporation, and transgenic animals are available to target particular populations. (3) Select genes of interest. Fluorescent proteins, imaging probes, optogenetic/chemogenetic tools, and recombinases can be expressed from the rabies viral vectors.

Table 1). Based on this specificity, we developed both higher-affinity and lower-affinity receptors, termed oTVB-H, oTVB-L, and oTVE-H. oTVB-H and oTVB-L are the respective higher and lower-affinity versions of TVB. oTVE-H is a receptor with higher affinity to oEnvE-RVΔG. Here, we propose to use optimal viral receptors depending on the purpose and design of experiments.
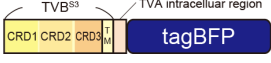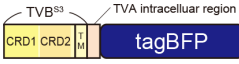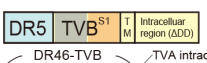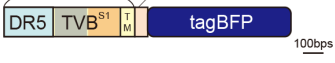
For cell-type-specific circuit tracing, using transgenic mouse lines expressing Cre, Flp, or tTA in a specific population is the most reliable and straightforward approach to define starter cells in mice. For that purpose, viral receptors with lower affinity, such as oTVA-L and oTVB-L, are recommended because leak expressions of viral receptors can be ignored. Typically, we used AAV vectors for introducing oTVA-L and oTVB-L and fluorescent reporters. In this study, we also developed high-affinity receptors, oTVB-H and oTVE-H. Higher-affinity versions are also applicable for

AAV-mediated circuit tracing along with the IRES system for introducing both receptors and fluorescent reporters as shown in **Figure 4E**. Notably, as TVA[800], high-affinity type TVA (Marshel et al., 2010; Gray et al., 2011; Rancz et al., 2011; Wertz et al., 2015), was used for labeling the single cell network *in vivo*; both oTVB-H and oTVE-H are suitable for single-cell electroporation followed by single-cell-initiated RVΔG tracing.

Application of Multiplex Circuit Tracing

Using the multiplex RVΔG tracing system that we developed in this study, various questions can be addressed regarding the circuit-level mechanisms of information processing. To utilize multiplex circuit tracing, we need to consider two parameters (**Figure 8**): (1) how to introduce the viral receptors (**Table 3**); and (2) which genes to express. (1) Using

TABLE 3 | oEnvX-RV∆G receptors which are available.

Chimeric Envelope	Receptor Name	Fused Reporter	Structure	Detectivity of Starter Neurons	Sensibility	References
oEnvA	TVA800	—		—	+++	Marshel et al. (2010)
	TVA950	mCherry EYFP		△	++	Watabe-Uchida et al. (2012) and Faget et al. (2016)
	TVA950E66T (oTVA-L)	mCherry EYFP		◎	+	Miyamichi et al. (2013) This study
oEnvB	TVB^S3	—		—	+	Brojatsch et al. (1996)
	TVB^S3-TVA (oTVB-H)	tagBFP		△	+++	This study
	TVB^S3ΔCRD3-TVA (oTVB-L)	tagBFP		○	++	This study
oEnvE	DR46-TVB	—		—	++	Klucking and Young (2004)
	DR46-TVB-TVA (oTVE-H)	mCherry tagBFP		△ (with IRES : ◎)	+++	This study

In the detectivity of starter neurons, each symbol indicates the following: ◎: the fluorescence reporter detects starter neurons almost perfectly at >90%. ○: the fluorescence reporter detects starter neurons at >80% and produces some pseudo-negative cells. △: the fluorescence reporter cannot easily detect starter neurons and produces many pseudo-negative cells.

transgenic mouse lines marking specific cell populations is the most straightforward and easiest method to introduce both viral receptors and RV-G. Tissue-specific promoters (e.g., human synapsin promoter; pan-neurons), brain-region-specific promoters (e.g., Ple67/FEV promoter; Raphe nuclei neurons, Ple155/PCP2 promoter; cerebellum Purkinje cells; de Leeuw et al., 2016), cell-type-specific promoters (e.g., CaMKlla promoter; excitatory neurons, Dlx promoter; inhibitory neurons; Yaguchi et al., 2013; Dimidschstein et al., 2016), or activity-dependent promoters (e.g., E-SARE promoter; Kawashima et al., 2009, 2013) are also powerful when used within viral vectors. In conjunction with the transgenic Cre/Flp mouse lines or Cre/Flp-expressing viral vectors, Cre/Flp-dependent viral vectors carry the Flex or DIO/DO cassette that restricts expression by recombination (Gradinaru et al., 2010; Saunders et al., 2012; Fenno et al., 2014). tTA/rtTA-expressing transgenic mouse lines can be interfaced with AAV or HIV vectors carrying the tetO promoter. When retrograde infective vectors (e.g., AAV2retro, RV-G-pseudotyped lentivirus, Canine adenovirus type 2) are used, local area neurons can be separated by their projection area. An intersectional strategy using a combination of recombinases and activators can increase the specificity of target cells. (2) RV∆G can be used for both anatomical and physiological studies (Osakada et al., 2011; Wertz et al., 2015; Tian et al., 2016). RV∆G can accommodate a larger size of transgenes in their viral genome than can AAVs. Further, multiple genes can be expressed without using IRES or 2A elements (Osakada et al., 2011; Osakada and Callaway,

2013). Genetically-encoded sensors for calcium, voltage, and neurotransmitters are powerful to characterize connectionally-defined circuits (Chen et al., 2013; Marvin et al., 2013, 2019; Dana et al., 2016; Jing et al., 2018; Patriarchi et al., 2018; Sun et al., 2018; Abdelfattah et al., 2019; Inoue et al., 2019). Chemogenetic manipulation using rabies-mediated DREADD expressions (e.g., hm3dq, hm4di, PSAM, and KORD) can relate neuronal connectivity and activity to behavior (Armbruster et al., 2007; Alexander et al., 2009; Magnus et al., 2011; Vardy et al., 2015). Combining optogenetic tools (e.g., Channelrhodpsin2, ChRmine), the manipulation of connected populations will be feasible with implantation of optic fibers in the brain (Osakada et al., 2011; Tian et al., 2016) and even with two-photon excitation with single-cell resolution (Mardinly et al., 2018; Carrillo-Reid et al., 2019; Marshel et al., 2019).

Limitations and Future Challenges

Optical techniques have become indispensable for neuroscientific research in recent decades (Yang and Yuste, 2017). Genetically-encoded indicators of calcium, voltage, neurotransmitters, and metabolism as well as optogenetic and chemogenetic actuators allow multi-modal interrogation of neural circuit function (Luo et al., 2018). These multi-colored, genetically-encoded biosensors and actuators will fit our multiplex rabies viral tracing. However, the number of colors we can use is limited due to the limitation of genetically-encoded fluorescent proteins. Accordingly, the number of circuits we can simultaneously analyze is also currently limited.

To overcome these limitations, chemical tags could be a viable alternative to fluorescent proteins. Genetically-encoded chemical tags, such as Halo-tag, SNAP-tag, CLIP-tag, and TMP-tag, were labeled with synthetic fluorophores (Gautier et al., 2008; Los et al., 2008; Chen et al., 2012; Kohl et al., 2014). Interfacing chemical tagging with rabies tracing is also powerful for multiplexed circuit analyses because more choices of fluorescence spectrum and photochemical properties are available with chemical tags than with fluorescent proteins. Synthetic fluorophores cover the spectrum from the UV to near-IR range, expanding the possible spectrum range and the repertoire of fluorescence labels. Thus, chemical tagging will enable advances in multiplexed capacity and the applications of neural circuit tracing.

Rabies viral vectors are powerful tools for labeling synaptically-connected neurons throughout the brain. Despite the strength in anatomical studies, application of RVΔG tracing in physiological and behavioral studies remains limited because of its toxicity (Osakada et al., 2011; Wertz et al., 2015; Tian et al., 2016), although the toxicity of RVΔG viral vectors is much lower than that of other replication-competent viruses such as HSV. The viral toxicity derives from the trans-synaptic spread feature of replication-competent rabies vectors. It is notable that viral toxicity and trans-synaptic spread are in tradeoff. Several approaches have been developed to reduce the toxicity of RVΔG vectors. Large protein (L) deleted rabies viral vectors expressing Cre recombinase minimized the toxicity in transgenic mice expressing a reporter in a Cre-dependent manner (Chatterjee et al., 2018). This is because L-deleted RVΔG vectors are deficient in the replication of the RV genome. However, L-deleted RVΔG vectors do not replicate in infected neurons, thereby failing in viral spread to their presynaptic neurons due to lack of replication ability. Although trans-complementation of L and G allows replication of L-deleted RVΔG vectors and viral spread from starter cells, L-deleted RVΔG vectors will kill the L-expressing starter cells. Self-inactivating RVΔG also controlled its viral replication using PEST and TEV signals to extend the survival periods of infected neurons (Ciabatti et al., 2017). However, numerous researchers failed to reproduce the original report by Ciabatti et al. (2017), probably because rabies viruses obtain new mutations in the viral genome (Matsuyama et al., 2019). Further improvements of rabies viral vectors that have lower toxicity but maintain trans-synaptic spread ability are required for neural circuit research.

Revealing parallel processing through independent circuits and integrated processing through interaction between circuits is pivotal in understanding how the brain functions. The multiplex RV tracing that we developed in the present study will facilitate the in-depth understanding of the principles of neural circuits and computations in the brain.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee in Nagoya University.

AUTHOR CONTRIBUTIONS

TS performed experiments. TS, NM, and FO analyzed the data. TS, NM, AA, and FO wrote the manuscript. FO designed the study and supervised this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncir.2019.00077/full#supplementary-material.

FIGURE S1 | Control injection of oEnvX-RVΔG in the absence of oTVX. No cells were infected with oEnvA-RVΔG-DsRed **(A)**, oEnvB-RVΔG-DsRed **(B)**, or oEnvE-RVΔG-GFP **(C)** without injection of AAV expressing corresponding receptor oTVX. These results indicate no sign of contamination by unpseudotyped RVΔG in oEnvA-RVΔG-DsRed **(A)**, oEnvB-RVΔG-DsRed **(B)**, and oEnvE-RVΔG-GFP **(C)**. Arrowheads indicate viral injection sites. Scale bar: 100 μm.

FIGURE S2 | Protein sequence alignment for TVB[S3], oTVB-H, and oTVB-L. Colored regions indicate functional domains: CRD1, CRD2, CRD3, and a transmembrane region. Gray boxes indicate absent or different amino acid.

FIGURE S3 | Protein sequence alignment for DR46-TVB and oTVE-H. Colored regions indicate the functional domains: death receptor 5, CRD2, CRD3, and a transmembrane region. Gray boxes indicate different or absent amino acid. Red characters indicate an essential amino acid for EnvE recognition.

FIGURE S4 | Trans-synaptic tracing using the oEnvE/oTVE system. **(A)** Monosynaptic tracing of layer 5 excitatory neurons in V1. Mixed AAVs were injected into the V1 of layer 5-specifc Tlx3-Cre mice: AAV2/9-CAG-DIO-H2B-mRuby3-IRES-oTVE-H and AAV2/9-CAG-DIO-oG. After the AAV

injection, oEnvE-RVΔG-GFP were co-injected into the same location of the V1. **(B–C)** Typical images of the monosynaptically restricted tracing to the layer 5 neurons of the mouse V1. H2B-mRuby3-expressing neurons (nuclear-localized mRuby3) of the mouse V1 were specifically infected with oEnvE-RVΔG-GFP. GFP signals were enhanced by immunostaining. **(B)** Starter neurons and their presynaptic neurons in the V1. Closed arrowheads indicate

H2B-mRuby3$^+$/oEnvE-RVΔG-GFP$^+$ starter neurons. Scale bar: 100 μm. **(C)** Presynaptic neurons in the dLGN. Scale bar: 50 μm.

TABLE S1 | Percentages of co-infected cells. Percentage of oEnvX-RVΔG-infected cells in oTVX-expressing cells in **Figures 5C–F** were shown. Data were obtained from three mice.

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Structural Neural Connectivity Analysis in Zebrafish With Restricted Anterograde Transneuronal Viral Labeling and Quantitative Brain Mapping

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The unique combination of small size, translucency, and powerful genetic tools makes larval zebrafish a uniquely useful vertebrate system to investigate normal and pathological brain structure and function. While functional connectivity can now be assessed by optical imaging (via fluorescent calcium or voltage reporters) at the whole-brain scale, it remains challenging to systematically determine structural connections and identify connectivity changes during development or disease. To address this, we developed Tracer with Restricted Anterograde Spread (TRAS), a novel vesicular stomatitis virus (VSV)-based neural circuit labeling approach. TRAS makes use of replication-incompetent VSV (VSVΔG) and a helper virus (lentivirus) to enable anterograde transneuronal spread between efferent axons and their direct postsynaptic targets but restricts further spread to downstream areas. We integrated TRAS with the Z-Brain zebrafish 3D atlas for quantitative connectivity analysis and identified targets of the retinal and habenular efferent projections, in patterns consistent with previous reports. We compared retinofugal connectivity patterns between wild-type and *down syndrome cell adhesion molecule-like 1* (*dscaml1*) mutant zebrafish and revealed differences in topographical distribution. These results demonstrate the utility of TRAS for quantitative structural connectivity analysis that would be valuable for detecting novel efferent targets and mapping connectivity changes underlying neurological or behavioral deficits.

Keywords: transsynaptic, zebrafish, VSV, brain mapping, viral tracing

INTRODUCTION

The function of the brain is closely linked to its structure, i.e., how its billions of constituent cells are wired and connected by trillions of synapses. Understanding how these connections are formed and maintained is key to gaining mechanistic insight toward brain function and identifying the causes and treatments for neuropsychiatric disorders (Belmonte et al., 2004; Lynall et al., 2010; Fornito et al., 2015). Techniques for mapping the structure and function of the brain have progressed

rapidly in past decades, from anatomical structural analysis to functional computation, and from human participants to animal models (Kasthuri et al., 2015; Glasser et al., 2016). However, the large number of neurons and vast spatial scale of neuronal structures (from meters to nanometers) of the mammalian brain makes mapping neuronal networks at the cellular level and correlating them with development and disease a daunting task (Swanson and Lichtman, 2016).

Zebrafish (*Danio rerio*), a small tropical fish, has emerged as an accessible model for studying behavior, neuronal networks, and cellular connectivity (Orger and de Polavieja, 2017). Zebrafish has analogous neuroanatomy and neurochemistry to mammals and can perform complex sensory, motor, and cognitive functions during larval stages (5–10 days post fertilization, dpf). Importantly, at this stage, there are only roughly 100,000 neurons in the brain, 80% of which can be imaged and physiologically recorded in live, behaving animals (Ahrens et al., 2013; Chen et al., 2018; Abdelfattah et al., 2019). As a result, zebrafish whole-brain functional imaging studies have been able to generate cellular resolution neuronal activity maps under different behavioral contexts and linking activity maps to pathological states associated with autism spectrum disorder, schizophrenia, and epilepsy (Sakai et al., 2018; Thyme et al., 2019). The ability to fully interpret patterns of functional connectivity and determine causality for disorders, however, is limited by the lack of detailed structural information on neuronal wiring in zebrafish, which still lags behind other commonly used model organisms like mice, *Drosophila*, and *C. elegans*. Efforts are ongoing to map the full complement of neuronal connections (i.e., connectome) with electron microscopy in the larval and adult zebrafish, but the time and labor-intensive nature of synapse-level reconstruction has thus far restricted investigations to connections within smaller brain regions (Wanner et al., 2016; Hildebrand et al., 2017; Vishwanathan et al., 2017; Svara et al., 2018).

To address this, we sought to develop a virus and light imaging-based structural mapping technique that would allow for quantitative brain-wide mapping of neuronal connectivity in larval zebrafish. Previously, we found that recombinant vesicular stomatitis virus (VSV) can function as an anterograde transsynaptic tracer in a wide range of organisms (Mundell et al., 2015). VSV is a negative-strand RNA virus in the *Rhabdoviridae* family, which also includes the rabies virus (RABV). In contrast to RABV, which spreads retrogradely (from dendrite to presynaptic afferent axons), VSV spreads anterogradely (from efferent axons to their postsynaptic targets) when enveloped by its endogenous glycoprotein (VSV-G) (Beier et al., 2011b; Mundell et al., 2015). VSV injection into the retina of mice, chicken, and larval zebrafish led to highly efficient labeling of the optic nerve and targets of the visual pathway, including both direct retinorecipient connections and third-order neurons in areas further downstream.

These findings open the door for utilizing VSV for zebrafish structural circuit mapping. Substantial limitations, however, still remain. First, the spread of VSV is unrestricted, making it difficult to disambiguate direct (monosynaptic) and indirect (polysynaptic) connections. Second, replication-competent VSV and VSV-G expression are cytotoxic and lead to rapid

deterioration of health in larval zebrafish (Hoffmann et al., 2010; Mundell et al., 2015). Finally, there is no established method for quantifying and annotating viral labeling in zebrafish, which is necessary to correlate anatomical tracing with functional imaging.

In this study, we developed a novel approach utilizing replication-incompetent VSV (VSVΔG) to achieve restricted anterograde transneuronal spread in zebrafish. We also developed an imaging and processing pipeline to register 3D image stacks to the widely used and extensively annotated Z-Brain digital atlas (Randlett et al., 2015). This method, termed TRAS (Tracer with Restricted Anterograde Spread), allows for a quantitative description of efferent connectivity based on neurotransmitter types and specific locations. We applied TRAS to investigate the axon projection patterns of retinorecipient cells and identified potential connectivity changes in zebrafish carrying a mutation in *down syndrome cell adhesion molecule-like 1 (dscaml1)*, a causal gene for autism spectrum disorder and human cortical abnormalities (Fuerst et al., 2009; Iossifov et al., 2014; Karaca et al., 2015; Galicia et al., 2018; Ma et al., 2020).

MATERIALS AND METHODS

Zebrafish Husbandry

Zebrafish (all ages) were raised under a 14/10 light/dark cycle at 28.5°C. Embryos and larvae were raised in water containing 0.1% Methylene Blue hydrate (Sigma-Aldrich). With the exception of *nacre* mutants, embryos were transferred to E3 buffer containing 0.003% 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) to prevent pigment formation at 24 h post-fertilization. Developmental stages are as described by Kimmel et al. (1995). Sex was not considered as a relevant variable for this study, as laboratory zebrafish remain sexually undifferentiated until 2 weeks of age, beyond the stages being used (0–9 dpf) (Maack and Segner, 2003; Wilson et al., 2014). All experimental procedures are performed in accordance with Institutional Animal Care and Use Committee guidelines at Augusta University and Virginia Tech.

Transgenic and Mutant Zebrafish Lines

The *dscaml1*[vt1] mutant line was generated by TALEN-targeted mutagenesis, which resulted in a seven base pair deletion and subsequent early translational termination (Ma et al., 2020). The *Tg(elavl3:H2B-GCaMP6f)* line was generously provided by E. Aksay at Weill Cornell Medicine, with permission from M. Ahrens at HHMI Janelia Research Campus (Kawashima et al., 2016). The *vglut2a:GFP* line [*Tg(slc17a6b:EGFP)*] was generously provided by J. Fetcho at Cornell University with permission from S. Higashijima at the National Institute for Basic Biology (Bae et al., 2009).

Preparation of VSVΔG

VSV was prepared using methods detailed by Beier et al. (2016). 293T cells (ATCC, #CRL-3216) were transfected at 80% confluency on 75 cm^2 flasks with 7 μg of *pCI-VSVG* plasmid (Addgene, #1733) and incubated overnight at 37°C. Afterward, cells were infected with VSVΔG-RFP (VSVΔG for short) (Beier et al., 2011b) at a multiplicity of infection (m.o.i.) of 0.1.

Viral supernatants were collected for the subsequent 3 days at 24-h intervals and combined. Cell debris was precipitated by centrifugation at 1,000 g for 20 min. To concentrate VSVΔG, viral supernatant was ultracentrifuged for 3 h at 80,000 g with an SW32Ti rotor, and the pellet was resuspended in 100 μl of culture medium. Viral stocks were titered by serial dilution on 90% confluent 293T cells. The number of fluorescent foci was calculated at 2 days post infection (dpi) by identifying RFP-positive cells. Typical viral titer was higher than 1×10^9 focus forming units/ml (ffu/ml).

For *in vitro* trans-complementation of VSVΔG with lentivirus, BHK-21 cells (ATCC, #CCL-10) were seeded into 96-well plates and incubated overnight to reach 20,000 cells per well. Cells were co-infected with VSVΔG (m.o.i. = 0.005) and VSV-G pseudotyped lentivirus (m.o.i. = 0–10,000) (lentivirus-SIN-CMV-eGFP or lentivirus-SIN-Ubi-iCre-mCherry; GT3 Core Facility of the Salk Institute). The typical viral titer for lentivirus stock was 10^{11}-10^{12} ffu/ml. At 2 h post-infection, cells were washed twice with PBS and incubated with fresh medium supplemented with 2% serum. At 2 dpi, the spread of VSV was visualized by fluorescent microscopy. The media in each well were collected and titered to evaluate viral yield.

Virus Injection

Viral injections were performed as previously described (Mundell et al., 2015; Beier et al., 2016). Briefly, glass capillaries (TW100F-4; World Precision Instruments) were pulled into injection needles with a pipette puller (P-97; Sutter Instruments). The tips of injection needles were trimmed to create a ~10 μm opening. Virus injection solution was made by diluting VSVΔG and lentivirus stock with tissue culture medium (DMEM; Fisher Scientific), with Fast Green dye (BP123-10; Fisher Scientific) as the injection marker. Two microliter of injection solution was loaded into the injection needle with a Microloader pipette tip (930001007; Eppendorf), and mounted into a microelectrode holder connected to a pneumatic PicoPump (PV820; World Precision Instruments). Injection volume was determined by calibrating the volume of the injected droplet on a stage micrometer (50-753-2911; Fisher Scientific). The hold pressure of the PicoPump was adjusted so that there was a slight outflow of virus solution when the needle tip was immersed in media.

For retina injection, 2.5 or 3 dpf larvae were anesthetized in Tricaine (0.013% w/v, AC118000500; Fisher Scientific) mounted laterally inside the center chamber of a glass-bottom dish (P50G-1.5-14-F; MatTek) with 1.5% low melting-point agarose (BP1360; Fisher Scientific). After the agarose has solidified, the dish is filled with Tricaine solution (0.013%) to maintain anesthesia. Under a stereo dissecting microscope (SMZ18; Nikon), the needle tip was moved with a micromanipulator (MN-151; Narishige) to approach the fish from the rear and penetrated the temporal retina, with the needle tip being in the neural retina. 0.25–0.5 nl of virus solution (concentrations as described in the Results section) were injected inside the retina. After injection, larvae were recovered from the agarose and returned to a 28°C incubator.

For TRAS labeling, we adjusted viral titer with the aim of achieving cellular labeling in areas innervated by the optic nerve and not in areas two synapses away (e.g., ipsilateral midbrain, hindbrain, cerebellum, habenula, telencephalon). VSVΔG injected without lentivirus at 10^6-10^9 ffu/ml resulted in robust retinal infection at the injection site. When lentivirus (2×10^9-10^{11} ffu/ml) was injected along with VSVΔG (10^6-10^8), transneuronal spread was observed. For quantitative analysis of retinorecipient cells, we used VSVΔG at 3×10^7 ffu/ml and lentivirus at 3×10^{10} ffu/ml. At these concentrations, we did not see any evidence for multisynaptic spread. It is worth noting that we did find sparse labeling of putative third-order neurons in one experiment that had extensive infection (potentially due to aliquot-specific variations in viral titer) with VSVΔG at 3×10^7 ffu/ml and lentivirus at 3×10^{10}-10^{11} (1–2 cells in 50% of injected fish, $n = 10$, **Supplementary Figure S1**). We only analyzed the experiments that had no third-order neuron labeling in this study.

For habenula injection, 3 dpf larvae were mounted with the dorsal side up. The agarose and skin above the left habenula were carefully removed with a sharpened tungsten needle (10130-05; Fine Science Tools). The injection needle tip was inserted into the left habenula and remained there for 5 s, allowing the slow outflow of virus solution (VSVΔG at 3×10^8 ffu/ml, lentivirus at 1×10^{11} ffu/ml) to immerse the surrounding tissue. At 1 dpi, larvae with habenula-specific RFP expression were screened and later fixed at 3 dpi for immunohistochemistry and confocal imaging.

Immunohistochemistry

Whole-mount immunohistochemistry was performed as described by Randlett et al. (2015). Zebrafish larvae were fixed overnight with 4% PFA and 0.25% Triton X-100 (Fisher Scientific) in 1X PBS (diluted from 10%PFA; Polysciences), then washed with 1X PBS and 0.25% Triton X-100. H2B-GCaM6f was stained with FluoTag-X4 anti-GFP (N0304-At488; NanoTag); GABA was stained with Rabbit anti-GABA (A2052; Sigma-Aldrich); ERK1/2 was stained with mouse anti-ERK1/2 (4696S; Cell Signaling Technology). The sRIMS solution, which is D-Sorbitol (Sigma-Aldrich) dissolved in PBS with 0.1% Tween-20 (Fisher Scientific) and 0.01% Sodium Azide, was used for optical clearing (Yang et al., 2014). Samples are immersed for 15 min (or until sunken to the bottom of the Eppendorf tube) through a gradient series (8.75–70%) of D-Sorbitol.

Image Acquisition

Epifluorescence images of cultured cells were acquired under a Nikon Eclipse Ts2 inverted fluorescent microscope. Images of zebrafish were acquired using a Nikon A1 laser scanning confocal system with a CFI75 Apochromat LWD 25x water-immersion objective. For TRAS quantification, image stacks were acquired at a standard resolution of $0.49 \times 0.49 \times 2.0$ μm^3 per voxel. For efferent tract-tracing, a standard resolution stack and a high-resolution stack ($0.38 \times 0.38 \times 0.5$ μm^3 per voxel) were acquired for each fish.

Efferent Tract Tracing

Standard resolution image stacks were morphed to the Z-Brain *elavl3:H2B-RFP* template using CMTK (Randlett et al., 2015). High-resolution stacks were then morphed to its

corresponding low-resolution stacks to register to Z-Brain coordinates. Morphed high-resolution stacks were imported into the neuTube software for tracing, in accordance with the neuTube online manual (https://www.neutracing.com/manual/) (Feng et al., 2015). The SWC files were saved and imported into Fiji plugin "Simple Neurite Tracer" then saved as an overlay.

TRAS Quantitation With Z-Brain

Image stacks from 24 wild-type and 26 *dscaml1-/-* larvae were morphed to the Z-Brain *elavl3:H2B-RFP* template using CMTK, using the GCaMP6f channel as reference (Randlett et al., 2015). For retinal ganglion cell arborization field (AF) fluorescent intensity analysis, the *ZBrainAnalysisOfMAPmaps.m* MATLAB script from Z-Brain was used to process each morphed stack individually (VSV∆G channel, no smoothing). From the output files, the total signal intensities from all Z-Brain annotated AFs (AF1-AF9 and the tectum neuropil) from each stack were then compiled and analyzed in the Prism statistical software (GraphPad).

For retinorecipient cell number and distribution analysis, the Fiji software's ROI manager (Analyze > Tools > ROI manager; extra-large size dot) was used on the morphed VSV∆G channel to mark all VSV∆G+ cells. The marked positions (ROIs) were saved into a zip file and overlaid onto the GCaMP6f channel. The ROIs that were GCaMP6f-negative were removed so that the remaining ROIs represented TRAS-labeled neurons (neuronal ROIs). Next, the neuronal ROIs were overlaid onto the GABA channel to create two subsets: the GABA+ ROIs (inhibitory neurons) and GABA- ROIs (excitatory neurons, created by subtracting the neuronal ROI with the GABA+ ROIs). Lastly, these two ROIs were overlaid onto a Z-brain reference-sized (X:Y:Z = 1,121 × 496 × 276 μm^3 at 0.8 × 0.8 × 2 μm^3 per voxel) blank stack, with inhibitory neuron ROIs pseudo-colored magenta and excitatory neuron ROIs pseudo-colored green. Then, we created .tif files with the overlaid ROIs.

To quantitate the anatomical distribution of retinorecipient cells, we used a modified Z-Brain workflow. Instead of using the *MakeTheMAPmap.m* MATLAB script, we used a custom Fiji macro script to create the output image file, which were then processed using a modified *ZBrainAnalysisOfMAPmaps.m* MATLAB script that quantifies sum pixel intensity values for neuronal ROIs within each region mask. The output intensity values were converted to cell counts, based on an estimate of the pixel values generated from a single-cell ROI (~18,265). For heatmap display and cohort-wise comparison of individual regions, the intensity signals for each fish were normalized by the sum signal from "Diencephalon" and "Mesencephalon." The numbers were then imported into MATLAB to make a scaled color map using the *imagesc* function.

Analysis of Topographical Distribution

To analyze the topographical distribution of wild-type and *dscaml1-/-* retinorecipient cells (**Figure 5**), the ROI files from all fish within a cohort were combined into a single zip file and overlaid into a Z-Brain compatible blank stack, as described previously for single fish ROI image files. The x-y-z coordinates of each ROI dot were used in the *scatter3* function in MATLAB

to create 3-D scatter plots. The same coordinates were imported into GraphPad for analysis of distribution properties. The cumulative frequency statistics were done using the K-S test provided within the Prism software (GraphPad).

RESULTS

Trans-complementation of VSV∆G by VSV-G Coated Lentivirus

For both VSV and RABV, the envelope glycoprotein (G) gene is essential for binding, internalization, membrane fusion, and release of the viral genome into the host cell (Albertini et al., 2012; Kim et al., 2017). A recombinant virus with genomic deletion of the G gene (∆G) can infect and replicate inside the cell but is unable to spread, unless the host cell complements the virus by providing G *in trans* (Wickersham et al., 2007; Beier et al., 2011b). Trans-complementation can, therefore, be utilized to restrict viral spread to direct synaptic partners. For instance, expressing the RABV glycoprotein (RABV-G) in neurons at the injection site (starter cells) allowed VSV∆G or RABV∆G to spread from the starter cells to their input neurons. Once inside the input neurons, the virus can no longer spread (Wickersham et al., 2007; Beier et al., 2013).

Given VSV's ability to spread anterogradely across synapses, we asked whether trans-complementing VSV∆G virus with VSV glycoprotein (VSV-G) could enable restricted anterograde spread (Mundell et al., 2015). Our efforts to express VSV-G *in vivo* through transgenesis were unsuccessful, possibly due to the pathogenic effects of VSV-G when persistently expressed (Yee et al., 1994). As an alternative, we tested whether VSV-G protein could be transduced to cells directly. We took advantage of the fact that most commercially available lentiviruses are coated with VSV-G and examined whether concomitant VSV∆G/lentivirus infection could provide sufficient VSV-G to trans-complement VSV∆G *in vitro*. 293T cells were co-infected with VSV-G enveloped, RFP-expressing VSV∆G at low density (m.o.i. = 0.005) and lentivirus at a range of densities (m.o.i. = 0–1,000). At 2 days post-infection (dpi), we visualized the spread of VSV∆G by fluorescent microscopy and determined the yield of newly synthesized VSV∆G in the media. Indeed, lentivirus complemented VSV∆G in a dose-dependent manner, indicating that VSV-G on the envelope of lentivirus could be taken up by VSV∆G to form functional virions (**Figures 1A–C**).

Lentivirus-mediated trans-complementation was also effective *in vivo*, enabling transneuronal spread. By itself, VSV∆G injection into the eye (0.5 nl at 10^8 ffu/ml) resulted in retina infection and RFP labeling of the optic nerve, but no cellular labeling in the brain (**Figures 1D,E**). This suggested that VSV∆G was not released from axon terminals to initiate a new cycle of infection in the brain. When low (5 × 10^9 ffu) or high titer lentivirus (2 × 10^{10} ffu) was co-injected with VSV∆G (10^8 ffu/ml), we observed cellular labeling in the brain in both conditions, with more spread in injections with high titer lentivirus (**Figures 1F,G**). This agrees with our *in vitro* results
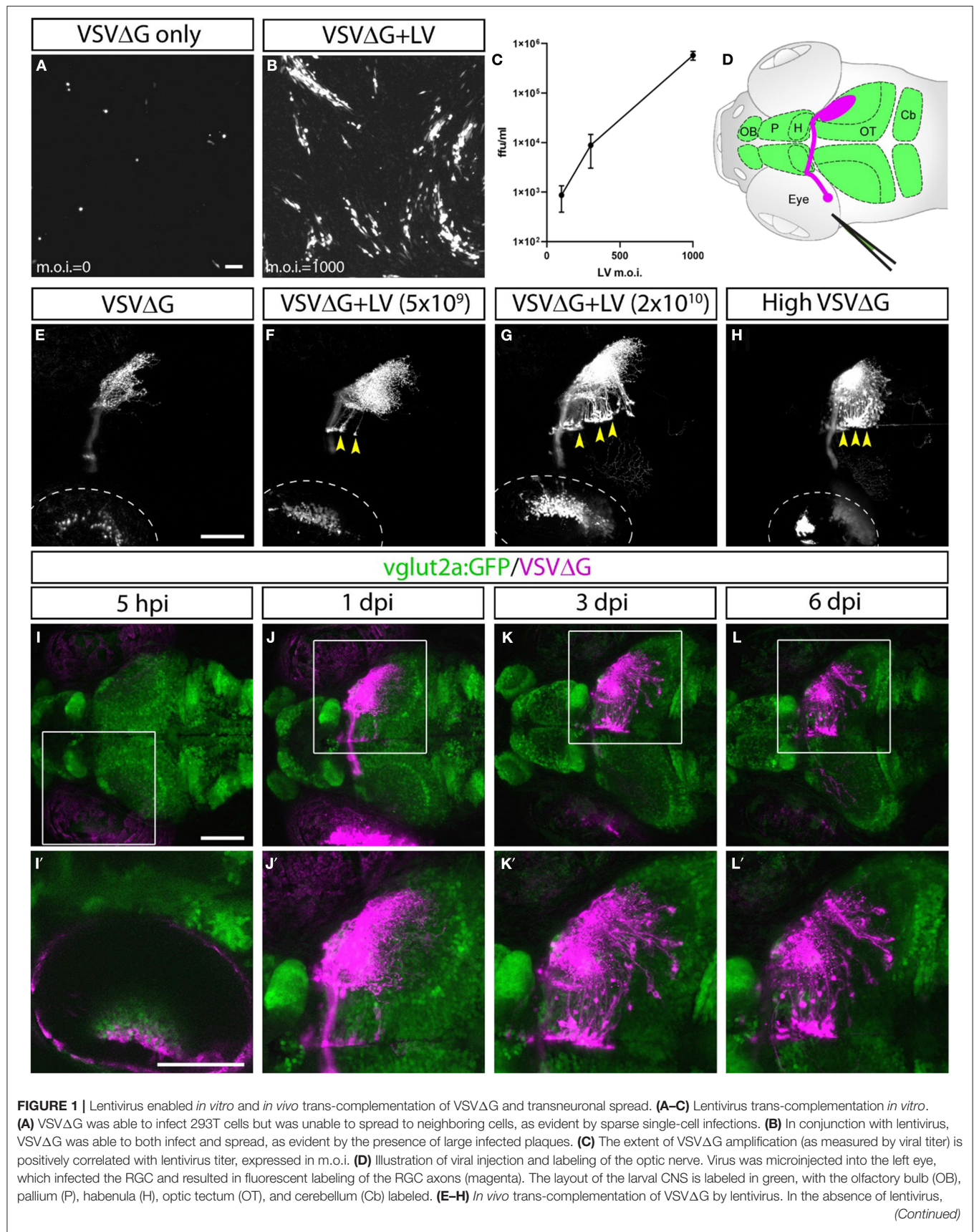
FIGURE 1 | Lentivirus enabled *in vitro* and *in vivo* trans-complementation of VSVΔG and transneuronal spread. **(A–C)** Lentivirus trans-complementation *in vitro*. **(A)** VSVΔG was able to infect 293T cells but was unable to spread to neighboring cells, as evident by sparse single-cell infections. **(B)** In conjunction with lentivirus, VSVΔG was able to both infect and spread, as evident by the presence of large infected plaques. **(C)** The extent of VSVΔG amplification (as measured by viral titer) is positively correlated with lentivirus titer, expressed in m.o.i. **(D)** Illustration of viral injection and labeling of the optic nerve. Virus was microinjected into the left eye, which infected the RGC and resulted in fluorescent labeling of the RGC axons (magenta). The layout of the larval CNS is labeled in green, with the olfactory bulb (OB), pallium (P), habenula (H), optic tectum (OT), and cerebellum (Cb) labeled. **(E–H)** *In vivo* trans-complementation of VSVΔG by lentivirus. In the absence of lentivirus,

(Continued)

FIGURE 1 | VSV△G infected RGCs and fluorescently labeled the optic nerve, but no spread in the CNS was observed **(E)**. **(F,G)** When lentivirus and VSV△G were coinjected, cellular labeling was observed in the CNS (yellow arrowheads), indicating transneuronal spread. **(H)** Similar patterns of spread was also seen at very high VSV△G titer (2 × 10^9 vs. 10^8 ffu/ml for **E–G**), suggesting that VSV△G was able to self-complement. **(I–L)** Time course of VSV△G infection and spread with lentivirus trans-complementation, with RFP expression from VSV△G (magenta) and GFP expression from the *vglut2a:GFP* transgene (green). Box regions are shown at higher magnifications below **(I'–L')**. Scale bars are 100 μm. Images in the same row are shown at the same scale.

and indicates that high titer lentivirus can trans-complement VSV△G, allowing viral spread from axon terminals.

The ability of lentivirus to trans-complement was not dependent on what the lentivirus genome encodes. Two types of VSV-G coated lentivirus were tested (lentivirus-SIN-CMV-eGFP and lentivirus-SIN-Ubi-iCre-mCherry). Both were able to mediate trans-complementation, and neither were able to drive transgene expression (eGFP and iCre-mCherry, respectively) on their own in fish at 6 dpi. Lastly, we asked whether VSV△G could self-complement at higher titer since VSV△G itself was also enveloped in VSV-G. Indeed, high titer VSV△G (2 × 10^9 ffu/ml) could spread from the retinal ganglion cell (RGC) to retinorecipient cells in the brain (**Figure 1H**). Together, these results show that VSV-G from different viral particles could be recycled to form infectious VSV particles. The use of lentivirus provides the additional flexibility to adjust VSV△G and lentivirus titers to optimize for the extent of starter cell infection and transneuronal spread for sparse or dense labeling (e.g., **Figures 1F,G**).

Restricted Anterograde Spread of VSV△G in the Zebrafish Visual Pathway

Since lentivirus was supplied at the injected site, only neurons at the injection site (starter cells) should be able to mediate spread. The spread from the starter cells should be limited to direct postsynaptic targets, i.e., anterograde monosynaptic spread. To test this, we examined whether the spatial and temporal patterns of VSV△G spread were consistent with monosynaptic spread from RGCs to retinorecipient neurons in the brain.

VSV△G and lentivirus were coinjected into the left temporal retina of anesthetized 3 dpf zebrafish larvae, followed by live confocal imaging at different time points ($n = 9$ animals). Initial RFP expression from VSV△G was present in the injected (left) eye as early as 5-h post infection (hpi) (**Figures 1I–I'**). Cellular labeling in the contralateral (right) brain was observed at 1 dpi, and more cells were labeled at 3 dpi (**Figures 1J–K'**). At 6 dpi, there was no further spread to other brain regions, compared to 3 dpi (**Figures 1L–L'**). This pattern of labeling is distinct from non-G-deleted (i.e., replication competent) VSV, which rapidly progressed from axonal labeling to cell body labeling in downstream areas like the cerebellum and habenula at 3 dpi (Mundell et al., 2015). These results suggest that lentivirus trans-complementation primarily mediated anterograde monosynaptic spread. We call this new technique Tracer with Restricted Anterograde Spread, or TRAS (pronounced like *trace*).

Efferent Projections of Retinorecipient Cells Were Revealed by TRAS

Retinorecipient cells extend axons to different parts of the brain to mediate visually guided cognitive, sensory, motor, and homeostatic functions. Previous studies have utilized transgenic reporter lines to characterize efferent projections of subsets of retinorecipient cells, but there has not been a method that could unbiasedly label retinorecipient cells in different brain regions and reveal their efferent projections (Zhang et al., 2017; Helmbrecht et al., 2018; Kramer et al., 2019).

With TRAS, we observed several prominent efferent tracts from retinorecipient neurons, innervating the telencephalon (6 of 9 animals), habenula (2 of 9), tegmentum (8 of 9), contralateral optic tectum (8 of 9), cerebellum (7 of 9), and along the ventral hindbrain (7 of 9) (**Figure 2**, image stack for **Figure 2A** is shown in **Supplementary Video 1**). The less frequently observed efferent projections (i.e., habenula) likely originate from a smaller retinorecipient cell population that was less frequently labeled by the stochastic spread of virus from RGC axon terminals. Overall, these projection patterns are reminiscent of the efferent projections of putative tectal/pretectal retinorecipient neurons identified by sparse transgenic labeling or single-cell photoconversion, further supporting the idea that TRAS primarily labels retinorecipient cells (Sato et al., 2007; Helmbrecht et al., 2018; Kramer et al., 2019). The projection into the telencephalon by retinorecipient neurons, to our best knowledge, has not been reported previously. These axon projections extend rostrally to enter the subpallium and then course dorsally to the caudal pallium. Some axons crossed near the anterior commissure. These pallium-projecting neurons may serve similar roles as the mammalian lateral geniculate neurons to relay sensory information to higher visual areas (Mueller, 2012). These results show that TRAS could be used to identify not only postsynaptic cells but also downstream areas innervated by these cells.

3D Mapping and Cell-Type Characterization

To quantify connectivity patterns, we registered TRAS-labeled image stacks to the Z-Brain zebrafish brain atlas (Randlett et al., 2015). Transgenic fish expressing neuronal-localized nuclear GCaMP6f (*elavl3:H2B-GCaMP6f*) were injected at 2.5 dpf, into the temporal region of the left eye (representing the frontal visual field). Under these conditions, viral infection in the retina is predominantly in the temporal quadrant of the eye (examples of starter cell infection in animals with non-pigmented retina are shown in **Supplementary Figure S2A**). Infected larvae were fixed at 3 dpi and stained with anti-GFP (to amplify the GCaMP6f signal) and anti-GABA. Stained samples were then cleared in sRIMS, a sorbitol-based mounting media that was crucial to resolving single cells and axon tracts in the ventral brain (Yang et al., 2014) (**Figures 3A,B**, **Supplementary Figures S2B,C**). By extracting the Z-Brain *elavl3:H2B-RFP* stack from ZBrainViewer as the reference, image stacks were morphed and aligned
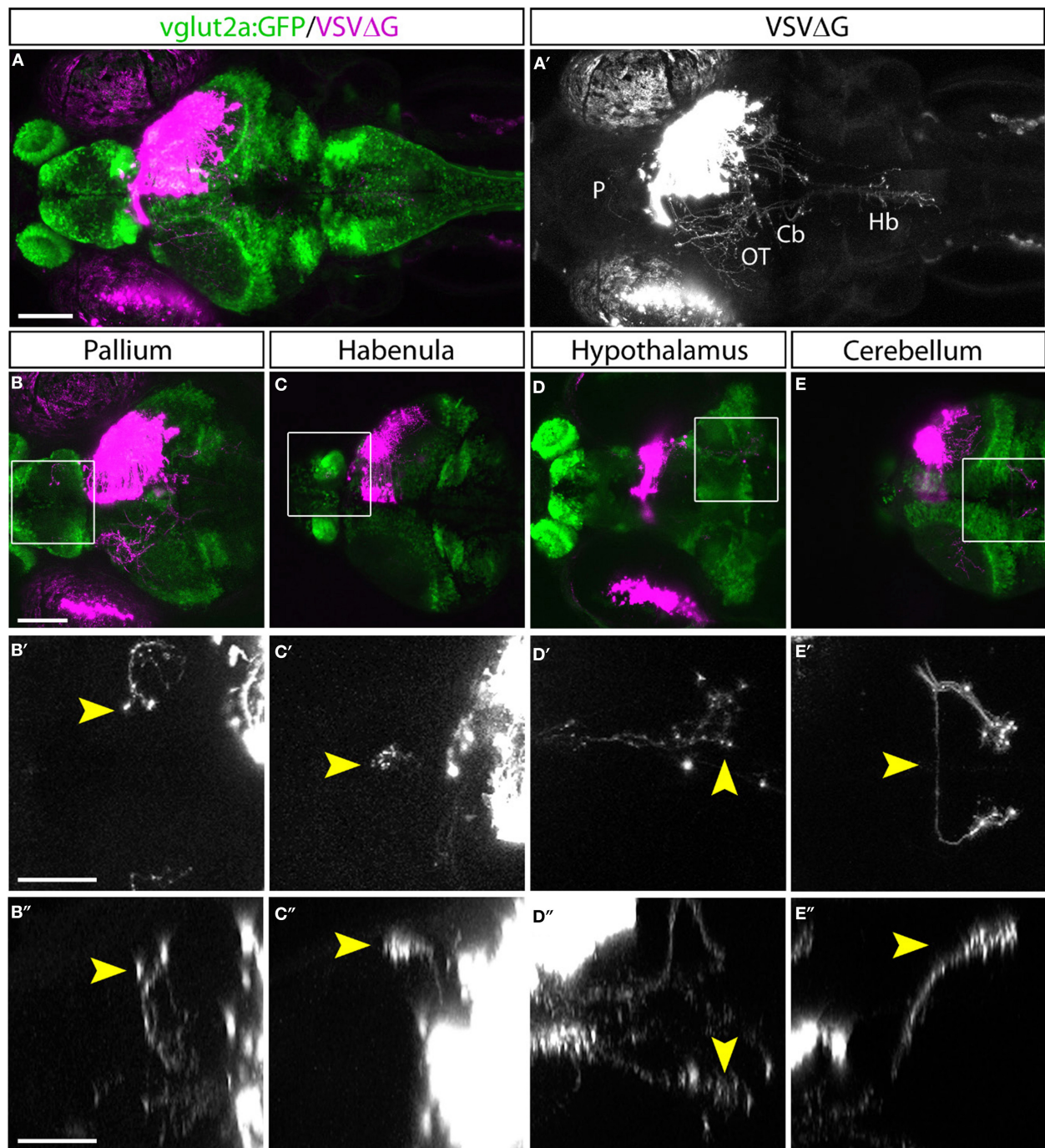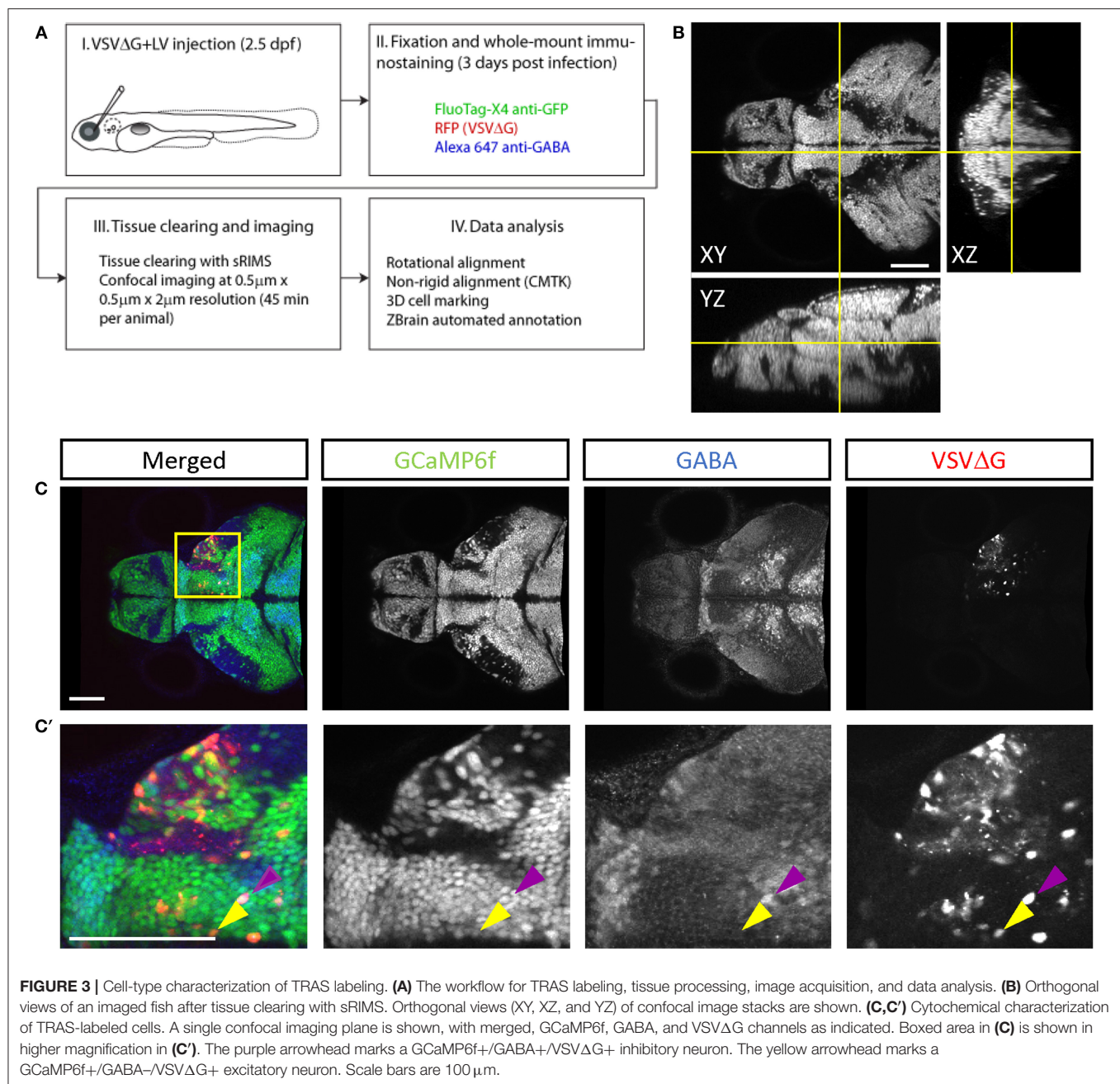
FIGURE 2 | Efferent projections of retinorecipient cells. **(A,A′)** Confocal maximal intensity projection (dorsal view) of TRAS-labeled larva, with RFP expression from VSVΔG in magenta **(A)** or white **(A′)**. Axon projections can be seen in the pallium (P), optic tectum (OT), cerebellum (Cb), and hindbrain (Hb). **(B–E)** Maximal intensity projection confocal substacks that contained the pallium **(B–B″)**, habenula **(C–C″)**, hypothalamus **(D–D″)**, and cerebellum **(E–E″)**. RFP expression from VSVΔG (magenta) and GFP expression from the *vglut2a:GFP* transgene (green) are shown in **B–E**, while boxed region is shown at higher magnification in **B′–E′** (dorsal view) and **B″–E″** (lateral view), with only the RFP channel (white). Yellow arrowheads point to the same efferent projections in the dorsal and lateral views. Scale bars are 100 μm in **(A,B)** and 50 μm in **(B′,B″)**. Images in the same row are shown at the same scale.

with the Computational Morphometry Toolkit (CMTK) (Rohlfing and Maurer, 2003; Jefferis et al., 2007; Randlett et al., 2015).

To distinguish different cell types, both transgenic and immunohistochemical markers were used. Nuclear GCaMP6f (expressed only in neurons) was used to distinguish between

FIGURE 3 | Cell-type characterization of TRAS labeling. **(A)** The workflow for TRAS labeling, tissue processing, image acquisition, and data analysis. **(B)** Orthogonal views of an imaged fish after tissue clearing with sRIMS. Orthogonal views (XY, XZ, and YZ) of confocal image stacks are shown. **(C,C′)** Cytochemical characterization of TRAS-labeled cells. A single confocal imaging plane is shown, with merged, GCaMP6f, GABA, and VSVΔG channels as indicated. Boxed area in **(C)** is shown in higher magnification in **(C′)**. The purple arrowhead marks a GCaMP6f+/GABA+/VSVΔG+ inhibitory neuron. The yellow arrowhead marks a GCaMP6f+/GABA–/VSVΔG+ excitatory neuron. Scale bars are 100 μm.

neuronal (GCaMP6f+) and GCaMP6f non-expressing (GCaMP6f–, non-neuronal cells and GCaMP6f-negative neurons) cells (**Supplementary Figure S3**). Anti-GABA staining was used to distinguish between non-GABAergic and GABAergic (inhibitory) neurons (Cui et al., 2005) (**Figures 3C–C′**). Since glycinergic neurons are not present in the retinorecipient areas, non-GABAergic retinorecipient neurons are mostly excitatory, with potential inclusion of some GABAergic neurons without soma-localized GABA. After image morphing with CMTK, all TRAS labeled cells were converted into Z-Brain coordinates and categorized into three types: excitatory neurons (GCaMP6f+, GABA–) inhibitory neurons (GCaMP6f+, GABA+), and

GCaMP6f– cells. In total, 24 wild-type and 26 *dscaml1-/-* fish (Ma et al., 2020) (see next section) were analyzed (**Figures 4A,B**, **Supplementary Video 2**). The overall ratio of excitatory vs. inhibitory cells was similar between wild-type and *dscaml1-/-* cohorts ($p = 0.515$, Chi-square = 0.424). The annotated stack will be made available in the Z-Brain on-line viewer (http://engertlab.fas.harvard.edu/Z-Brain/) (Randlett et al., 2015).

The extent of starter cell (RGC) infection and subsequent spread was not significantly different between wild-type and *dscaml1-/-* cohorts. To estimate the number of starter cells, we quantified the RFP fluorescence signal in the RGC arborization fields (AF1-10) (Burrill and Easter, 1994; Robles
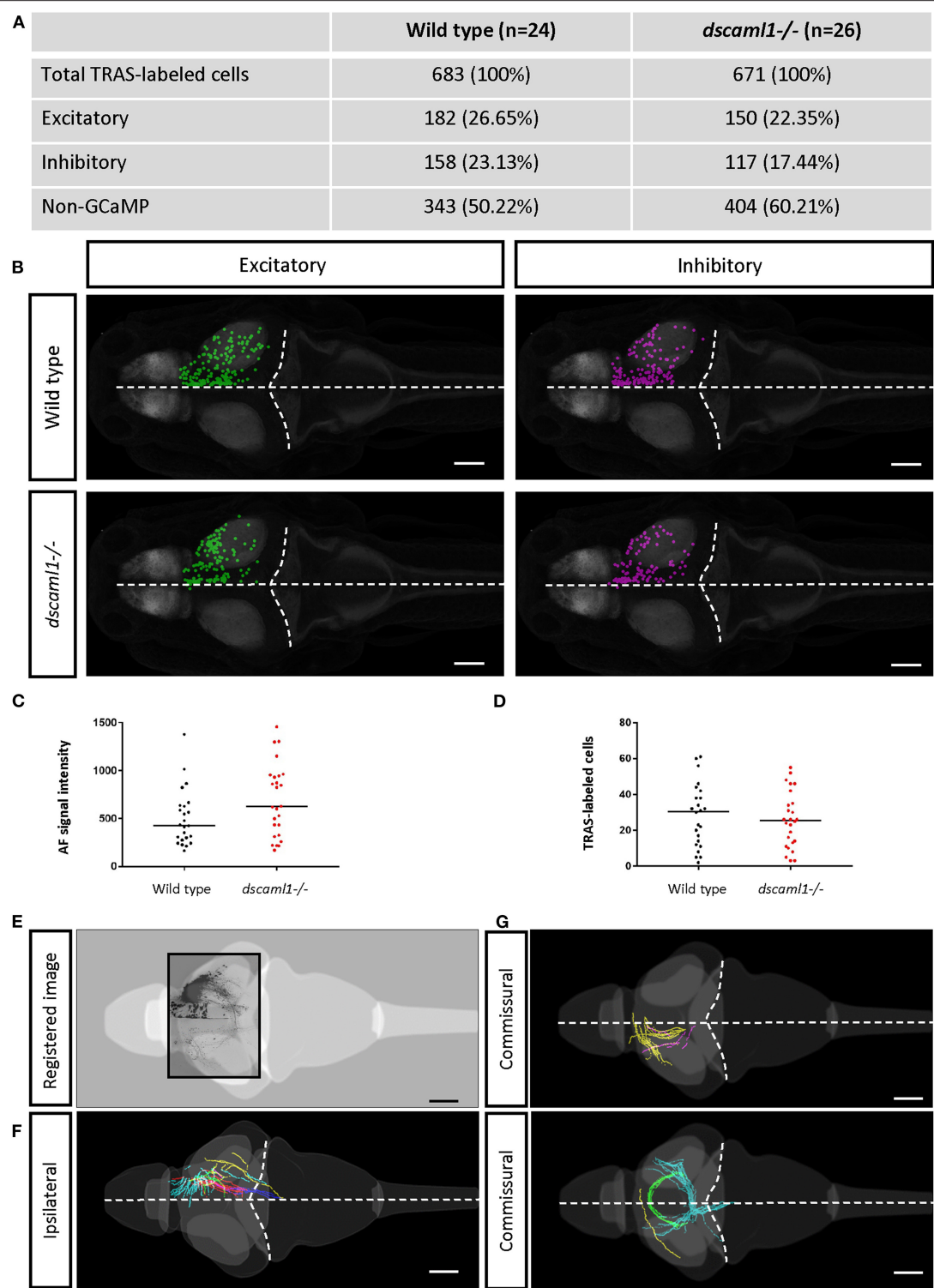
FIGURE 4 | Annotation of TRAS-labeled neurons and efferent axons in the Z-Brain standard brain reference. **(A)** Overview of all annotated TRAS-labeled retinorecipient cells within the wild-type and *dscaml1-/-* cohorts. **(B)** Spatial layout of TRAS-labeled neurons (dorsal view, rostral to the left) overlaid onto the Z-brain reference brain scale, for wild-type (top row) and *dscaml1-/-* (bottom row) cohorts. Green dots mark excitatory neurons, and magenta dots mark inhibitory neurons. **(C)** RGC arborization field (AFs) fluorescent intensity (a proxy for starter cell number) was similar between cohorts. **(D)** The total number of TRAS-labeled cells per fish was not significantly different between cohorts. **(E–G)** Efferent tract tracing from wild-type larvae (*n* = 10). Maximum Z-projection is shown for confocal image **(E)**, traced ipsilateral tracts **(F)**, and commissural tracts **(G)**. Axons with similar trajectories are displayed in the same color. In **(B,E–G)**, the horizontal dashed lines indicate the midline and the curved dashed lines indicate the optic tectum/hindbrain boundary. Scale bars are 100 μm.

FIGURE 5 | Topographical organization of retinorecipient neurons in wild-type and *dscaml1-/-* cohorts. Graphs show the topographical distribution of excitatory **(A,B)** and inhibitory neurons **(C,D)** in wild-type (black, *n* = 24) and *dscaml1-/-* (red, *n* = 26) cohorts. Axes are relative distances (pixels) within the Z-Brain reference brain stack. **(A,C)** Three-dimensional distribution of excitatory **(A)** and inhibitory **(C)** neurons. **(B,D)** Frequency distributions of excitatory **(B)** and inhibitory **(D)** neurons in the rostral-caudal, dorsal-ventral, and lateral-medial axes. Dashed lines indicate the midpoints of the distributions. K-S test, *P < 0.05; ***P < 0.001.

et al., 2014), which are mostly derived from RGC axon terminals. There was no significant difference in arborization field fluorescence between cohorts, suggesting that the extent of starter cell infection was similar (Mann-Whitney *U, p* = 0.0932) (**Figure 4C**). The total number of TRAS-labeled retinorecipient cells were also not significantly different, suggesting similar

efficiencies in viral spreading between cohorts (Mann-Whitney *U, p* = 0.6335) (**Figure 4D**).

We verified that Z-Brain registered stacks could also be used as templates for tracing the efferent projections of retinorecipient cells (**Figures 4E–G**). We acquired high-resolution and low-resolution image stacks for the same fish

and used the high-resolution image stacks for tracing and low-resolution image stacks as the template to register to Z-Brain. We observed ipsilateral and commissural axon tracts, with morphologies that are similar to the tectal efferent tracts described in previous studies (Sato et al., 2007; Helmbrecht et al., 2018).

Comparative Analysis of Retinofugal Connectivity

In addition to normal patterns of retinofugal connectivity, TRAS and Z-Brain can be used to investigate retinofugal connectivity patterns in mutants with visual deficits. We focused on *Down Syndrome Cell Adhesion Molecule Like-1* (*DSCAML1*), a gene mutated in patients with autism spectrum disorder, cortical abnormalities, and developmental disorders (Iossifov et al., 2014; Karaca et al., 2015; Deciphering Developmental Disorders Study, 2017). In zebrafish, *dscaml1* is broadly expressed in visual areas and required for visual and visuomotor behaviors, suggesting an underlying visual circuit deficit (Ma et al., 2020). Therefore, we compared the retinofugal connectivity patterns between 5.5 dpf wild-type fish and their *dscaml1* mutant (*dscaml1-/-*) siblings.

We asked whether the loss of *dscaml1* affected the topographical distribution of retinorecipient neurons (340 and 267 in wild type and *dscaml1-/-*, respectively) (**Figure 5**). As the initial site of viral infection was in the temporal retina, these retinorecipient neurons likely respond to frontal visual stimuli. The overall distribution was similar between cohorts along the three cardinal axes, but the proportion of retinorecipient cells was significantly shifted in the rostral-caudal and lateral-medial axes. Along the rostral-caudal axis, both excitatory and inhibitory retinorecipient neurons from the *dscaml1-/-* cohort were more rostrally distributed, compared to wild type ($p < 0.001$ and $p < 0.05$ for excitatory and inhibitory neurons, respectively, K-S test). Along the dorsal-ventral axis, the effect of *dscaml1* deficiency was milder. *dscaml1-/-* retinorecipient cells were more dorsally distributed, compared to wild type, but only for excitatory neurons ($p < 0.05$, K-S test). There were no differences in lateral-medial distribution. These results suggest that loss of *dscaml1* may affect the topographic mapping of visual inputs, particularly along the rostral-caudal axes.

Next, we focused on the distribution of retinorecipient cells within specific annotated brain regions. We adapted the Z-Brain quantification tools to measure the sum pixel intensity derived from TRAS-labeled cells for each region (see methods). Among regions defined by anatomy (i.e., not defined by transgene expression), 16 were found to contain, on average, at least 1 retinorecipient cell per animal in the wild-type cohort (**Figures 6A,C**, **Supplementary Figure S4**). Two major brain divisions, the mesencephalon and diencephalon, encompassed all of the retinorecipient cells. Within these divisions, the retinorecipient cells are located within subregions corresponding to known to receive retinofugal input, including the preoptic area, hypothalamus, thalamus, eminentia thalami, pretectum, and optic tectum (tectum neuropil, tectum stratum periventriculare, and medial tectal band) (Burrill and Easter, 1994; Zhang et al., 2017; Helmbrecht et al., 2018; Kramer

et al., 2019). We saw no cellular labeling in the olfactory bulb (which innervates the retina), indicating that lentivirus complementation did not facilitate retrograde spread (Li and Dowling, 2000; Mundell et al., 2015). We also identified several retinorecipient areas that, to the best of our knowledge, had not previously been identified (torus semicircularis, tegmentum, posterior tuberculum). Some of these regions also receive retinorecipient efferent projections (tegmentum, poster tuberculum), as mentioned earlier.
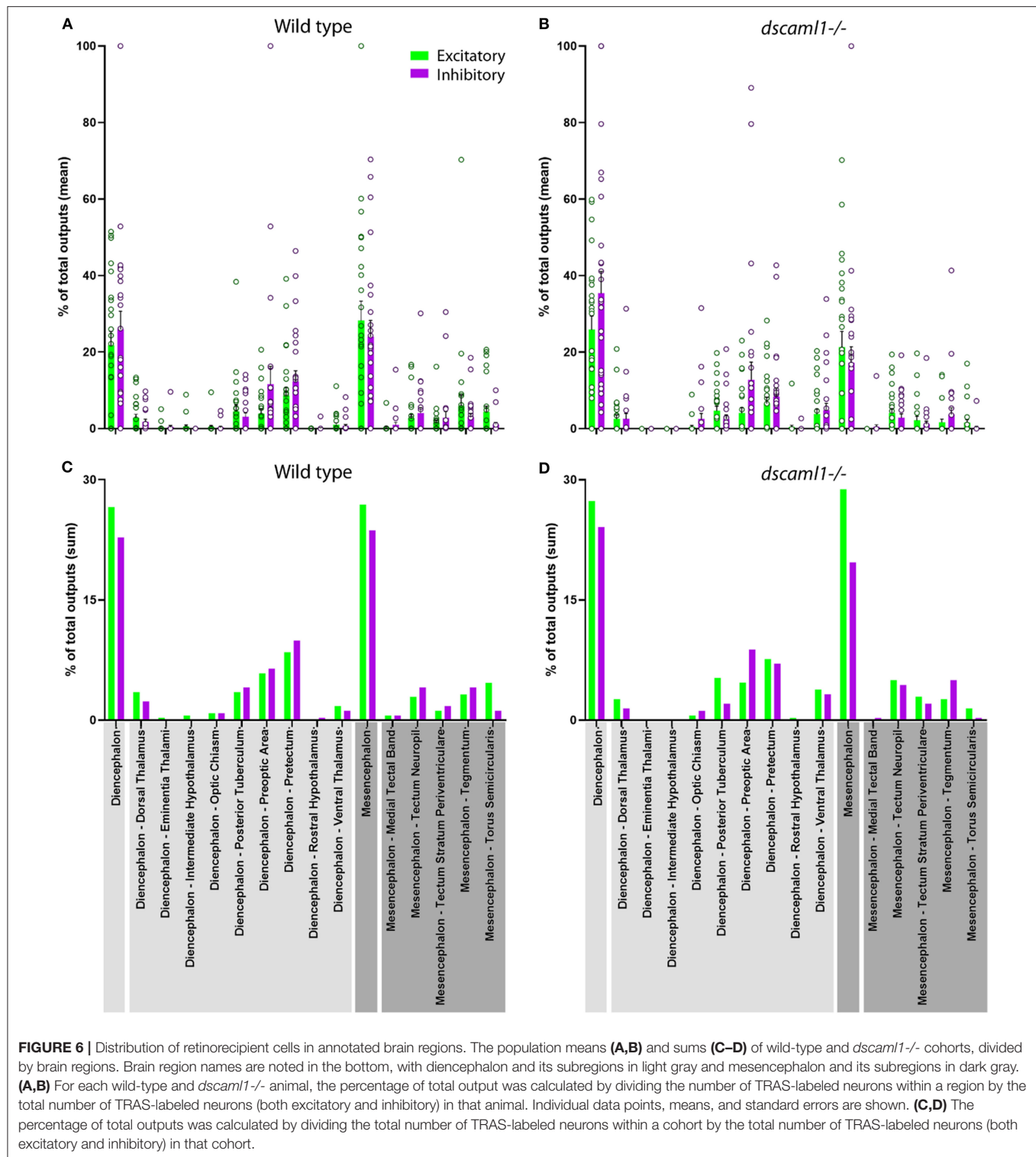
In general, the same areas were innervated in both wild-type and *dscaml1-/-* cohorts, except for two smaller areas that were not innervated in the *dscaml1-/-* cohort (eminentia thalami and intermediate hypothalamus) (**Figures 6A,C** vs. **Figures 6B,D**). This result indicates that major retinorecipient areas are innervated by the optic nerve in the *dscaml1-/-* animals.

TRAS Mapping of Habenular-Recipient Neurons

Finally, to test whether TRAS can be applied more generally to other CNS neuronal populations besides RGCs, we examined efferent targeting from the left habenula (Bianco and Wilson, 2009; Amo et al., 2010; Lee et al., 2010; Dreosti et al., 2014; Duboue et al., 2017; Zhang et al., 2017). The bilaterally asymmetrical habenula receives many different sensory cues and is involved in processing social cues, fear learning, and avoidance. The left habenula is known to project to the interpeduncular nucleus (IPN) and superior raphe, providing a suitable pathway to test TRAS mapping in the CNS (Bianco and Wilson, 2009; Amo et al., 2010). VSVΔG and lentivirus were injected into the left habenula of 3 dpf wild-type animals. At 3 dpi, animals for processed for anti-ERK1/2 immunostaining, confocal imaging, and image registration (see methods). Registered image stacks from five animals with selective left habenula labeling were combined (**Figures 7A–C′**). We observed consistent labeling of the habenular axon tract and the characteristic annular axon terminals in the IPN. Cell bodies near IPN axon terminals were manually marked (**Figures 7D–E′**). Consistent with previous findings, habenular target cells we labeled within the IPN and raphe nucleus (Amo et al., 2010). We did not observe efferent axons emanating from the habenular-recipient cells (Chou et al., 2016), which suggests that habenular-recipient projection neurons may be relatively rare at this stage. Overall, TRAS labeling in the retina and the habenula demonstrates the general applicability of TRAS for mapping the targets of efferent axons.

DISCUSSION

In this study, we developed TRAS, a new method for monosynaptic anterograde labeling in larval zebrafish. This method was applied to the retinofugal pathway and also validated in the habenula efferent pathway. We showed that TRAS could be combined with the Z-Brain image registration and quantitation pipeline to identify changes in retinofugal connectivity patterns caused by the loss of *dscaml1*. These results demonstrate the broad utility of TRAS for neural circuit studies in zebrafish.

FIGURE 6 | Distribution of retinorecipient cells in annotated brain regions. The population means **(A,B)** and sums **(C–D)** of wild-type and *dscaml1-/-* cohorts, divided by brain regions. Brain region names are noted in the bottom, with diencephalon and its subregions in light gray and mesencephalon and its subregions in dark gray. **(A,B)** For each wild-type and *dscaml1-/-* animal, the percentage of total output was calculated by dividing the number of TRAS-labeled neurons within a region by the total number of TRAS-labeled neurons (both excitatory and inhibitory) in that animal. Individual data points, means, and standard errors are shown. **(C,D)** The percentage of total outputs was calculated by dividing the total number of TRAS-labeled neurons within a cohort by the total number of TRAS-labeled neurons (both excitatory and inhibitory) in that cohort.

Trans-complementation of VSVΔG by Lentivirus

The structure and function of VSV-G have been extensively studied in the context of viral entry, membrane fusion, toxicity, and subcellular transport (Dotti and Simons, 1990; Thomas et al., 1993; Ang et al., 2004; Hoffmann et al., 2010; Albertini et al., 2012; Fossati et al., 2014; Kim et al., 2017). VSV-G also determines the infectivity of VSV-G coated viruses (VSV, RABV, lentivirus, retrovirus), which is crucial for their research and clinical applications (Wickersham et al., 2013; Amirache
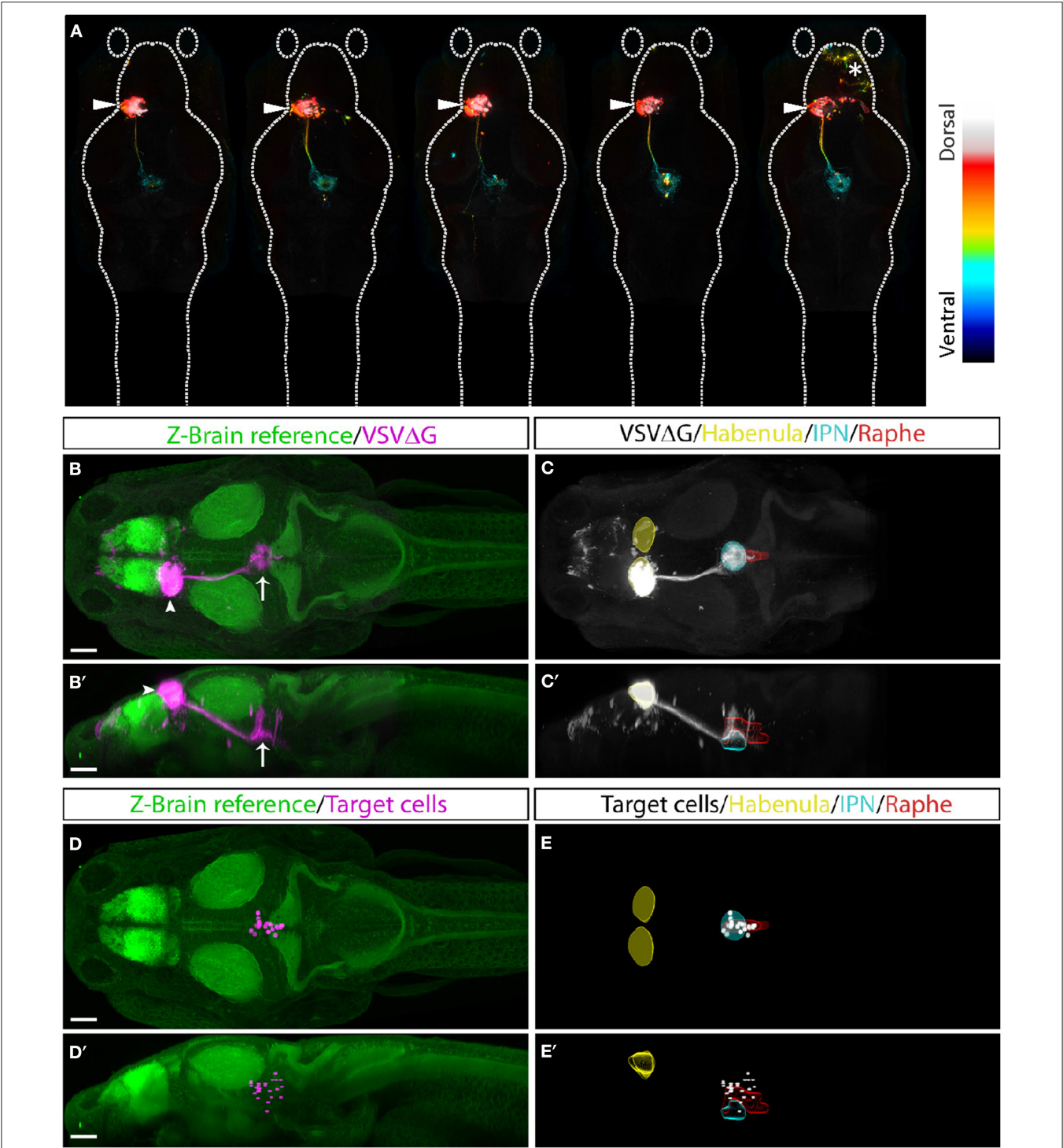
FIGURE 7 | TRAS labeling of habenular target cells. **(A)** Five fish with TRAS tracing from the left habenula (arrowhead), viewed dorsally. The z-dimension is color-coded, as indicated in the lookup table on the right. In one fish (far right), we observed infection in the telencephalon (asterisk). Given the absence of habenula to telencephalon efferents, these cells were likely infected by virus diffusion after injection. **(B–C')** Combined maximal projection of registered image stacks from the animals shown in **(A)**. The left habenula (arrowhead) projects into the IPN (arrow). RFP expression from VSVΔG infection is shown in magenta **(B,B')** or white **(C,C')**. For anatomical reference, images are overlaid on top of the Z-Brain ERK1/2 reference stack (green, **B,B'**), or region outlines for the habenula (yellow), IPN (cyan), and raphe nucleus (red) **(C,C')**. Dorsal **(B,C)** and lateral **(B',C')** views are shown. **(D–E')** Manually marked habenular target cells (magenta in **D,D'**, white in **E,E'**) are overlaid on top of anatomical references, as described for **(A,B)**. Dorsal **(D,E)** and lateral **(D',E')** views are shown. Scale bars are 100 μm. All images are shown at the same scale.

et al., 2014; Mundell et al., 2015; Kobayashi et al., 2016). Our findings revealed a new aspect of VSV-G function, where VSV-G protein from a different viral species can be recycled to generate infectious VSV.

The spread of VSVΔG from the retina to CNS neurons indicates that VSV-G on the lentivirus surface remained functional after lentivirus infection, and a portion of it was transported anterogradely from the cell body to the axon terminal. At the axon terminal, lentivirus-derived VSV-G was able to re-encapsulate the VSV nucleocapsid and mediate subsequent infection. Although it is possible to achieve transneuronal labeling with high titer of VSVΔG, TRAS's two-virus system provides the flexibility of adjusting the titer of VSVΔG based on the demand of the experiment (e.g., sparse vs. dense labeling), while maintaining robust transneuronal spread.

Additionally, the strategy of using lentivirus as a tool for glycoprotein complementation could potentially be applied more broadly. For example, current strategies for RABV monosynaptic tracing utilizes AAV to express RABV glycoprotein, which usually takes several weeks for sufficient glycoprotein expression (Miyamichi et al., 2011). It will be interesting to test whether rabies glycoprotein-coated lentivirus could be a more rapid method to provide glycoprotein for retrograde tracing.

Applying TRAS for Zebrafish Neural Connectivity Analysis

Advances in viral engineering have led to new neural circuit tracing strategies utilizing replication-incompetent viruses (e.g., RABV, AAV, HSV) that are safer to use, less toxic to host cells, and have restricted (mostly monosynaptic) spread (Wickersham et al., 2007; Zingg et al., 2017; Chatterjee et al., 2018; Beier, 2019). Unfortunately, many of the transsynaptic viruses used in mammalian systems either do not infect zebrafish (e.g., AAV) or have low efficiency for transsynaptic spread (e.g., RABV) (Zhu et al., 2009; Dohaku et al., 2019). VSV, in contrast, can infect larval zebrafish and spreads robustly both anterogradely and retrogradely. However, replication-competent VSV has high cytotoxicity and can spread across multiple synapses, making it difficult to distinguish between direct vs. indirect connections (Mundell et al., 2015).

To address these limitations and provide a tool for neural circuit mapping for larval zebrafish, we developed TRAS. TRAS utilizes recombinant VSV with genomic deletion of the glycoprotein gene (VSVΔG). VSVΔG can infect cells at the injection site but cannot spread. Although wild-type VSV does not cause serious illness to humans, the use of VSVΔG further reduces the risk of exposure (Spickler, 2016). The lack of VSV-G expression from the viral genome also helps reduces toxicity to the host cell, as long-term VSV-G expression is known to be cytotoxic (Yee et al., 1994). To complement VSVΔG, we directly provided VSV-G protein, utilizing lentivirus as the transducing reagent. Compared to transgenic or virus-induced expression, this approach is rapid and transient, therefore minimizing the cellular exposure to VSV-G. Both VSVΔG and VSV-G coated lentivirus are available from a commercial source, making TRAS an easy method to adopt in a typical neuroscience laboratory.

To expand the utility of TRAS, we developed procedures to register brain images to the Z-Brain anatomical template (Randlett et al., 2015). The combination of neural circuit tracing within a standard 3D-brain atlas is the current state of the art approach for understanding neural network connections, both in zebrafish and mammalian models (Watabe-Uchida et al., 2012; Oh et al., 2014; Helmbrecht et al., 2018; Kramer et al., 2019; Kunst et al., 2019). This approach provides a more objective way to map cells and pathways onto specific brain regions across different experimental animals and promotes cross-referencing between research findings.

We demonstrated that TRAS and Z-Brain could be used for neural circuit mapping in efferent pathways originating from the retina and the left habenula. These are two of the better-studied pathways in larval zebrafish, which allowed us to assess the specificity of TRAS for anterograde labeling of direct postsynaptic targets. By applying TRAS to the habenula, we showed that TRAS could be used to map connections between regions within the central nervous system. It is interesting that we did not observe any efferent axons emanating from the habenular-recipient cells, given the known IPN output pathways described in the adult zebrafish (Chou et al., 2016). Further studies are needed to determine whether any habenular-recipient cells are projection neurons. Overall, TRAS identified all of the target regions described in previous studies, which gives confidence to the future application of TRAS to map unknown neural connections in zebrafish. Furthermore, given that VSV is also an anterograde tracer in mice and chicken, it will be interesting to test whether TRAS can be applied to these experimental systems for neural circuit mapping (Mundell et al., 2015).

Limitations and the Future Development of TRAS

While TRAS offers many advantages as a neural circuit mapping tool, it has similar limitations as other viral tracing techniques (Beier, 2019). In each injected animal, only a subset of the targeted starter cell population is infected, and only a subset of all connections from those starter cells are labeled by trans-neuronal spread. Therefore, the absence of TRAS labeling in a particular region in one animal does not equate an absence of connection in that animal. At the population level, patterns of spread may be affected by synaptic activity, physical distance, the age of the animal, selective biases of viral infection, and viral titer (Callaway and Luo, 2015). Here, we will discuss the limitations specific to TRAS and areas for further technological development.

Specificity of Initial Infection

Since VSV-G binds to a receptor that is widely expressed (LDL receptor) (Finkelshtein et al., 2013), VSV-G coated viruses can infect most cell types. Therefore, the specificity of TRAS depends on precise injection into the brain region of interest. For brain regions smaller than the habenula, a compound microscope with DIC optics would be necessary. To restrict infection to a particular cell type, it may be possible to make use of ASLV-A pseudotyped VSVΔG that can selectively target neurons expressing an exogenous receptor, TVA (Beier et al., 2011b;

Dohaku et al., 2019). However, potential interactions between virions with different envelope glycoproteins may interfere with the specificity of VSVΔG infection (Beier et al., 2011a).

Labeling of Direct vs. Indirect Synaptic Targets

Our analysis of retinorecipient cells indicates that TRAS spread anterogradely from the afferent axons to their direct targets. This is based on the fact that lentivirus is only present near the starter cells and that third-order neurons in downstream areas were rarely labeled. The pattern of TRAS labeling is notably more restricted compared to polysynaptic VSV, which labels third-order neurons within 3 days after initial infection (Mundell et al., 2015). However, we cannot rule out that some of the TRAS-labeled cells in primary retinorecipient areas may be indirectly connected to RGCs. Multiple approaches (e.g., electron microscopy, electrophysiology) will be needed to confirm findings from viral tracing.

Physiological Effects of Infection

While the lack of genomic VSV-G expression from VSVΔG reduces toxicity, other genes expressed from the viral genome can still change the metabolism of the host cell. For instance, the VSV M protein is capable of altering host cell transcription and translation (Ahmed and Lyles, 1998; Connor and Lyles, 2005). Chronic VSVΔG infection would likely affect the survival of infected neurons and impair its neurophysiological functions. Several approaches for reducing the toxicity of RABV have been reported recently to reduce the function or expression of viral proteins, such as destabilizing the RABV nucleoprotein or deleting the RABV L gene (Ciabatti et al., 2017; Chatterjee et al., 2018). Similar manipulations may also reduce the toxicity of VSV.

Cell-Type Characterization

We have used the *elavl3:H2B-GCaMP6f* transgene and GABA immunoreactivity as markers for post-mitotic neurons and inhibitory neurons, respectively. These markers are widely used in the zebrafish field (Mueller et al., 2006; Mueller and Wullimann, 2015), but it is likely that not all neurons express *elavl3*, and not all GABAergic neurons have GABA immunoreactivity in the soma. Further characterization of the *elavl3* and GABA-immunoreactive populations using alternative excitatory and inhibitory neuron markers (e.g., *vglut2*, *gad1b*) is needed to provide clarity on this issue (Satou et al., 2013).

Optimization of Viral Titer

The efficiency of TRAS depends on the titer of VSVΔG and lentivirus as well as other factors specific to each experiment, including the condition and duration of virus storage, the tropism of virus to specific cell types, and the age/genetic background of the host. The viral titers for each type of application will need to be adjusted so that VSVΔG by itself only labels the starter cells, and the addition of lentivirus enables robust monosynaptic spread (**Figures 1E–G**) (see methods). Low viral titer may result in the absence of labeling in more sparsely connected areas, whereas high viral titer increases the chance of VSVΔG self-complementation at the axon terminal and multisynaptic spread.

Labeling of Retinorecipient Cells

With TRAS labeling, retinorecipient cells were strongly fluorescent, allowing us to observe and trace the retinorecipient cell efferent tracts. However, the dendritic morphologies of the retinorecipient cells were harder to discern, as the RGC afferents in the vicinity are labeled by the same fluorophore. This also made it difficult to trace the retinorecipient efferent axons back to their cellular origins. For mapping studies where the local projections or morphologies of the recipient cells are important, it will be beneficial to express a distinct fluorophore in the starter cells (e.g., from a transgene).

Mapping With Z-Brain

Z-Brain analysis depends on morphing and registration of image stacks to a reference template, followed by manual identification of labeled neurons. This approach is suitable to test the effects of single genes or pathological states, but likely too laborious as a screening tool to identify candidate genes or screen drugs. Selective fluorescent labeling of neuronal cell bodies (without labeling neurites) and automation of cell detection would be a crucial next step to improve the utility of TRAS.

Connectivity Patterns Associated With *dscaml1* Deficiency

The ability to quantitate efferent connections prompted us to investigate whether TRAS can be used to identify connectivity deficits caused by *dscaml1* deficiency. As mentioned previously, human DSCAML1 mutations are believed to be causative for neurodevelopmental disorders. Additionally, our recent work has found that loss of *dscaml1* significantly impaired visuomotor function associated with light perception and eye movements, suggesting a possible underlying deficit along the visual pathway (Ma et al., 2020).

Using TRAS and Z-Brain quantification, we found that *dscaml1* deficiency might have a role in refining the retinofugal topography and cell-type specificity. On a broader scale, we saw similar patterns of topographic and region-specific projections between wild-type and *dscaml1-/-* cohorts (**Figures 5, 6**). This indicated that RGC axonal targeting was mostly intact in the *dscaml1* mutants. Interestingly, there was a significant rostral shift in the position in both excitatory and inhibitory retinorecipient cells. Given that RGC axon terminals and retinorecipient cells are both topographically organized, this shift in positioning may result in diminished spatial perception (Stuermer, 1988; Muto et al., 2013; Robles et al., 2014). Further physiological studies will be needed to formally test whether *dscaml1* affects spatial perception in the visual pathway.

CONCLUSIONS

Here, we present the development of TRAS, a new technique that is suitable for mapping neural connectivity in zebrafish. TRAS makes use of a novel lentivirus trans-complementation approach to enable restricted anterograde transneuronal spread by recombinant VSV. We have validated this method in two efferent pathways and identified potential connectivity pattern changes caused by a genetic deficiency in *dscaml1*, a neuronal cell adhesion molecule associated with human neurodevelopmental

disorders. The ability of TRAS to map structural connectivity would enable the discovery of new neural connections and complement existing brain mapping efforts.

DATA AVAILABILITY STATEMENT

The image stacks and other data supporting the findings of this study are available from the corresponding author upon reasonable request. Custom Fiji and MATLAB scripts used of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committees at Augusta University and Virginia Tech.

AUTHOR CONTRIBUTIONS

MM, SK, and YP conceived the study, performed the experiments, and analyzed the data. SK prepared and characterized recombinant viruses. MM and YP wrote the manuscript, with contributions from SK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncir.2019.00085/full#supplementary-material

Supplementary Figure S1 | Examples of third-order neuron labeling. Red arrowheads indicate third-order neurons labeled in the hindbrain by TRAS. Yellow dash line marks the border of mesencephalon and cerebellum. Scale bar: 50 μm.

Supplementary Figure S2 | Retina injection and tissue clearing. **(A)** Examples of RFP expression in the retina of unpigmented (PTU-treated) animals 3 days after virus injection into the temporal retina. Images are lateral views, with rostral side to the left and dorsal side to the top. Yellow dashed lines outline the eye (outer oval) and lens (inner oval). **(B,C)** Tissue clearing with sRIMS solution. Whole-mount ERK1/2 immunolabeling without **(B)** or with sRIMS clearing **(C)**. Orthogonal views (XY, XZ, and YZ) of confocal image stacks are shown, centered just bellowed the cerebellum (intersect of yellow lines). Ventral structures are not visible without sRIMS clearing. Scale bars are 100 μm.

Supplementary Figure S3 | TRAS labeling of GCaMP6f positive and negative cells. A single confocal imaging plane is shown, with merged, GCaMP6f, GABA, and VSVΔG channels as indicated. Boxed area in **(A)** is shown in higher magnification in **(A')**. The purple arrowheads mark two GCaMP6f+/GABA+/VSVΔG+ inhibitory neurons. The orange arrowhead marks a GCaMP6f–/GABA–/VSVΔG+ cell. Scale bars are 100 μm.

Supplementary Figure S4 | Signals detected via Z-brain in wild-type and *dscaml1-/-* fish. **(A,B)** Heat map of normalized signals in major anatomical regions from Z-brain showing normalized signals detected within each sample used for analysis, wild-type ($n = 24$) and *dscaml1-/-* ($n = 26$), and their corresponding neuron types **(A)** is excitatory while **(B)** is inhibitory. All signals were descended aligning to Diencephalon.

Supplementary Video 1 | Image stack of TRAS-labeled zebrafish larva, 3 days after the initial infection. VSVΔG labeling is shown in magenta and *vglut2a:GFP* labeling in green.

Supplementary Video 2 | Excitatory (green) and inhibitory (magenta) retinorecipient cells in wild type **(Left)** and *dscaml1-/-* **(Right)** cohorts. ERK1/2 immunolabeling (white) from Randlett et al. (2015) is overlaid to serve as an anatomical reference.

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Neural Correlates of Learning Pure Tones or Natural Sounds in the Auditory Cortex

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Associative learning of pure tones is known to cause tonotopic map expansion in the auditory cortex (ACx), but the function this plasticity sub-serves is unclear. We developed an automated training platform called the "Educage," which was used to train mice on a go/no-go auditory discrimination task to their perceptual limits, for difficult discriminations among pure tones or natural sounds. Spiking responses of excitatory and inhibitory parvalbumin (PV$^+$) L2/3 neurons in mouse ACx revealed learning-induced overrepresentation of the learned frequencies, as expected from previous literature. The coordinated plasticity of excitatory and inhibitory neurons supports a role for PV$^+$ neurons in homeostatic maintenance of excitation–inhibition balance within the circuit. Using a novel computational model to study auditory tuning curves, we show that overrepresentation of the learned tones does not necessarily improve discrimination performance of the network to these tones. In a separate set of experiments, we trained mice to discriminate among natural sounds. Perceptual learning of natural sounds induced "sparsening" and decorrelation of the neural response, consequently improving discrimination of these complex sounds. This signature of plasticity in A1 highlights its role in coding natural sounds.

Keywords: auditory, mice, automated training, interneurons, perceptual learning

INTRODUCTION

Learning is accompanied by plastic changes in brain circuits. This plasticity is often viewed as substrate for improving computations that sub-serve learning and behavior. A well-studied example of learning-induced plasticity is following perceptual learning where cortical representations change toward the learned stimuli (Gilbert et al., 2001; Roelfsema and Holtmaat, 2018). Whether such changes improve discrimination has not been causally tested and remains debated, and the mechanisms of change are still largely unknown.

Perceptual learning is an implicit form of lifelong learning during which perceptual performance improves with practice (Gibson, 1969). Extensive psychophysical research on perceptual learning tasks led to a general agreement on some attributes of this type of learning (Hawkey et al., 2004). For example, perceptual learning has been shown to be task specific and poorly generalized to other senses or tasks. It is also largely agreed upon that gradual training is essential for

improvement (Lawrence, 1952; Ramachandran and Braddick, 1973; Ball and Sekuler, 1987; Berardi and Fiorentini, 1987; Karni and Sagi, 1991; Irvine et al., 2000; Wright and Fitzgerald, 2001; Ahissar and Hochstein, 2004; Ericsson, 2006; Kurt and Ehret, 2010). Given the specificity observed at the behavioral level, functional correlates of perceptual learning are thought to involve neural circuits as early as primary sensory regions (Gilbert et al., 2001; Schoups et al., 2001). In auditory learning paradigms, changes are already observed at the level of primary auditory cortex (Weinberger, 2004). Learning to discriminate among tones results in tonotopic map plasticity toward the trained stimulus (Recanzone et al., 1993; Rutkowski and Weinberger, 2005; Polley et al., 2006; Bieszczad and Weinberger, 2010; reviewed in Irvine, 2017). Notably, however, not all studies could replicate the learning-induced changes in the tonotopic map (Brown et al., 2004). Furthermore, artificially induced map plasticity was shown to be unnecessary for better discrimination performances *per se* (Talwar and Gerstein, 2001; Reed et al., 2011). Our understanding of the mechanisms underlying auditory cortex plasticity remains rudimentary, let alone for more natural stimuli beyond pure tones.

To gain understanding of learning-induced plasticity at single neuron resolution, animal models have proven very useful. Mice, for example, offer the advantage of a rich genetic experimental toolkit to study neurons and circuits with high efficiency and specificity (Luo et al., 2018). Historically, the weak aspect of using mice as a model was its limited behavioral repertoire to learn difficult tasks. However, in the past decade, technical difficulties to train mice to their limits have been steadily improving with increasing number of software and hardware tools to probe mouse behavior in high resolution (de Hoz and Nelken, 2014; Egnor and Branson, 2016; Murphy et al., 2016; Aoki et al., 2017; Francis and Kanold, 2017; Krakauer et al., 2017; Cruces-Solis et al., 2018; Erskine et al., 2018). Here, we developed our own experimental system for training groups of mice on an auditory perceptual task—an automatic system called the "Educage." The Educage is a simple affordable system that allows efficient training of several mice simultaneously. Here, we used the system to train mice to discriminate among pure tones or complex sounds.

A1 is well known for its tonotopic map plasticity following simple forms of learning in other animal models (Irvine, 2017). An additional interest in primary auditory cortex is its increasing recognition as a brain region involved in coding complex sounds (Bizley and Cohen, 2013; Kuchibhotla and Bathellier, 2018). We thus asked what are the changes single neurons undergo following training to discriminate pure tones or natural stimuli. We describe distinct changes in the long-term stimulus representations by L2/3 neurons of mice following perceptual learning and assess how these contribute to information processing by local circuits. Using two-photon targeted electrophysiology, we also describe how L2/3 parvalbumin-positive neurons change with respect to their excitatory counterparts. Our work provides a behavioral, physiological, and computational foundation to questions of auditory-driven plasticity in mice, from pure tones to natural sounds.

MATERIALS AND METHODS

Animals

A total of $n = 88$, 10- to 11-week-old female mice were used in this work as follows. Forty-four mice were C57BL/6 mice and 44 mice were a crossbreed of PV-Cre mice and tdTomato reporter mice (PV × Ai9; Hippenmeyer et al., 2005; Madisen et al., 2010). All experiments were approved by the Hebrew University's IACUC.

Behavioral Setup

The "Educage" is a small chamber (10 × 10 × 10 cm), opening on one end into a standard animal home cage where mice can enter and exit freely (**Figure 1A** and **Supplementary Figure 1a**). On the other end, the chamber contains the hardware that drives the system, hardware for identifying mice and measuring behavioral performance. Specifically, at the port entrance there is a coil radio antenna (ANTC40 which connected to LID665 stationary decoder; Dorset) followed by infrared diodes used to identify mice individually and monitor their presence in the port. This port is the only access to water for the mice. Water is delivered *via* a solenoid valve (VDW; SMC) allowing precise control of the water volume provided on each visit. Water is delivered via a water spout, which is also a lickometer (based on 1 microampere current). An additional solenoid valve is positioned next to the water spout in order to deliver a mild air puff as a negative reinforcement, if necessary. For sound stimulation, we positioned a speaker (ES1; TDT), at the center of the top wall of the chamber. Sound was delivered to the speaker at 125 kHz sampling rate *via* a driver and a programmable attenuator (ED1, PA5; TDT). For high-speed data acquisition, reliable control, on-board sound delivery, and future flexibility, the system was designed via a field programmable gate array (FPGA) module and a real-time operating system (RTOS) module (CompactRIO controller; National Instruments). A custom-made A/D converter was connected to the CompactRIO controller, mediated signal from infrared diodes and lickometer and controlled the valves. A custom code was written in Labview to allow an executable user-friendly interface with numerous options for user-input and flexibility for designing custom protocols. All software and hardware design are freely available for download at https://github.com/MizrahiTeam/Educage.

Training Paradigm

Prior to the training, each mouse was implanted, under light and very short period of isoflurane anesthesia, with a radio frequency identification (RFID; Trovan) chip under its scruff. RFID chips allow identification of mice individually, which is then used by the system to control the precise stimulus delivery and track behavioral performance, on a per-mouse basis. Food and water were provided *ad libitum*. While access to water was only in the Educage, mice could engage the water port without restriction. Thus, mice were never deprived of food or water. At the beginning of each experiment, RFID-tagged mice were placed in a large home cage that was connected to the Educage. Before training, we removed the standard water bottle
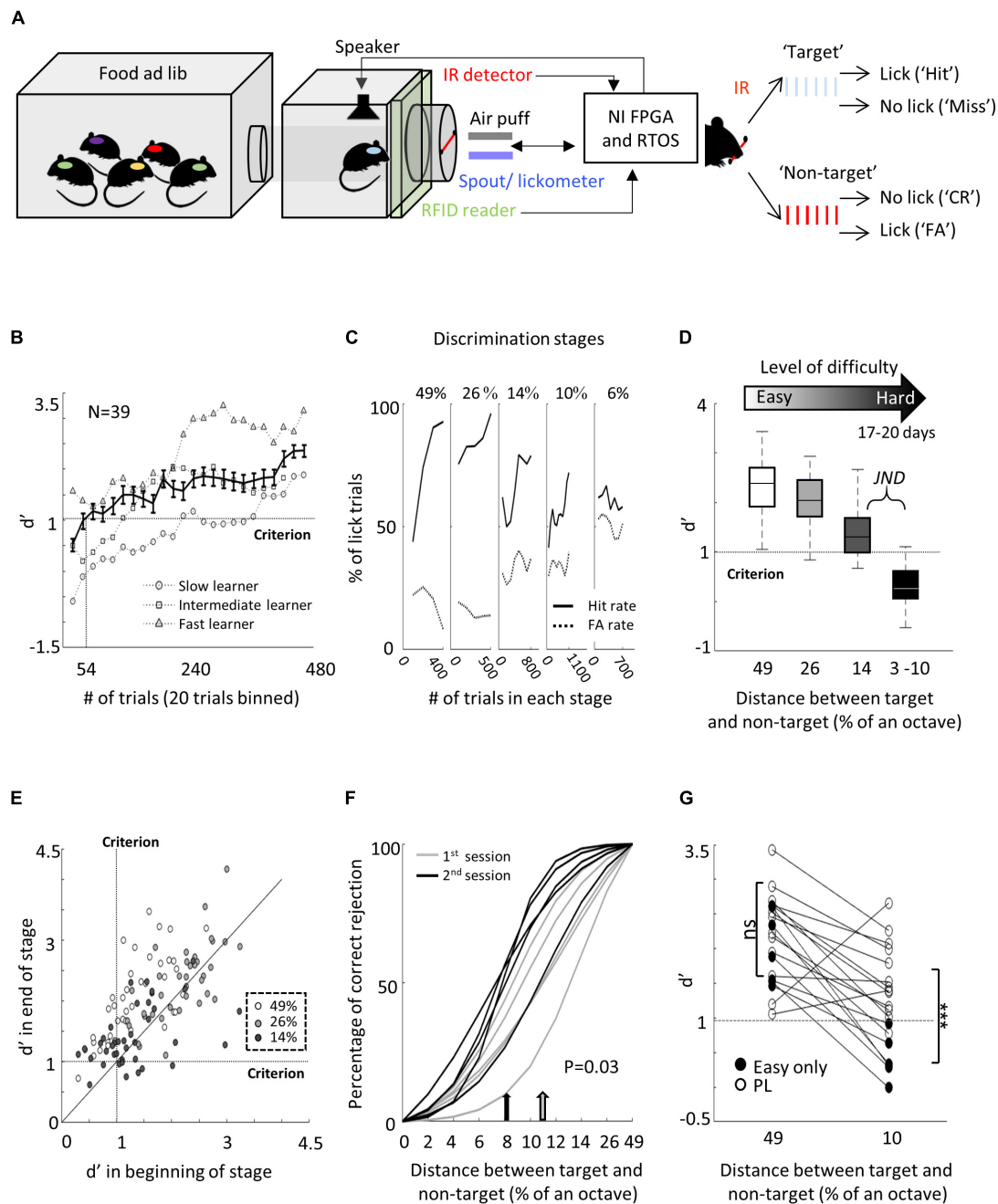
FIGURE 1 | Perceptual learning in the "Educage." **(A)** Left: Schematic design of the "Educage" system and its components. Colored ovals on the back of each mouse represent the different radio frequency identification (RFID) chip implanted prior to the experiment, which allows identification of mice individually. For high-speed data acquisition, system control, on-board sound delivery, and future flexibility, the system was designed via a field programmable gate array (FPGA) module and a real-time operating system (RTOS) module. Right: Schematic representation of the go/no-go auditory discrimination task. CR, correct rejection; FA, false alarm. **(B)** d' values of three representative mice and the population average $\pm$ s.e.m. for the first stage of discrimination. Learning criterion is represented as dashed line ($d' = 1$). **(C)** Lick responses to the target tone (solid line) and non-target tone (dashed line) of one representative mouse along different discrimination stages. Proportions of lick responses were calculated over 100 trials/bin. This mouse improved his discrimination (hit rate went up and FA rate went down) within a stage but his discrimination deteriorated as task became more difficult. **(D)** Population average d' values for the different discrimination stages. $N = 39$ mice (mean $\pm$ s.e.m.). Shades denote the level of difficulty (from 49% to 4–10% octave). **(E)** Individual d' values at the end of each level as a function of d' in the beginning of that level. Shades denote the level of difficulty. Learning criterion represented as dashed lines ($d' = 1$). **(F)** Normalized psychometric curves of five mice calculated from the first (light curves) and the second (dark curves) 14%/octave session. Light and dark arrows indicate average decision boundaries in the first and second sessions respectively (Mann–Whitney U-test on criteria: $p = 0.03$). **(G)** d' values in easy (49%/octave) and more difficult (10% /octave) discrimination stages of individual mice from the "Easy only" group (filled circles) and from the perceptual learning group (blank circles). d' values are significantly different between groups only for the hard discrimination level (Mann–Whitney U test: ***$p < 0.001$).

from the home cage. Mice were free to explore the Educage and drink the water at the behavioral port. Every time a mouse entered the behavioral port, it was identified individually by the antenna and the infrared beam and a trial was initiated. Before learning, any entry to the port immediately resulted in a drop of water, but no sound was played. Following 24 h of exploration and drinking, mice were introduced for the first time to the "target" stimulus—a series of six 10-kHz pure tones (100 ms duration, 300 ms interval; 3 ms on and off linear ramps; 62 dB SPL) played every time the mouse crossed the IR beam. To be rewarded with water, mice were now required to lick the spout during the 900-ms response window. This stage of operant conditioning lasted for 2–3 days until mice performed at >70% hit rates.

We then switched the system to the first level of discrimination when mice learned to identify a non-target stimulus (a 7.1 kHz pure tone series at 62 dB SPL) from the already known target stimulus (i.e., tones separated by 49%/octave). Thus, on each trial, one of two possible stimuli were played for 2.1 s—either a target tone or a non-target tone. Mice were free to lick the water spout anytime, but for the purpose of evaluating mouse decisions, we defined a response window and counted the licking responses only within 900 ms after the sound terminated. Target tones were played at 70% probability and non-target tones were played at 30% probability, in a pseudorandom sequence. A "lick" response to a target was rewarded with a water drop (15 μl) and considered as a "Hit" trial. A "no lick" response to a target sound was considered as a "Miss" trial. A lick response to the non-target was considered a "False Alarm" (FA) trial, which was negatively reinforced by a mild air puff (60 PSI; 600 ms) followed by a 9-s "timeout." A "no lick" response to the non-target was considered a correct rejection (CR) and was not rewarded.

Once mice learned the easy discrimination (reached 70% correct ratio), we switched the system to the second discrimination stage. Here, we increased task difficulty by changing the non-target tone to 8.333 kHz, thus decreasing the inter tone distance to 26%/octave. In each training stage, only one pair of stimuli was presented. Then, at the following stage, the inter tone distance was further decreased to 14%/octave and then down to 6%/octave. This last transition was often done in a gradual manner (»12%/octave » 10%/octave » 8%/octave » 6%/octave). In some of the animals ($n = 25$), we trained mice to their just noticeable difference (JND) and then changed the task back to an easier level. In order to extract psychometric curves, for some of the mice ($n = 5$), we played "catch trials" during the first and second sessions of the 14%/octave discrimination stages. In catch trials, different tones spanning the frequency range of the whole training (7–10 kHz) were presented to the animals in low probability (6% of the total number of sounds), and were neither negatively nor positively reinforced. We have recorded from 21 mice that underwent the behavioral training. Their discriminability indexes in the 49, 26, and 14% octave discrimination levels were: 2.4 ± 0.8, 1.9 ± 0.6, and 1.4 ± 0.5, respectively.

For the vocalizations task, we used playback of pups' wriggling calls (WC) as the target stimulus. These vocalizations were recorded with a one-quarter-inch microphone (Brüel and Kjær) from P4–P5 PV × Ai9 pups ($n = 3$), sampled at 500 kHz, and identified offline (Digidata 1322A; Molecular Devices). As the non-target stimulus, we used manipulations of the WC. During the first stage of the operant learning, mice learned to discriminate between WC and a fully reversed version of this call. Then, the second manipulation on the non-target stimulus was a gradual change of the frequency modulation (FM) of all but the last syllable in the call while leaving the temporal structure of the full call intact. To manipulate the syllable FM, we used a dynamic linear FM ramp. This operation multiplies each sampling interval within the syllable by a dynamic speeding factor, which changed according to the relative distance from the start and end of the syllable, and generated a new waveform by interpolation from the original waveform. For example, for a 0.6 speeding factor, the beginning of each syllable was slower by a factor of 0.4 while the end of each syllable accelerated by a factor of 0.4. The range of sound modulation used here was 0.66–0.9. A value of 0.66 is away from the WC; 0.9, similar to the WC; and 1, exactly the same as the WC. The basic task design for the non-target sound was as follows: Reverse » 0.66 » 0.81 » 0.9.

Surgical Procedure

Mice were anesthetized with an intraperitoneal injection of ketamine and medetomidine (0.80 and 0.65 mg/kg, respectively) and a subcutaneous injection of Carprofen (0.004 mg/g). Additionally, dextrose–saline was injected to prevent dehydration. Experiments lasted up to 8 h. The depth of anesthesia was assessed by monitoring the pinch withdrawal reflex, and ketamine/medetomidine was added to maintain it. The animal's rectal temperature was monitored continuously and maintained at 36 ± 1°C. For imaging and recording, a custom-made metal pin was glued to the skull using dental cement and connected to a custom stage to allow precise positioning of the head relative to the speaker (facing the right ear). The muscle overlying the left auditory cortex was removed, and a craniotomy (∼2 × 2 mm) was performed over A1 (coordinates, 2.3 mm posterior and 4.2 mm lateral to bregma) as described previously (Stiebler et al., 1997; Cohen et al., 2011; Maor et al., 2016).

Imaging and Electrophysiology

Cell-attached recordings were obtained using targeted patch-clamp recording by a previously described procedure (Margrie et al., 2003; Cohen and Mizrahi, 2015; Maor et al., 2016). For visualization, the electrode was filled with a green fluorescent dye (Alexa Flour-488; 50 μM). Imaging of A1 was performed using an Ultima two-photon microscope from Prairie Technologies equipped with a 16 × water-immersion objective lens (0.8 numerical aperture; CF175; Nikon). Two-photon excitation at wavelength of 930 nm was used in order to visualize both the electrode, filled with Alexa Flour-488, and PV$^+$ somata, labeled with tdTomato (DeepSee femtosecond laser; Spectraphysics). The recording depths of cell somata were restricted to subpial depths of 180–420 μm, documented by the multiphoton imaging. Spike waveform analysis was performed on all recorded cells, verifying that tdTomato+ cells in L2/3 had faster/narrower

spikes relative to tdTomato-negative (tdTomato-) cells (see also Cohen and Mizrahi, 2015).

Auditory Stimuli

The auditory protocol comprised 18–24 pure tones (100 ms duration, 3 ms on and off linear ramps) logarithmically spaced between 3 and 40 kHz and presented at four sound pressure levels (72–42 dB SPLs). Each stimulus/intensity combination was presented 10–12 times at a rate of 1.4 Hz. The vocalizations protocol comprised the playback of pups' wriggling calls (WC) and three additional FM calls, presented at 62 dB SPL for 16 repetitions.

Behavioral Data Analysis

To evaluate behavioral performance, we calculated, for different time bins (normally 20 trials), Hit and FA rates, which are the probability to lick in response to the target and non-target tones, respectively. In order to compensate for the individual bias, we used a measure of discriminability from signal detection theory—d-prime (d'). d' is defined as the difference between the normal inverse cumulative distribution of the "Hit" and FA rates, $d' = z(\text{hit}) - z(\text{FA})$ (Nevin, 1969). d' for each discrimination stage was calculated based on trials from the last 33% of the indicated stage. Psychometric curves were extracted based on mouse performance in response to the catch trials. By fitting a sigmoidal function to these curves, we calculated decision boundaries as the inflection point of each curve. Detection time was calculated for each mouse individually, by determining the time in which lick patterns in the correct reject *vs.* the hit trials diverged (i.e., the time when significance levels crossed $p < 0.001$ in a two-sample t-test).

Data Analysis—Electrophysiology

Data analysis and statistics were performed using custom-written code in MATLAB (MathWorks). Spikes were extracted from raw voltage traces by thresholding. Spike times were then assigned to the local peaks of suprathreshold segments and rounded to the nearest millisecond. For each cell, we obtained peri-stimulus time histogram (PSTH) and determined the response window as the 100 ms following stimulus onset that evoked the maximal response integral. Only neurons that had tone-evoked response ($p < 0.05$; two sample t-test) were included in our dataset. Based on this response window, we extracted the cell's tuning curve and frequency-response area (FRA). Evoked firing rate was calculated as the average response to all frequencies that evoked a significant response. Firing rate in the training band was calculated as the response to frequencies inside the training band (7–10 kHz), averaged across all intensities. Best frequency (BF) of each cell was determined as the tone frequency that elicited the strongest responses averaged across all intensities. The selectivity of the cell is the % of all frequency–intensity combinations that evoked significant response (determined by a two-sample t-test followed by Bonferroni correction). Pairwise signal correlations (r_{sc}) were calculated as Pearson correlation between FRA's matrices of neighboring cells (<250 μm apart; Maor et al., 2016). The spontaneous firing rate of the cell was calculated based on the 100 ms preceding each stimulus presentation. Response latency

is the time point after stimulus onset at which the average spike count reached maximum.

Statistical Model Based on the Independent Basis Functions (IBF) Method

Since the measured responses before and after learning are not from the same cells, we cannot estimate the changes of individual tuning curves due to learning. Instead, we must rely on estimated learning-induced changes in the *ensemble* of single-neuron responses. Our goal, therefore, was to build a statistical model of single-neuron tuning curves before and after learning. The models were based on the statistics of each experimental group separately and used to estimate the learning-induced changes in the population of responses in each condition. Furthermore, we used this model as a generative model that allowed us to generate a large number of "model neurons" with statistically similar response properties as the measured ones.

In principle, one could use a parametric model, by fitting each observed tuning curve to a specific shape of functions (e.g., Gaussian tuning curves). However, since the tuning curves of neurons to tone frequencies do not have symmetric "Gaussian" shapes, and some are bimodal, fitting them to a parametric model has not been successful. Instead, we chose to model each single neuron response as a weighted sum of a small set of orthogonal basis functions.

$$r_i(f) = \sum_{l=1}^{K} a_l^i g_l(f) \qquad (1)$$

Here, $r_i(f)$ is the firing rate (i.e., the trial-averaged spike count) of the ith neuron in response to the stimulus with frequency f; K is the number of orthogonal basis functions denoted by $g_l(f)$ (dependencies in f are in log scale). In order to determine the basis functions and the coefficients, a_l^i, we performed singular value decomposition (SVD) of the matrix of the measured neuronal firing rates for the 18 values of f. Our model (1) uses a subset of the K modes with the largest singular values (the determination of K is described below). The SVD yields the coefficients $a_{l,\ data}^i$ for the N *observed* neurons and (2) smoothes the resultant SVD f-dependent vectors using a simple "moving average" technique to generate the basis functions, $g_l(f)$. (3) Importantly, to use the SVD as a generative model, for each l, we compute the histogram of the N $a_{l,\ data}^i$ coefficients. To generate "new neurons," we sample each coefficient independently from the corresponding histogram. In other words, we approximate the joint distribution of the coefficients by a factorized distribution. This allowed us to explore the effect of changing the number of neurons that downstream decoders use in order to perform the perceptual task.

Model (1) describes the variability of the population responses to the stimulus, in terms of tuning curves of the trial averages firing rates. Additional variability in the data is the single trial spike count. We model these as independent Poisson random variables with means given by $r_i(f)$. Since neurons are not simultaneously recorded, we do not include noise correlations in the model. We performed this procedure for the *naïve*

and *expert* measured responses separately, so that both the basis functions and the coefficient histograms are evaluated for the two conditions separately. Note that we do not make Gaussian assumptions about the coefficient histograms. In fact, the observed histograms are in general far from Gaussian.

The Choice of Number of Basis Functions

Due to a limited number of trials that we sampled for each neuron, taking a large value of K can result in overfitting the model to the noise caused by the finite number of trials. To estimate the optimal number of basis functions, we evaluated the percentage of response firing rate variability of the population (i.e., the fraction of the sum of the squared SVD eigenvalues) as a function of K. We also evaluated the parameters of model (1) based on a subset of trials and checked how well it accounts for the observed tuning curves that are calculated from the test trials. We took K that produces the smallest test error and saturated the fractional variance.

We used model (1) with the above choice of K in order to evaluate the discrimination ability of the population of A1 neurons, by creating *an ensemble* of single-neuron responses for the naïve and expert conditions. To generate the model neurons, we sampled the coefficients of the basis function independently from the corresponding histogram of the measured neurons and used these neurons for the calculations depicted below.

Fisher Information

We calculated the Fisher Information (FI) for each condition (naïve vs. expert) using our model (1). FI measure bounds the mean squared error of an (unbiased) estimator of the stimulus from the noisy single trial neuronal responses. When the neuronal population is large (and they are noise- independent) FI also determines the discriminability d' of a maximum likelihood discriminator between two nearby values of the stimulus (Seung and Sompolinsky, 1993). Under the above Poisson assumption, the FI for the ith neuron is equal to $I_i = \frac{r_i'(f)^2}{r_i(f)}$, where $r_i'(f)$ is the derivative of the firing rate with respect to the stimulus value f. The total FI is the sum of the FIs of individual neurons (Seung and Sompolinsky, 1993). This has been evaluated in both naïve and expert conditions. Note that the FI are functions of the stimulus value f, around which the discrimination task is performed.

Discrimination by Linear Readout

We applied a linear decoder to assess the ability to discriminate between nearby stimuli on the basis of the neuronal population responses. We trained a support vector machine (SVM) with a linear kernel, which finds an "optimal" linear classifier that discriminates between two nearby frequencies on the basis of single-trial vectors of spike counts generated with our generative model (1) and Poisson variability. We then evaluated the probability of classification errors to test trials, in both naïve and expert conditions. Since our training set is not linearly separable, we used SVM with slack variables (Vapnik, 1998), which incorporates a "soft" cost for classification errors. Each classification was iterated a maximum 500 times (or until

converged). In each iteration, 16 trials were used for training the classifier and four trials were used to test the decoder accuracy. Classification performance of the decoder was tested separately for discrimination between nearby frequencies which lay inside the training band or near the training band (0.4396 octave apart).

Data Analysis—Vocalization Responses

Similarity of response to different vocalizations was calculated as Pearson correlation between the PSTHs of the different stimuli. To quantify lifetime sparseness, we used the following measure: $S = (1 - [(\Sigma r_i/n)2/\Sigma (r_i2/n)])/[1 - (1/n)]$, where r_i is the response to the ith syllable in the original vocalization (averaged across trials) and n is the number of syllables. Values of S near 0% indicate a dense code, and values near 100% indicate a sparse code (Vinje and Gallant, 2000). Population sparseness was calculated as 100—the percent of cells that evoked a significant response to each syllable in the call (Willmore and Tolhurst, 2001). Classification of vocalization identity based on population activity was determined using the SVM decoder with a linear kernel and slack variables. The decoder was tested for its accuracy to differentiate between responses to two different vocalizations. The input to the SVM consisted of the spike count of each neuron in the syllable response window. The same number of neurons (37) was used in both groups to avoid biases. We then evaluated the probability of classification errors to test trials, using leave-one-out cross-validation. Each classification was iterated 1000 times. In each iteration, 15 trials were used for training the classifier and one trial was used to test decoder accuracy. The number of syllables utilized in the decoder was increased cumulatively.

Statistical Analysis

All statistical analysis was performed with MATLAB (Mathworks). Rank-sum test was used for comparison unless otherwise noted. In cases where the same data sample was used for multiple comparisons, we used the Holm-Bonferroni correction to adjust for the increased probability of Type I error. Statistical significance was defined as $p < 0.05$.
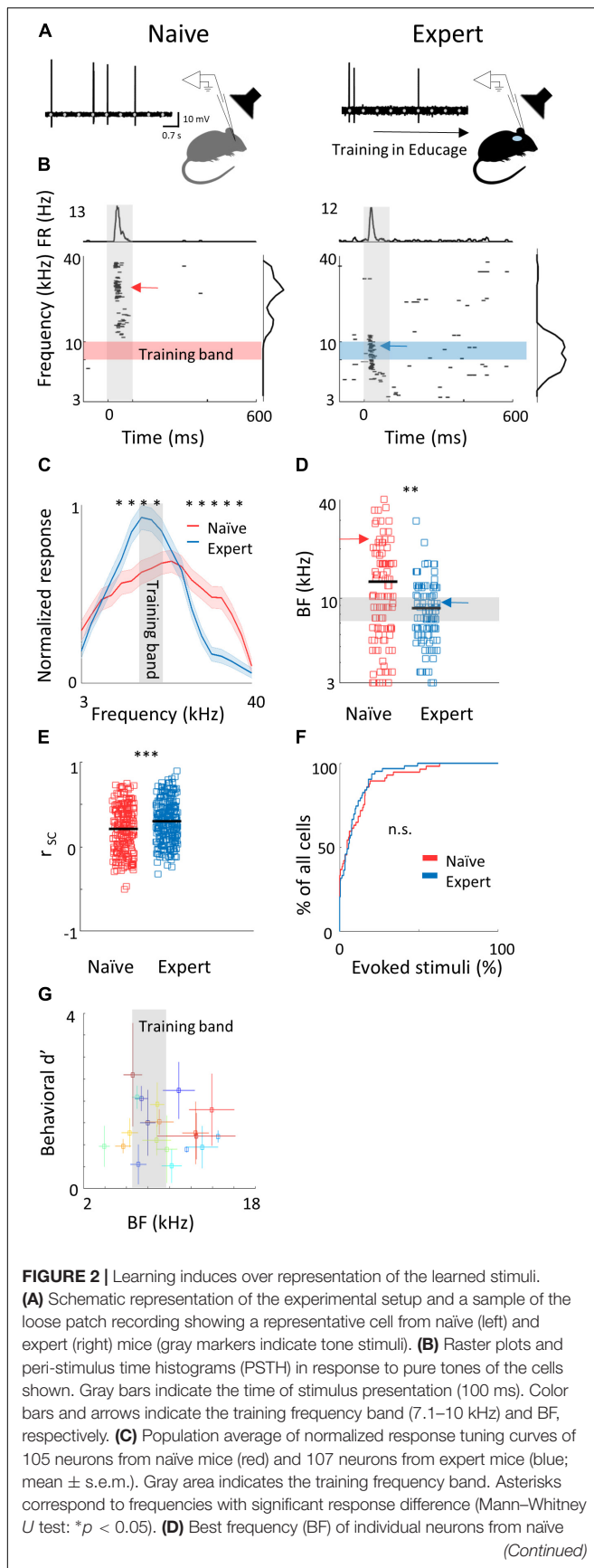
Code Accessibility

All software and hardware design for the Educage system are available for download at https://github.com/MizrahiTeam/Educage.

Codes used for data analysis are available from the corresponding author upon request.

RESULTS

Behavior—Discrimination of Pure Tones

To study perceptual learning in mice, we developed a behavioral platform named the "Educage" (all software and hardware design are freely available for download at https://github.com/MizrahiTeam/Educage). The Educage is an automated home-cage training apparatus designed to be used simultaneously with several mice (up to 8 animals). One advantage of the Educage over other procedures is that human interference is brought

FIGURE 2 | Learning induces over representation of the learned stimuli.
(A) Schematic representation of the experimental setup and a sample of the
loose patch recording showing a representative cell from naïve (left) and
expert (right) mice (gray markers indicate tone stimuli). **(B)** Raster plots and
peri-stimulus time histograms (PSTH) in response to pure tones of the cells
shown. Gray bars indicate the time of stimulus presentation (100 ms). Color
bars and arrows indicate the training frequency band (7.1–10 kHz) and BF,
respectively. **(C)** Population average of normalized response tuning curves of
105 neurons from naïve mice (red) and 107 neurons from expert mice (blue;
mean $\pm$ s.e.m.). Gray area indicates the training frequency band. Asterisks
correspond to frequencies with significant response difference (Mann–Whitney
U test: *$p < 0.05$). **(D)** Best frequency (BF) of individual neurons from naïve
(Continued)

to minimum and training efficiency increases. The "Educage"
is a small modular chamber ($10 \times 10 \times 10$ cm), opening
on one end into a standard animal home cage where mice
can enter and exit freely (**Figure 1A**). On its other end, the
chamber contains the hardware that drives the system, hardware
for identifying mice and measuring behavioral performance
(**Figure 1A** and **Supplementary Figure 1**, see section "Materials
and Methods"). Mice were free to engage the behavioral port
at their own will, where they consume all of their water intake.
Following habituation, mice were trained on a go/no-go auditory
discrimination task to lick in response to a target tone (a series
of 10 kHz pure tones) and withhold licking in response to the
non-target tone. A "lick" response to a target was rewarded with
a water drop (15 μl) and considered as a "Hit" trial. A "no
lick" response to a target sound was considered as a "Miss"
trial. A lick response to the non-target was considered a "False
Alarm" (FA) trial, which was negatively reinforced by a mild air
puff (60 PSI; 600 ms) followed by a 9-s "timeout." A "no lick"
response to the non-target was considered a correct rejection
(CR) and was not rewarded (**Figure 1A**). On average, mice
performed 327 ± 71 trials per day, mainly during dark hours
(**Supplementary Figure 1b**).

The initial level of learning was to identify a non-target
stimulus (a series of 7.1 kHz pure tones) from the 10 kHz target
stimulus (**Figure 1A** right and **Supplementary Movie 1**). These
stimuli are separated by 49% of an octave and are perceptually
easily separated by mice. Discrimination performances were
evaluated using d'—a measure that is invariant to individual
bias (Nevin, 1969). Despite the simplicity of the task, behavioral
performance varied widely between mice (**Figure 1B** and
Supplementary Figure 1c). On average, it took mice 54 ± 38
trials to cross our criterion of learning, which was set arbitrarily
at $d' = 1$ (**Figure 1B**; dotted line), and gradually increased
to plateau at $d' = 2.35 \pm 0.64$ (**Figure 1D**). To extend the
task to more challenging levels, we gradually increased task
difficulty by changing the non-target tone closer to the target
tone. The target tone remained constant at 10 kHz throughout
the experiment and only the non-target stimulus changed.
The lowest distance used between target and non-target was
3%/octave (9.6 kHz vs. 10 kHz). A representative example from
one mouse's performance in the Educage throughout a complete
experiment is shown in **Figure 1C**. The JND for each mouse was
determined when mice could no longer discriminate (e.g., the

JND of the mouse shown in **Figure 1C** was determined between 6 and 10%/octave). The range of JNDs was 3–14%/octave and averaged 8.6 ± 4.7%/octave. These values of JND are typical for frequency discrimination in mice (Ehret, 1975; de Hoz and Nelken, 2014). Most mice improved their performance with training (**Figures 1E,F**), showing improved perceptual abilities along the task. The duration to reach JND varied as well, ranging 3069 ± 1099 trials (14 ± 3 days). Detection times, defined as the time in which lick patterns in the correct reject trials diverged from the lick patterns of the hit trials, increased monotonically by ∼177 ms for each step of task difficulty, demonstrating the increased perceptual load during the harder tasks (**Supplementary Figure 1d**).

To show that gradual training is necessary for perceptual learning (Ahissar and Hochstein, 2004; Ericsson, 2006; Kurt and Ehret, 2010), we trained groups of littermate mice on different protocols simultaneously. In one group of mice, we used a standard protocol and the animals were trained on the gradually increasing task difficulty described above. Simultaneously, in the second group of mice—termed "easy only"—animals were trained continuously on the easy task. Although both groups of mice trained together, only the mice that underwent gradual training were able to perform the hard task (**Figure 1G**). Taking together, these data demonstrate the efficiency of the Educage to train groups of mice to become experts in discriminating between a narrowband of frequencies in a relatively short time and with minimal human intervention.

Representation of Pure Tones in L2/3 Neurons Following Perceptual Learning

To evaluate cortical plasticity following perceptual learning, we compared how pure tones are represented in A1 of expert mice, trained to discriminate between narrowband frequencies, and age-matched naive mice who were never introduced to these sounds. We used *in vivo* loose patch recording of L2/3 neurons in anesthetized mice to record tone-evoked spiking activity in response to 3–40 kHz pure tones (**Figures 2A,B** and **Table 1**). We targeted our recording electrode to the center of A1 based on previously validated stereotactic coordinates (Maor et al., 2016). Response latencies further support that our recordings are from primary auditory cortex (range of minimal latencies: 20–44 ms; mean ± sd: 30 ± 6 ms). Loose patch recording enables superb spatial resolution, is not biased to specific cell types, and has high signal-to-noise ratio for spike detection. However, one caveat of this technique is a potential bias of recording sites along the tonotopic axis. In order to overcome it, we measured from neurons in a large number of animals, such that possible biases are likely averaged out. In naïve mice, responses were highly heterogeneous, with best frequencies covering a significant frequency range, as expected from the heterogeneous functional microarchitecture of L 2/3 neurons in A1 (**Figures 2C,D**; $n = 105$ neurons, $n = 22$ mice, red). In expert mice, best frequencies of tuning curves were biased toward the frequencies that were presented during learning (**Figures 2C,D**; $n = 107$ neurons, $n = 20$ mice, blue). These data show, as expected from previous literature, that learned frequencies in A1 become overrepresented at least as measured by the neuron's best frequency (BF). We next showed that this overrepresentation was specific to the learned tone by training mice on 4 kHz as the target tone and recording neurons in a similar manner to the abovementioned experiment. Indeed, L2/3 neurons in A1 of mice training on 4 kHz showed BF shifts toward 4 kHz (**Supplementary Figure 2a**). These results are largely consistent with previous studies in monkeys, cats, rats, and gerbils (reviewed in Irvine, 2017), extending this phenomenon of learning-induced changes in tuning curves to the mouse, to L2/3 neurons and to local circuits.

To study neuronal changes further, we analyzed response dynamics. Temporal responses to the trained frequencies were only slightly different between naive and expert mice. Specifically, average spiking responses were slightly but significantly faster and stronger in experts (**Supplementary Figure 2b** and **Table 1**). In addition, we recorded the responses to pure tones at different intensities and constructed frequency response areas (**Supplementary Figure 2c**). The average pairwise signal correlation of neighboring neurons, calculated from these frequency response areas, was high in naïve mice (0.2 ± 0.28) but even higher in experts (0.3 ± 0.24; **Figure 2E**). Notably, the increased signal correlation was not an artifact of differences in response properties between naïve and expert group (**Supplementary Figure 2d**) but reflected true similarity in receptive fields (**Supplementary Figure 2e**). Thus, the basal level of functional heterogeneity in A1 (Bandyopadhyay et al., 2010; Rothschild et al., 2010; Maor et al., 2016) is reduced following learning. This learning-induced increase in functional homogeneity of the local circuit, emphasizes the kind of shift that local circuits undergo. Since neurons in expert mice did not have wider response areas (**Figure 2F** and **Table 1**), our data suggests that neurons shifted their response properties toward the learned tones at the expense of frequencies outside the training band.

TABLE 1 | Learning-induced physiological changes.

Group	Animals (n)	Cells (n)	BF (kHz)	Spontaneous spike rate (Hz)	Evoked firing rate in BF (Hz)	FR in T.B. (Hz)	Response latency (ms)	Selectivity (% of all stimuli)	"Lifetime sparseness" (%)
Naïve	22	105	11.3 ± 8.4	0.45 ± 0.58	15.5 ± 5.3	3.4 ± 5	35.2 ± 8.7	9.7 ± 13.9	88 ± 1
Expert	20	107	8.6 ± 4	0.59 ± 0.65	13.2 ± 3.4	4.2 ± 4	32.6 ± 9.7	8.5 ± 9.9	88 ± 1
Rank-sum test			0.004	0.06	0.01	0.02	0.007	0.58	0.35

A summary table of the complete dataset of recordings from excitatory neurons in naïve and expert mice after perceptual learning of pure tones. Columns show different parameters of the dataset or property tested. The third row shows the statistical p-value between naïve and experts using a Mann–Whitney U test. T.B., Training Band.

Finally, the representation of sounds by the neuronal population was not clearly related to behavioral performance of individual mice (**Figure 2G**), raising the possibility that changes in BF are not necessarily related to better performance. Alternatively, the full breadth of the tuning curve is not faithfully reflected in the BF alone and a more detailed analysis of the tuning curves is necessary.

A Generative Model of A1 Population Responses to Pure Tones

To what extent does overrepresentation of a learned stimulus sub-serve better discrimination by the neural population? To answer this question, we built a statistical model of tuning curves of neurons in A1 using six basis functions (**Supplementary Figures 3a–f**) that correspond to the six largest SVD vectors of the population responses (IBF method; see section Materials and Methods). In **Figure 3A**, we show two representative examples of tuning curves and their reconstruction by our model. In contrast to Gaussian fits used previously (Vapnik, 1998; Briguglio et al., 2018), our model captures the salient features of the shape of the auditory tuning curves (asymmetry, multimodality), yet also smoothed the *raw* response vectors to reduce overfitting due to finite sampling.

In order to choose the appropriate number of basis functions, we determined the minimal number of basis functions that achieves good performance in reconstructing test single trial responses. In **Figure 3B**, we show the fraction of explained variance as a function of the number of basis functions, K. For both the naïve and expert groups, the explained variance reaches above 96% after five basis functions. **Figure 3C** shows the mean square error (MSE) on the unseen trials as a function of K in data from both the naïve and expert animals. The MSE exhibits a broad minimum for K in a range between 6 and 13. Interestingly, both naïve and experts achieve roughly the same MSE values, although for experts, the MSE values at both low and large values of K are considerably larger than that of the naïve. Taken together, we conclude that for these conditions, six basis functions are the appropriate number and we used this value for our calculations.

Based on the IBF method described above, we generated a population of tuning curves (500 "new neurons") and estimated their total FI (see section Materials and Methods). **Figure 3D** shows the FI as a function of the stimulus f for both the naïve and expert conditions. Surprisingly, the FI of the neurons from expert animals was enhanced relative to the naïve group, but only for stimuli at both flanks of the training band. Importantly, the FI within the band of the trained frequencies remained unchanged (**Figure 3D**, within the black lines). The same result holds true for a performance of a SVM classifier. Using SVM to separate any two frequencies that are 0.2198 octave apart, discriminability (d') values derived from the classifier's error show similar results to the FI (**Figure 3E**; Seung and Sompolinsky, 1993). The value of d' is larger in the expert groups as compared to the naïve but only outside the training band, whereas within the band, discriminability is not improved (or even slightly compromised).

The results shown in **Figure 3** do not change qualitatively if we use our SVD model for the recorded neurons, as opposed to newly modeled neurons, nor if we compute discriminability index directly from the neuronal firing rates (**Supplementary Figure 3g**). One advantage of having a generative model for the population responses is that we can generate an unlimited number of trials and tuning curves. We took advantage of this to explore whether the results of the FI and SVM change with population size. To answer this question, we evaluated the mean discrimination performance (over test neurons) as a function of the number of sampled cells, N, which increases as expected. Consistent with the results of the SVM, the performances in the naïve and expert groups are similar with slight tendency for a higher accuracy in the naive population at large Ns (**Figure 3F**). In contrast, for frequencies near the training band, the accuracy is substantially larger in the expert than in the naïve group for virtually all N (**Figure 3G**). Thus, it seems that learning-induced changes in tuning curves do not improve discriminability of the learned stimuli.

Perceptual Learning of Natural Sounds

Natural sounds are characterized by rich spectro-temporal structures with frequency and amplitude modulations over time (Mizrahi et al., 2014). Discrimination of such complex stimuli could be different from that of pure tones. Thus, we next designed a task similar to that with the pure tones but using mouse vocalizations as the training stimuli. We used playback of pups' wriggling calls (WC) as the target stimulus (**Figure 4A**, top). As the non-target stimuli, we used frequency modulations of the WC, a manipulation that allowed us to morph one stimulus to another by a continuous metric (**Figure 4A**). The range of sound modulation used here was indexed as a "speeding factor" (see section "Materials and Methods" for details). In short, a modulation factor of 0.66 affected the original WC more than a modulation factor of 0.9 did, and is therefore easier to discriminate (**Figure 4B**). To reach perceptual limits, we trained mice gradually, starting with an easy version of the task (WC vs. a temporally reversed version of the WC) and then gradually to modulated calls starting at 0.66 modulation. Once mice reached > 80% hit rates, we changed the non-target stimulus to more difficult stimuli until mice could no longer discriminate (**Figure 4C**). Mice ($n = 9$) learned the easy task, i.e., discriminating WC from a 0.66 modulated call, with average d' values of 2.5 ± 0.4 (**Figure 4D**). On average, mice could only barely discriminate between a WC and its 0.9 modulation (d' at 0.9 was 1 ± 0.8; **Figure 4D**). While these discrimination values were comparable to the performance of pure tones, detection times were substantially slower (**Supplementary Figure 4a**). For similar d' values, discriminating between the vocalizations took 300–1000 ms longer as compared to the pure tone tasks (**Figure 4E**). In addition, learning curves were slower for the vocalization task as compared to the pure tones task. The average number of trials to reach $d' = 1$ for vocalizations was 195 trials, more than three times longer as compared with pure tones (compare **Supplementary Figure 4b** and **Figure 1B**, respectively). These differences may arise from the difference in the delay, inter-syllable interval, and temporal modulation of each stimulus type, which we did not further explore. Taken together, these behavioral results demonstrate a
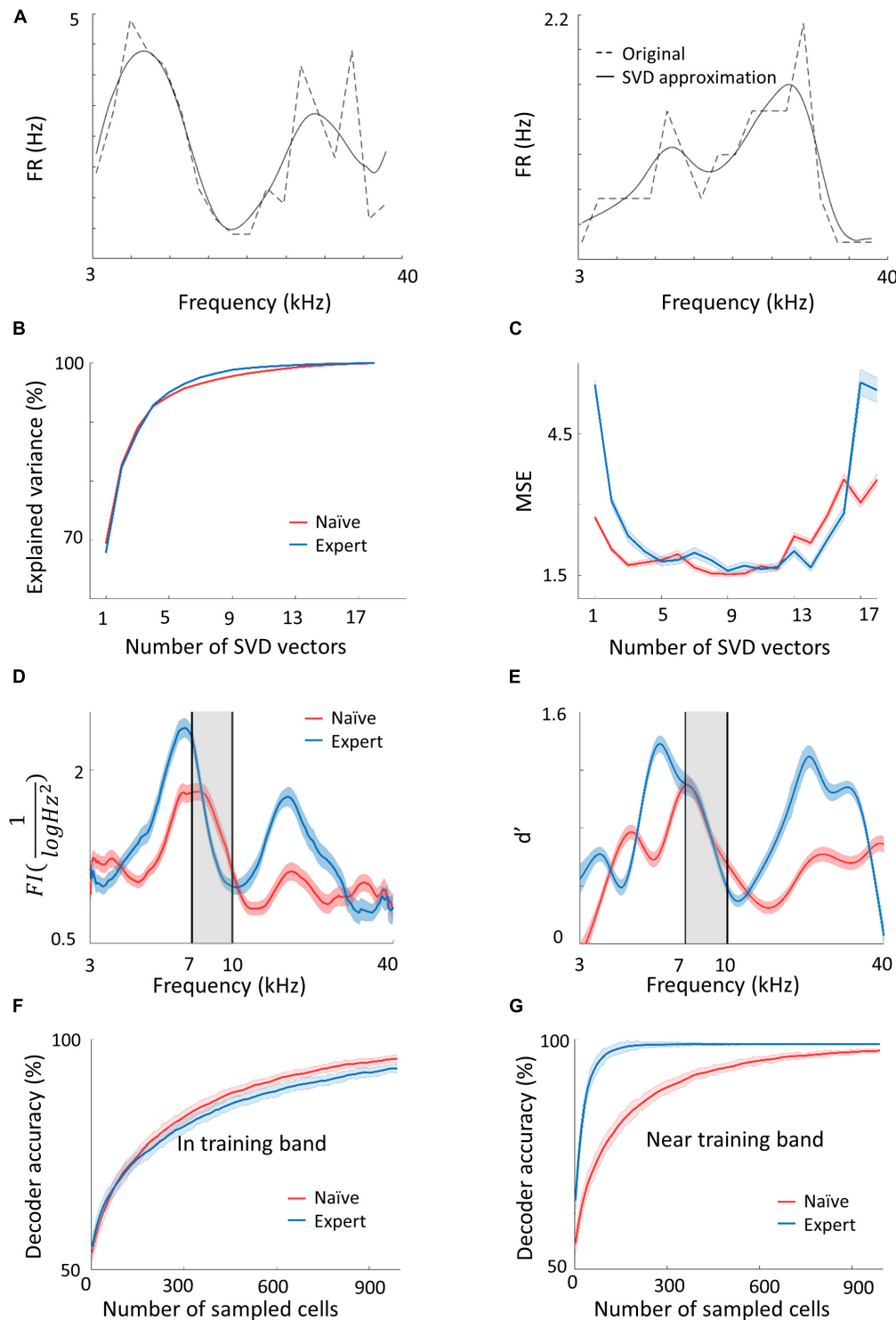
FIGURE 3 | Plasticity in A1 does not improve the discrimination of the learned tones. **(A)** Two representative examples of tuning curves of neurons recorded in A1. Average spike rates and the SVD approximation of the particular curves are shown in dashed and solid lines respectively. Note that although the SVD approximation is smooth, it captures the irregular dynamics (i.e., non-Gaussian) of the tuning curves. **(B,C)** The explained variance and error as a measure of the number of SVD vectors used in the model. **(D)** Fisher Information calculated from the tuning curves of both populations along the frequency dimension. Note the increased FI for the expert neurons in the flanks of the training band but not within it (gray band). **(E)** Discriminability (*d'*) of SVM decoder along the range of frequencies. Pairwise comparison along the continuum is performed for frequencies 0.2198 octave apart. In accordance with *d'*, the decoder does not perform better in the training band (gray shade). **(F,G)** Classification performance of the decoder as a function of the number of neurons in the model. In the training band **(F)**, the performance is similar for both naïve and expert mice. Outside the training band (0.4396 octave apart; **(G)** performance improved rapidly in the expert mice.
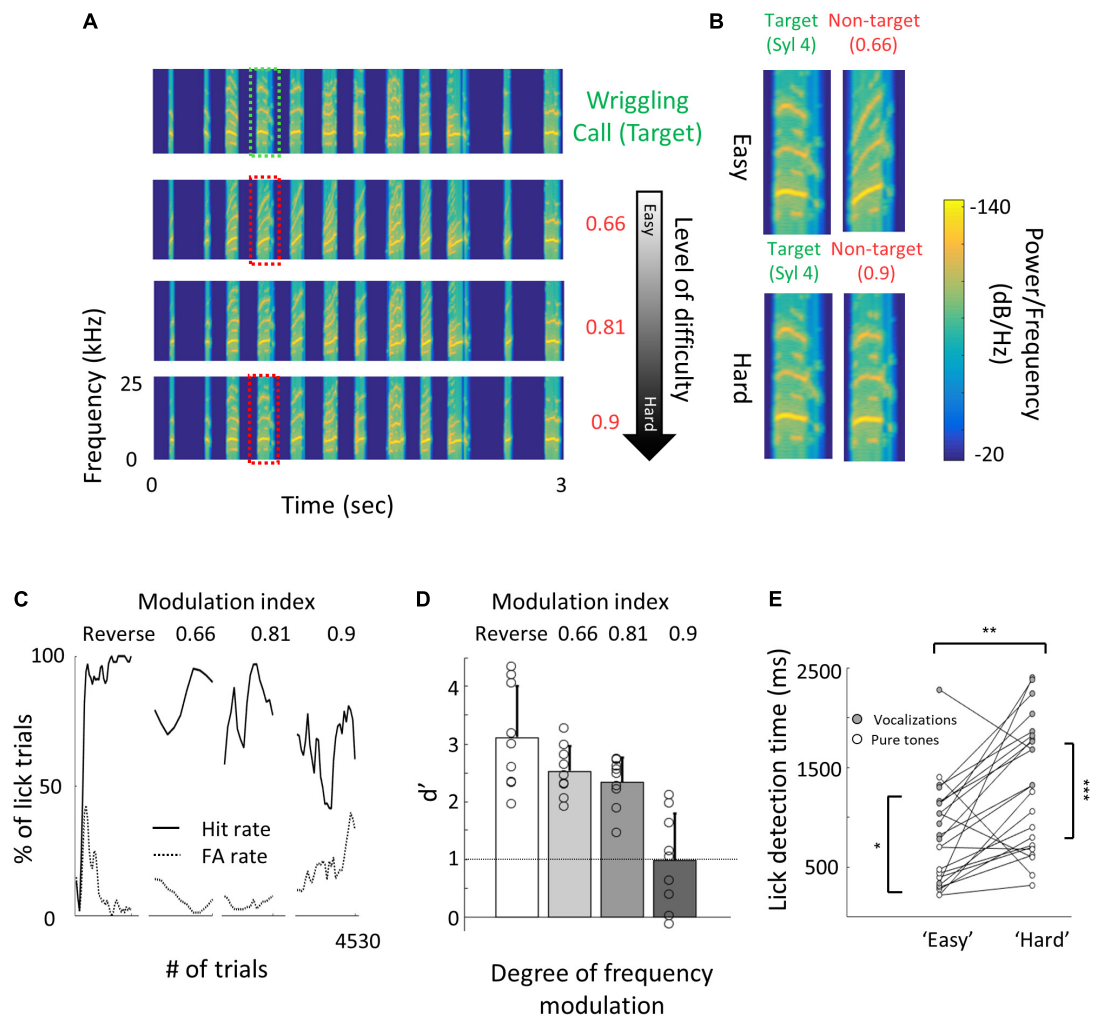
FIGURE 4 | Perceptual learning of vocalizations. **(A)** Spectrograms of the wriggling call (WC, "target" stimulus, top panel) and the manipulated WC's ("non-target" stimuli, bottom panels). **(B)** Enlargement of the spectrogram's 4th syllable of the target WC and the manipulated calls. Top, a large manipulation (speeding factor, 0.66), which is perceptually easy to discriminate from the WC. Bottom, a minor manipulation (speeding factor, 0.9), which is perceptually closer to the WC. **(C)** Lick responses to the target tone (solid line) and non-target tone (dashed line), binned over 50 trials, of one representative mouse along the different discrimination stages. The task of the first stage was to discriminate between WC vs. reversed playback of the call ("Reverse"). The following stages are different degrees of call modulation. Titles correspond to the speeding factors used for the non-target stimulus. **(D)** Population average d' values for the different discrimination levels. $N = 9$ mice (mean ± s.e.m). Shades denote the level of difficulty. **(E)** Comparison between detection times during the easy and difficult stages of pure tone (blank circles) and vocalizations (filled circles) discrimination tasks. Detection times are significantly different between all groups (Mann–Whitney U test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

gradual increase in perceptual difficulty using a manipulation of a natural sound.

Sparser Response in L2/3 Neurons Following Perceptual Learning of Natural Sounds

To study the neural correlates in A1 that follow natural sound discrimination, we recorded L2/3 neurons in response to the learned stimuli (**Figure 5A**), expecting increased representation of these particular stimuli (e.g., that more neurons will respond to the calls or that firing rates will increase). Surprisingly, we did not find an increase in the representation of the

learned stimuli. The fraction of cells responding to the trained vocalization remained constant (**Supplementary Figure 5a**) as well as the evoked firing rate for the preferred vocalization or the preferred syllable within a vocalization (**Table 2**). Instead, representation in expert mice became sparser. Here, we measured the "lifetime sparseness" (Vinje and Gallant, 2000; Willmore and Tolhurst, 2001) of each neuron to determine how selective its responses is to a given syllable. Sparse representation can be a result of having a smaller fraction of neurons responding to a given stimulus and/or a decrease responsiveness in the call. We found that the population of neurons in expert mice were sparser (**Figure 5B**; Naïve: 45% ± 20%; Expert: 64% ± 15%; $p < 0.001$), even
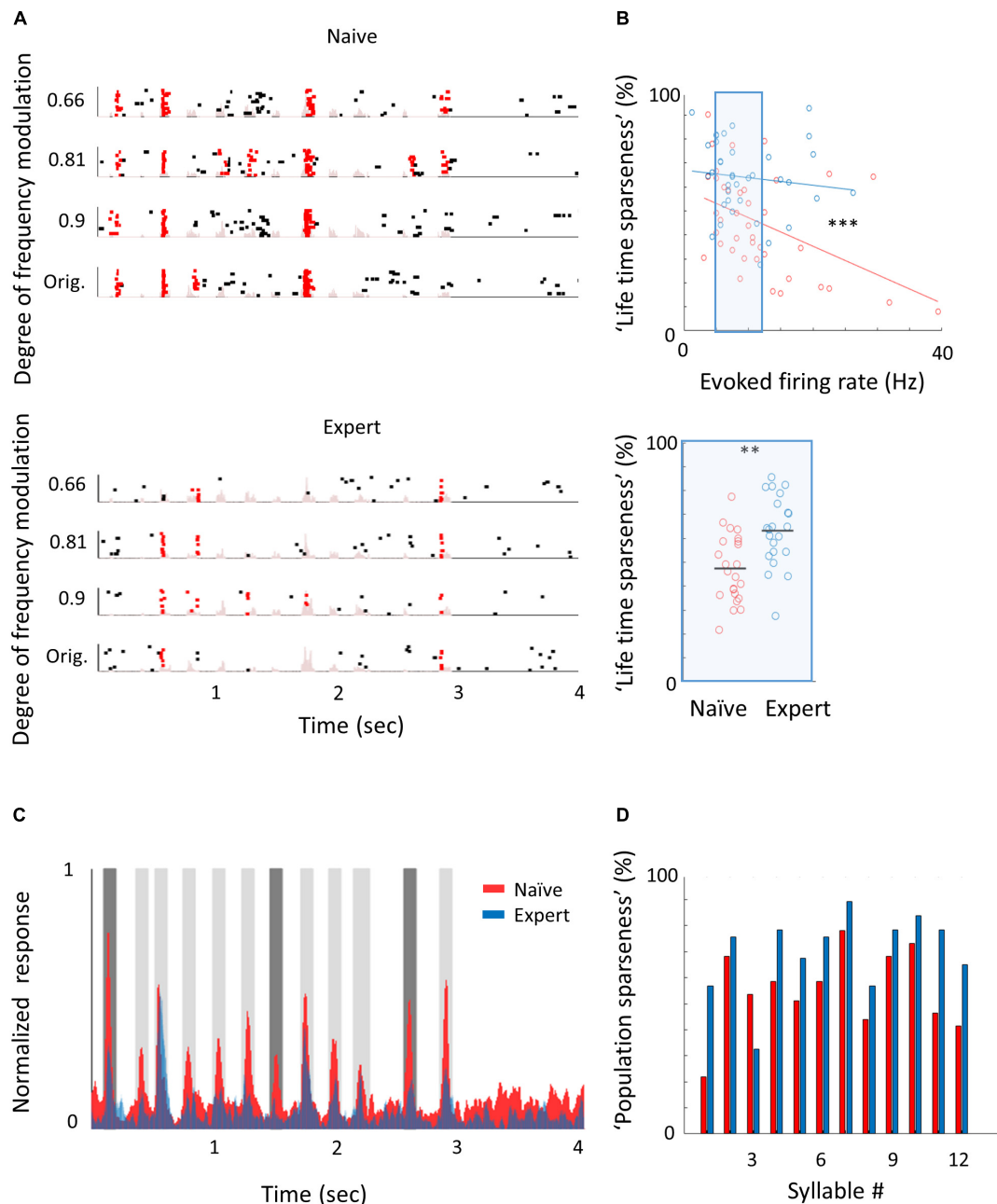
FIGURE 5 | Learning complex sounds induces "sparsening." **(A)** Representative examples of raster plots in response to 4 modulated wriggling calls from naïve (top) and expert (bottom) mice. Red lines indicate spikes in response windows that are significantly above baseline. The stimulus power spectrum shown in light pink in the background. **(B)** Top: Scatter plot of "lifetime sparseness" (along the vocalization, see methods) and evoked firing rate for individual neurons from naïve (red circles) and expert (blue circles) mice. Sparseness is significantly higher in the expert group (Mann–Whitney U test; ***$p < 0.001$). The middle range of firing rate distribution (0.5 SD bellow and above the median) is indicated as a blue rectangle. Bottom: Lifetime sparseness for all cells from the middle range of the distribution shows significant difference between the groups (Mann–Whitney U test; **$p = 0.001$). **(C)** Average normalized PSTHs calculated from all neurons in response to the original WC. Data are shown overlaid for naïve (red) and expert (blue) mice. In expert mice, only syllables 1, 7, and 11 evoked significantly weaker responses as compared to naïve mice (dark gray bars, Mann–Whitney U test followed by Bonferroni correction; $p = 0.03, 0.001, 0.03$). **(D)** Population sparseness (% of cells that evoked a significant evoked response to a given syllable) of all neurons in naïve (red) and expert (blue) for the different syllables in the call.

regardless of their evoked firing rate (**Figure 5B**; Naïve: 47% ± 14%; Expert: 63% ± 15%; $p = 0.001$). Increased sparseness was not apparent following pure tone learning (**Table 1**). Sparseness was also evident from the average population

response to the vocalization (**Figure 5C**). Nearly all syllables had weaker responses, three of which were statistically weaker (**Figure 5C**, dark gray bars). Moreover, the population sparseness (Willmore and Tolhurst, 2001), derived from the fraction of

Group	Animals (n)	Cells (n)	Spontaneous firing rate (Hz)	Evoked firing rate (Hz)	Response latency (ms)	Response selectivity (% of evoked stimuli)	Fano-Factor
Naïve	8	41	1.8 ± 2.9	7.7 ± 5.9	36 ± 11	25.5 ± 21.6	1 ± 0.45
Expert	6	37	0.78 ± 0.74	6.7 ± 3.6	43 ± 13	15.4 ± 11	1.1 ± 0.35
Rank-sum test			$p = 0.1$	$p = 0.6$	$p = 0.02$	$p = 0.05$	$p = 0.12$

A summary table of the complete dataset of recordings from excitatory neurons in naïve and expert mice after perceptual learning of vocalizations. Columns show different parameters of the dataset or property tested. The third row shows the statistical p-value between naïve and experts using a Mann–Whitney U test.

active neurons at any time, was higher in expert animals for almost all syllables in the call (**Figure 5D**; Naïve: 55 ± 16%; Expert: 70 ± 15%; $p = 0.026$). Notably, the smaller fraction of responses was not just an apparent sparseness due to increase in trial-to-trial variability, as reliability of responses by neurons in the expert group remained similar to reliability of responses in the naïve group (**Table 2**). Thus, "sparsening" of A1 responses is a main feature of plasticity following learning to discriminate complex sounds.

We next asked whether the "sparsening" described above bears more information to the learned stimulus. We analyzed population responses by including all neurons from all mice as if they are a single population (naïve: $n = 41$ neurons from 8 mice; expert: $n = 37$ neurons from 6 mice). We calculated the Pearson correlation of the population response to all responses in a pairwise manner (**Figure 6A** and **Supplementary Figure 5b**). As compared to naïve mice, the absolute levels of correlations in expert mice were significantly lower for nearly all pairs of comparisons (**Figure 6A**, asterisks). As expected, weaker modulations of the call and, hence, high similarity among stimuli, were expressed as higher correlations in the neuronal responses (**Figure 6B**). The pairs of stimuli that mice successfully discriminated in the behavior (0.66 *vs.* the original WC and 0.81 *vs.* the original WC) had significantly lower correlation in the expert mice (**Figure 6B**, rank sum test, $p < 0.05$). Responses to the more similar stimuli that were near perceptual thresholds (i.e., 0.9 *vs.* the original WC) were lower in expert mice, but not significantly (**Figure 6B**, rank sum test, $p > 0.05$). This reduced correlation suggested that plasticity in A1 supports better discrimination among the learned natural stimuli. Indeed, a SVM decoder performed consistently better in expert mice, discriminating more accurately the original WC from the manipulated ones (**Figure 6C**). As expected, the decoder performance monotonically increased when utilizing the responses to more syllables in the call. However, in the expert mice, performances reached a plateau already halfway through the call, suggesting that neuronal responses to the late part of the call carried no additional information useful for discrimination. Similarly, the correlation of the population responses along the call shows that responses were separated already following the first syllable, but that the lowest level of the correlation was in the 5th to 7th syllable range, which then rapidly recovered by the end of the call (**Supplementary Figure 5c**). These findings are also consistent with the behavioral performance of the mice as decisions are made within the first 1.5 s of the trial (corresponding to the first seven syllables of the call).

Specifically, the head of the mouse is often retracted by the time the late syllables are played (**Figure 4E**). Taken together, "sparser" responses improve neural discrimination of learned natural sounds.

Learning-Induced Plasticity of Parvalbumin Neurons

The mechanisms responsible for the learning-induced changes are currently unknown. We used mouse genetics and two-photon targeted patch to ask whether local inhibitory neurons could contribute to the observed plasticity we describe above. To this end, we focused only on parvalbumin inhibitory (PV⁺) interneurons as they are the most abundant inhibitory cell type with the strongest direct silencing effect on pyramidal cells (Avermann et al., 2012; Hu et al., 2014). In addition, recent evidence points to their role in a variety of learning-related plasticity processes (Letzkus et al., 2011; Wolff et al., 2014; Kaplan et al., 2016; Lagler et al., 2016; Goel et al., 2017; Lee et al., 2017). Here, we probe the role of inhibition in perceptual learning by measuring learning-induced plasticity in the response properties of the PV⁺ neurons. We trained PV⁺-Cre x Ai9 mice (i.e., mice with PV⁺ neurons expressing tdTomato) in the Educage and then patched single neurons under visual guidance (**Figure 7A**; Cohen and Mizrahi, 2015; Maor et al., 2016). In order to increase the sample of PV⁺ neurons, we used targeted patch and often patched both PV⁺ and PV⁻ neurons in the same mice. PV⁻ neurons were used as proxy for excitatory neurons (these neurons were also included in the analysis shown in **Figures 2–6**). All the TdTomato⁺ neurons that we patched were also verified as having a fast spike shape (**Figure 7A**), a well-established electrophysiological signature of PV⁺ cells, while PV⁻ neurons verified as having regular spike shape. PV⁺ neurons had response properties different from PV⁻ neurons in accordance with our previous work (Maor et al., 2016). For example, PV⁺ neuron responses were stronger and faster to both pure tones and natural sounds (**Figures 7B,C** and **Table 3**; see also Maor et al., 2016).

Following pure tone learning, PV⁺ neurons also changed their response profile. On average, the BF of PV⁺ neurons shifted toward the learned frequencies, similar to what we described for PV⁻ neurons (**Figures 8A,B**). This result is consistent with recent evidence from the visual cortex showing increased selectivity of PV⁺ to trained stimuli following learning (Khan et al., 2018). The shift in tuning curves of PV⁺ neurons was also accompanied by a significant widening of their receptive fields, unlike the PV⁻ population (**Supplementary Figure 6a**). When we compared the
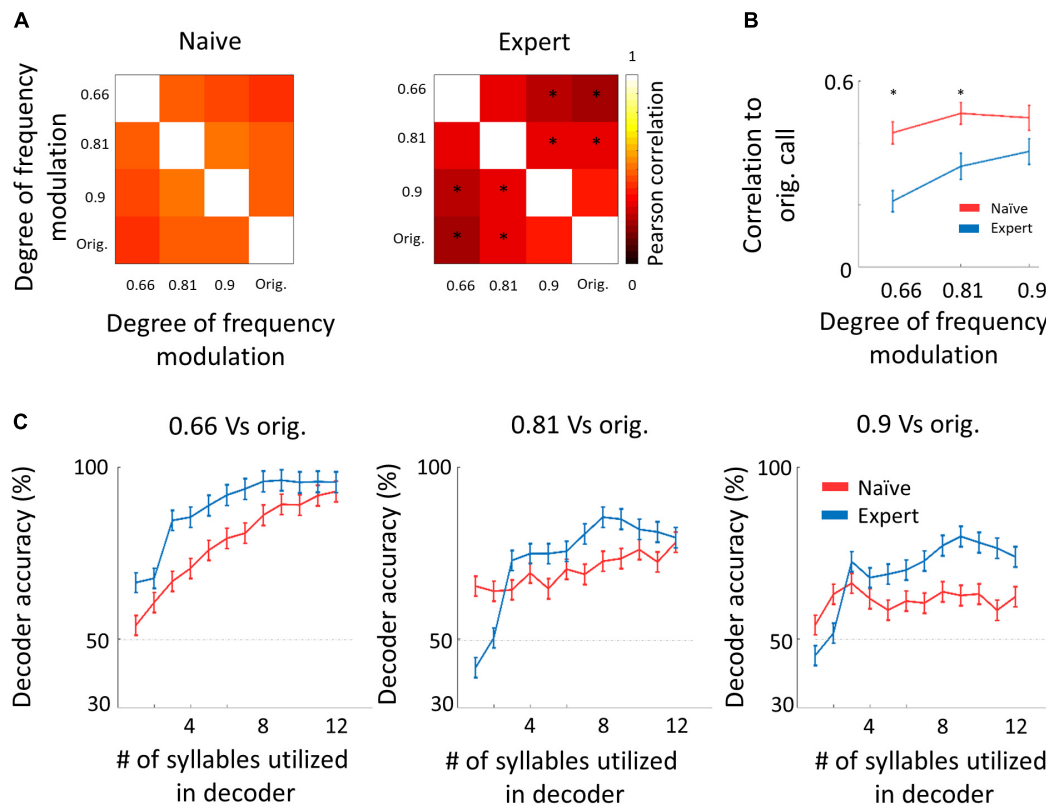
FIGURE 6 | Decorrelation improves coding. **(A)** Matrices describing the average response similarity of individual neurons between all combinations of stimuli in naïve (left) and expert (right) mice. Each pixel indicates the average Pearson correlation value calculated from all syllables' evoked spike rate from all neurons to two different calls. Neurons from expert mice have lower correlation between responses to different modulated calls (asterisks indicate significant differences between naïve and expert groups; Mann–Whitney U test followed by Bonferroni correction: $*p < 0.05$). **(B)** Pearson correlation between responses to modulated WCs and responses to the original WC in naïve (red) and expert (blue) mice (mean ± s.e.m.). Correlations are significantly different in the 0.66 and 0.81 modulation (Mann–Whitney U test: $*p < 0.05$). **(C)** Classification performance of a support vector machine (SVM) decoder. The decoder was tested for its accuracy to differentiate between the modulated WC stimuli against the original WC. The performance of the decoder is shown for neurons from the naïve (red) and expert (blue) groups. Decoder performance is plotted separately for the three different pairs of stimuli. Each point in each graph shows the number of syllables the decoder was trained on and allowed to use. Error bars are SEM for 1000 repetitions of leave-one-out cross-validation.

BFs of PV^- and PV^+ neurons within the same brain (within 250 microns of each other), we found that excitatory and inhibitory neurons became more functionally homogeneous as compared to naïve mice (**Figure 8C**). As both neuronal groups show similar trends in the shift of their preferred frequencies, we rule out a simple scenario whereas parvalbumin neurons increase their responses in the sidebands of the learning frequency. In other words, plasticity does not seem to be induced by lateral inhibition *via* parvalbumin neurons, but rather maintains a strict balance between excitation and inhibition, regardless of whether they are naïves or experts (Wehr and Zador, 2003; Zhou et al., 2014). Note that the peak of the PV^+ population response and their BF distribution was on the outskirts of the training band, rather than within it (compare **Figures 8A,B** with **Figures 2C,D**), and concomitantly, the slope of the population responses at the trained frequency band increased due to learning (**Figure 8A**). To assess the computational effect of the plasticity in the inhibitory neurons' responses, we have applied on their responses the same d' and FI calculated for the PV^- neurons

(**Figures 8D,E**). Overall, the discrimination performance of the two cell populations (when equalized in size) is similar. However, the PV^+ population shows a significant learning related increase in tone discrimination performance by these cells within the training band (**Figures 8D,E**) in contrast to the results for PV^- neurons (**Figures 3D,E**). This result is consistent with the abovementioned increase in their response slopes in the trained frequency.

Following natural sound learning, we found no significant changes in basic response properties of the PV^+ neurons (**Table 3**), or in the degree of sparseness of their representation of the learned vocalizations (**Supplementary Figure 6b**). The relationship between the responses of PV^+ and their PV^- neighbors remained constant as reflected in the similar slopes of the functions describing PV^- firing versus PV^+ firing (**Supplementary Figure 6c**). This result suggests that the excitation–inhibition balance, as reflected in the responses of PV^- versus PV^+, remains. In PV^+ neurons, the temporal correlation along the call as well as the decoding performance
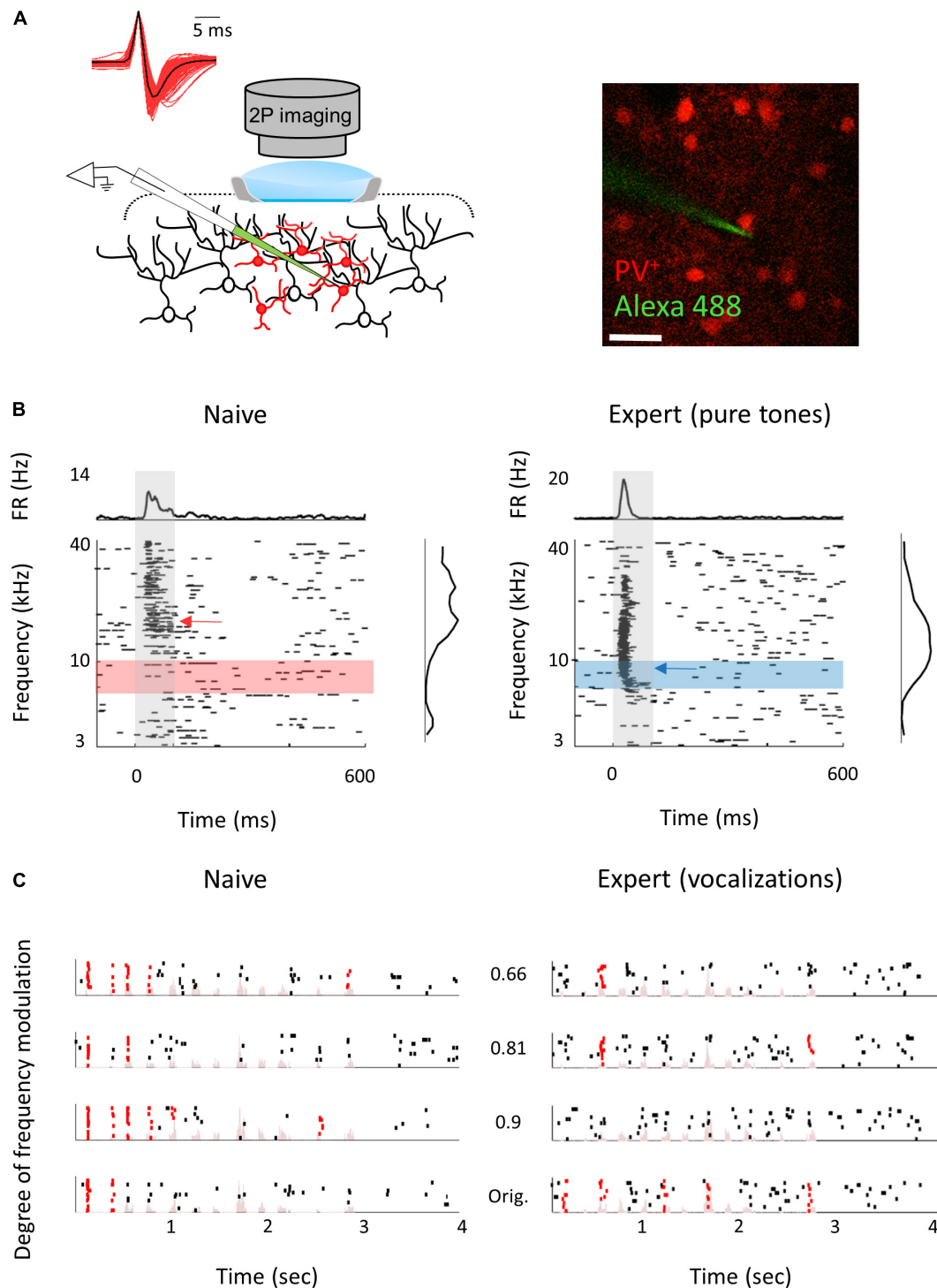
FIGURE 7 | Response properties of PV^{+} neurons. **(A)** Left: Schematic representation of the experimental setup for two-photon targeted patch and average spike waveform of 203 PV^{+} neurons. Right: Representative two-photon micrograph (projection image of 120 microns) of tdTomato+ cells (red) and the recording electrode (Alexa Fluor-488, green). **(B)** Raster plots and peri-stimulus time histograms (PSTH) in response to pure tones of a representative PV[+] neuron from naïve (left) and expert (right) mice. Gray bars indicate the time of stimulus presentation (100 ms). Color bars and arrows indicate the training frequency band (7.1–10 kHz) and BF, respectively. **(C)** Representative examples of raster plots from PV^{+} neurons in response to four modulated wriggling calls from naïve (left) and expert (right) mice. Red lines indicate spikes in response windows that are significantly above baseline. Stimulus power spectrum shown in light pink in the background.

TABLE 3 | Physiological changes in PV$^+$ neurons.

Group	Animals (n)	Cells (n)	BF (kHz)	Spontaneous firing rate (Hz)	Evoked firing rate in BF (Hz)	FR in training band (Hz)	Response latency (ms)	Response selectivity (% of evoked stimuli)
Naïve FS PT	21	120	16.2 ± 10.4	2.2 ± 2.1	21 ± 9	9.2 ± 9	31.1 ± 6.7	17 ± 17
Expert FS PT	18	83	11.8 ± 8.1	1.7 ± 1.8	22 ± 10	11.9 ± 10	33.2 ± 9.3	25 ± 18
Rank-sum test			0.006	0.05	0.7	0.02	0.2	<0.001
Naïve FS vocalizations	6	17		4 ± 3.7	14.3 ± 10.8		31.5 ± 8.6	27 ± 14.9
Expert FS vocalizations	5	18		3.4 ± 3.9	14.9 ± 12.9		32 ± 8	25.6 ± 15.6
Rank-sum test				0.4	0.97		1	0.72

A summary table of the complete dataset of recordings from PV neurons in naïve and expert mice after perceptual learning of pure tones (rows 1 and 2) and vocalizations (rows 4 and 5). Columns show different parameters of the dataset or property tested. The white color row shows the statistical p value between naïve and experts using a Mann–Whitney U test for each group separately.

from these neurons showed changes that are qualitatively similar to their PV$^-$ counterparts, but the data across the population were noisier (**Figures 8F,G** and **Supplementary Figure 6d**) perhaps due to the smaller sample of the PV$^+$ dataset.

DISCUSSION

Plasticity in Frequency Tuning Following Perceptual Learning

Shifts in the average stimulus representation toward the learned stimuli are not a new phenomenon. Similar findings were observed in numerous studies, multiple brain areas, animal models, and sensory systems, including in auditory cortex (Karni and Sagi, 1991; Buonomano and Merzenich, 1998; Weinberger, 2004). In fact, the model of learning-induced plasticity in A1, also known as tonotopic map expansion, is an exemplar in neuroscience (Bakin and Weinberger, 1990; Recanzone et al., 1993; Rutkowski and Weinberger, 2005; but see Crist et al., 2001; Ghose et al., 2002; Kato et al., 2015). Although we did not measure tonotopic maps, our results support the observations of others that tuning curves are plastic in primary sensory cortex. Specifically, we show here an average shift in the tuning of L2/3 neurons in A1 in mice.

Since we sampled only a small number of neurons, our observations cannot be inferred as direct evidence for tonotopic map expansion. Rather, our data emphasize that plastic shifts occur in local circuits (**Figure 2E**). Given that neurons in A1 are functionally heterogeneous within local circuits (Maor et al., 2016), any area in A1 that represents a range of frequencies prior to learning could become more frequency-tuned once learned. Such a mechanism allows a wide range of modifications within local circuits to enable increased representation of the learned stimuli without necessarily perturbing gross tonotopic order. One advantage of local circuit heterogeneity is that it allows circuits to maintain a dynamic balance between plasticity and stability (Mermillod et al., 2013).

It is often assumed that the learning-induced changes in tuning properties improve the accuracy of coding of the trained stimuli. In particular, perceptual learning theory predicts that sharpening the slope of the tuning curves improves the discriminability of the relevant stimuli

(Seung and Sompolinsky, 1993). However, the observed increased representation of the BFs toward the training band in expert mice may not increase over all tuning slopes and may even decrease them, especially since the slopes tend to be small at the BFs. A closer look at the tuning curves in A1 shows that they are often irregular with multiple slopes and peaks (i.e., not having simple unimodal Gaussian shapes). Furthermore, the learning-induced changes in the ensemble of tuning curves are not limited to shifting the BF; hence, a more quantitative approach was required to assess the consequences of the observed learning-induced plasticity on discrimination accuracy. Our new, SVD-based, generative model (**Figure 3**) allowed us to assess the combined effects of changes in BFs as well as other changes in the shapes of the tuning curves. Surprisingly, both FI analysis and estimated classification errors of an optimal linear classifier show that learning-induced changes in tuning curves do not improve tone discriminability at trained values. This conclusion is consistent with previous work on the effect of exposure to tones during development that has been argued to decrease tone discriminability for similar reasons (Han et al., 2007). However, in that work, the functional effect of tuning curve changes was consistent with an observed impaired behavioral performance, suggesting that plasticity in A1 sub-serves discrimination behavior. In contrast, the stable (or even reduced) accuracy in the coding of the trained frequency we observed occurs despite the improved behavioral performance after training. To test whether behavioral performances of individual mice are correlated with coding accuracy, we plotted neuronal d' with behavioral d' on a mouse by mouse basis. Although we recorded high d' values in the few mice that performed particularly well, we did not find a significant correlation between these two measures across mice (**Supplementary Figure 7a**). Mice with similar neuronal d' values often differed as much as twofold in their behavioral performance (**Supplementary Figures 7a,b**).

One possibility for the observed changes in the tuning of pure tones in A1 are the result of unsupervised Hebbian learning induced by overexposure to the trained tones during the training period, similar to the reported results in early overexposure (Han et al., 2007). Unsupervised learning signals are not driven by task-related reward and punishment *per se* and may increase representation rather than discriminability. Increased representation of trained stimuli may lead to improved
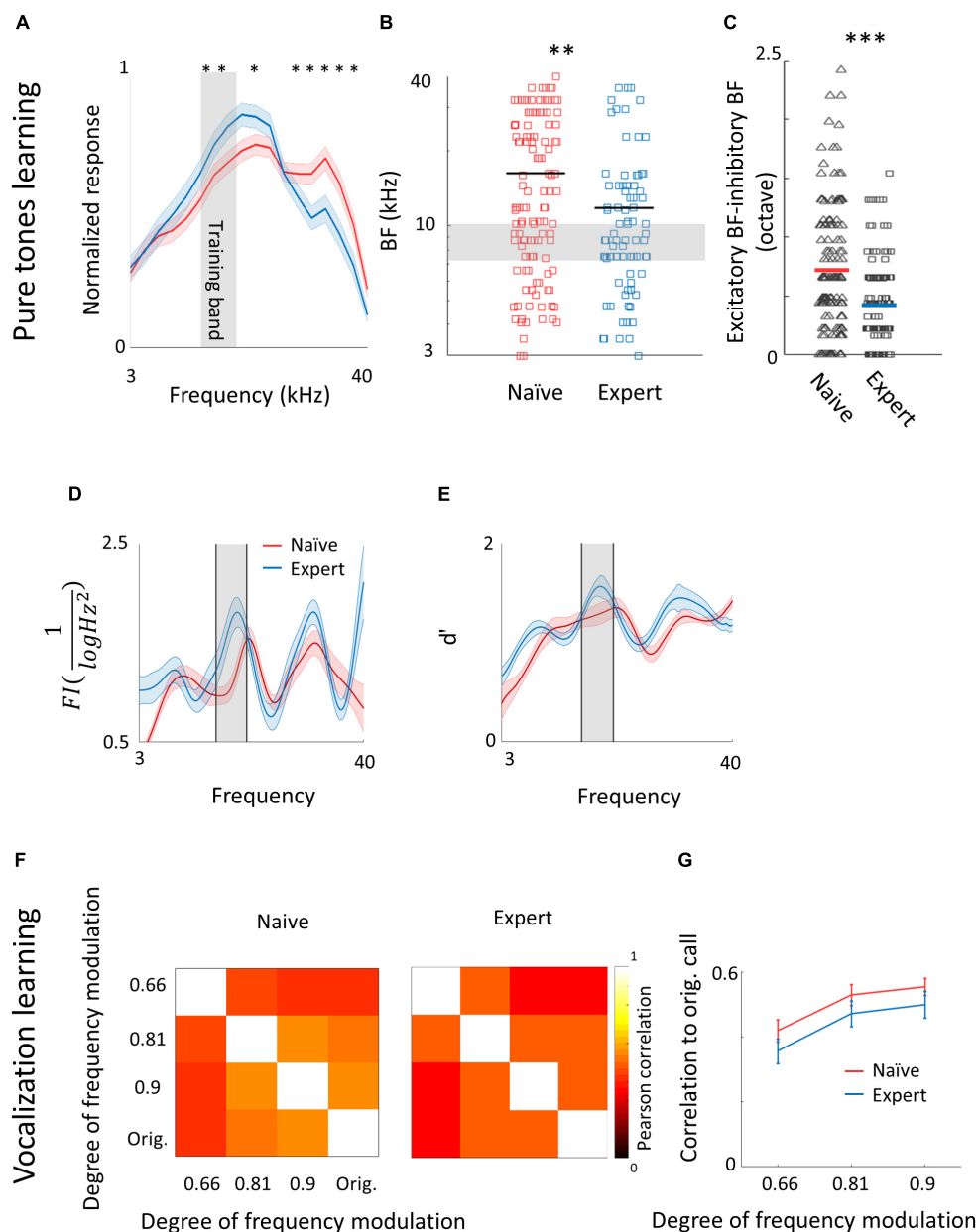
FIGURE 8 | Plasticity of PV+ neurons after learning. **(A)** Population average of normalized response tuning curves of n = 120 PV+ neurons from naïve mice (red) and 83 PV+ neurons from expert mice (blue; mean ± s.e.m). Gray area indicates the training frequency band. Asterisks correspond to frequencies with significant response difference (Mann–Whitney U test: *p < 0.05). **(B)** Best frequency (BF) of individual PV+ neurons from naïve mice (red) and expert mice (blue; Mann–Whitney U test: **p < 0.01). **(C)** Average distance in BF between PV- and PV+ neurons from the same penetration sites. Distances were significantly smaller in expert mice (Mann–Whitney U test: ***p < 0.001). **(D)** Fisher Information calculated from the tuning curves of PV+ neurons **(E)** Discriminability (d') of SVM decoder along the range of frequencies. **(F)** Matrices describing the average response similarity of individual PV+ neurons between all combinations of different stimuli in naïve (left) and expert (middle) mice. Each pixel indicates the average Pearson correlation value calculated from all syllables evoked spike rate from all neurons to two different calls. There was no significant difference between correlations of responses to different modulated calls in naïve and expert mice (Mann–Whitney U test, p > 0.05). **(G)** Pearson correlation between responses to modulated WCs versus the responses to the original WCs in naïve (red) and expert (blue) mice (mean ± s.e.m). Correlations are not significantly different for all comparisons (Mann–Whitney U test: p = 0.4, 0.5, 0.5).

discriminability to untrained tones, as observed experimentally. Both reward and punishment can drive associative learning and do so *via* non-overlapping neuronal pathways (Cohen and Blum, 2002; Seymour et al., 2007). Here, we used both reward and punishment to drive mice to their perceptual

limits. Fear conditioning has been shown to induce receptive field plasticity along the auditory pathway (Diamond and Weinberger, 1986; Kim and Cho, 2017), but so did positive reinforcement (Rutkowski and Weinberger, 2005). More recently, David et al. (2012) showed that positive and negative

reinforcements drive cortical plasticity in opposite directions—negative reinforcement boosts but positive reinforcement reduces responses of the target frequency (David et al., 2012). Notably, if both mechanisms act simultaneously, as it is in our case, it would be difficult to tease out their individual contribution to discrimination. In our data, the FI of expert mice peaked near 7 kHz (**Figure 3D**), leading us to speculate that aversion effects may have predominated. Indeed, the effects of the negative reinforcement on mouse behavior were strong. Specifically, following non-target stimuli (both FA and CR trials), mice returned back to the home cage and initiated their next trial only > 100 s later (**Supplementary Figure 7c**). Thus, we speculate that both unsupervised learning and the negative reinforcement may have affected cortical responses to pure tones.

Our protocol to push mice to their limits thus involves several processes acting simultaneously. The relative contribution of each—unsupervised learning or supervised learning (positive or negative)—utilized to drive the behavior and its resultant neural correlate remains to be elucidated.

Plasticity Following Natural Sounds Learning

Unlike pure tones, learning-induced changes in A1 improved the discriminability of learned natural sounds. Although auditory cortex is organized tonotopically, it may not be critical for processing simple sounds, as these stimuli are accurately represented in earlier stages in the auditory hierarchy (Nelken, 2004; Mizrahi et al., 2014). While the causal relationships between A1 and pure tones discrimination are still debatable (Ohl et al., 1999; LeDoux, 2000; Weible et al., 2014; O'Sullivan et al., 2019), the role of A1 in processing and decoding complex sounds is becoming increasingly more evident (Letzkus et al., 2011; Ceballo et al., 2019).

Neurons in A1 respond to sounds in a non-linear fashion (Bathellier et al., 2012; Harper et al., 2016; Angeloni and Geffen, 2018). These can be selective to harmonic content that are prevalent in vocalizations (Feng and Wang, 2017). Other neurons show strong correlations to global stimulus statistics (Theunissen and Elie, 2014). Furthermore, A1 neurons are sensitive to the fine-grained spectrotemporal environments of sounds, expressed as strong gain modulation to local sound statistics (Williamson et al., 2016), as well as to sound contrast and noise (Rabinowitz et al., 2011, 2013). These features (harmonics, globally and locally rich statistics, and noise), as well as other unique attributes such as frequency range, amplitude modulations, frequency modulations, inter syllable interval and duration variability, are well represented in the WCs. Importantly, WCs have been shown to drive strong responses in mouse A1 (Maor et al., 2016; Tasaka et al., 2018). Which of these particular sensitivities changes after perceptual learning and what is the contribution of the different attributes to the plasticity observed following vocalization learning is not yet known. However, one expression of this plasticity can be the increased sparse sound representation we found here (**Figure 5**). Sparseness can take different forms (Barth and Poulet, 2012). Here, sparseness was expressed as reduced number of neurons in the network that respond to any of

the 12 syllables played (**Figures 5B–D**). Such increase could arise from disparate mechanisms, and changes in the structure of local inhibition was one suspect that we tested (Froemke, 2015).

Inhibitory Plasticity Follows Excitatory Plasticity

Cortical inhibitory neurons are central players in many forms of learning (Kullmann et al., 2012; Hennequin et al., 2017; Sprekeler, 2017). Inhibitory interneurons have been implicated as important for experience-dependent plasticity in the developing auditory system (Hensch, 2005), during fear learning in adulthood (Letzkus et al., 2011; Courtin et al., 2014), and following injury (Resnik and Polley, 2017). Surprisingly, however, and despite the numerous studies on parvalbumin neurons, we could not find any references in the literature of recordings from parvalbumin neurons after auditory perceptual learning. Two simple (non-mutually exclusive) hypotheses are naively expected. One is that plasticity in inhibitory neurons is a negative mirror of the plasticity in excitatory neurons. This would predict that inhibitory neurons would increase their responses to the stimuli for which responses of excitatory neurons are downregulated, as found in the plasticity of somatostatin expressing (SOM) neurons following passive sound exposure (Kato et al., 2015), or in multisensory plasticity in mothers (Cohen and Mizrahi, 2015). The second is that inhibitory neurons enhance their response to "lateral" stimuli, thus enhancing selectivity to the trained stimulus, as suggested by the pattern of maternal-related plasticity to pup calls (Galindo-Leon et al., 2009). Our study provides a first test of these hypotheses in the context of perceptual learning. We found no evidence for these scenarios. Instead, a common motif in the local circuit was that parvalbumin neurons changed in a similar manner to their excitatory counterparts. These results are in line with the observation that PV neurons in V1 following visual discrimination task become as selective as their pyramidal neighbors (Khan et al., 2018).

The cortex hosts several types of inhibitory cells (Hattori et al., 2017; Zeng and Sanes, 2017), presumably serving distinct roles. While PV neurons are considered a rather homogeneous pool of neurons based on molecular signature, their role in coding sounds is not (Seybold et al., 2015; Phillips and Hasenstaub, 2016). In the visual cortex, PV$^+$ cells' activity (and presumably its plasticity) was correlated with stimulus-specific response potentiation but not in ocular dominance plasticity (Kaplan et al., 2016), again suggesting that PV$^+$ neurons are not necessarily involved in all forms of experience-dependent plasticity. Inhibitory neurons have been suggested to play a key role in enhancing the detection of behaviorally significant vocalization by lateral inhibition (Galindo-Leon et al., 2009). But recent imaging data argue that somatostatin interneurons rather than PV$^+$ interneurons govern lateral inhibition in A1 (Kato et al., 2017). Our results are also consistent with the observation that in contrast to the SOM neurons, the changes in responses following sound exposure are similar in PV$^+$ and pyramidal neurons (Kato et al., 2015; Khan et al., 2018). To what extent somatostatin or other interneurons subtypes

contribute to excitatory plasticity after auditory perceptual learning remains to be studied.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Hebrew University's IACUC.

AUTHOR CONTRIBUTIONS

IM and AM designed the experiments. RS-Z and HS analyzed data. LF conducted the experiments. YE constructed the vocalization stimuli and software for their delivery. IM conducted the experiments and analyzed the data. IM, HS, and AM wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncir.2019.00082/full#supplementary-material

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Common Principles in Functional Organization of VIP/Calretinin Cell-Driven Disinhibitory Circuits Across Cortical Areas

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In the brain, there is a vast diversity of different structures, circuitries, cell types, and cellular genetic expression profiles. While this large diversity can often occlude a clear understanding of how the brain works, careful analyses of analogous studies performed across different brain areas can hint at commonalities in neuronal organization. This in turn can yield a fundamental understanding of necessary circuitry components that are crucial for how information is processed across the brain. In this review, we outline recent *in vivo* and *in vitro* studies that have been performed in different cortical areas to characterize the vasoactive intestinal polypeptide (VIP)- and/or calretinin (CR)-expressing cells that specialize in inhibiting GABAergic interneurons. In doing so, we make the case that, across cortical structures, interneuron-specific cells commonly specialize in the synaptic disinhibition of excitatory neurons, which can ungate the integration and plasticity of external inputs onto excitatory neurons. In line with this, activation of interneuron-specific cells enhances animal performance across a variety of behavioral tasks that involve learning, memory formation, and sensory discrimination, and may represent a key target for therapeutic interventions under different pathological conditions. As such, interneuron-specific cells across different cortical structures are an essential network component for information processing and normal brain function.

Keywords: hippocampus, cerebral cortex, vasoactive intestinal polypeptide, interneuron, microcircuit, disinhibition, behavior

INTRODUCTION

Interneuron-specific (I-S) cells are inhibitory interneurons that are specialized to primarily target other interneurons. Most often they are identified via expression of vasoactive intestinal polypeptide [VIP; a neuropeptide that was initially localized in gastrointestinal nerves and ventromedial hypothalamus (Larsson et al., 1976)], and calretinin [CR; a calcium-binding protein (Rogers, 1987)]. Some CR+ cells and VIP+ cells in hippocampus and cortex also express serotonin (5HT) receptor mRNA, including 5HT2, 5HT3A receptor (Lee et al., 2010; Tremblay et al., 2016; Prönneke et al., 2019) and 5HT6 receptor (Helboe et al., 2015). As well, VIP has been shown to modulate the effects of 5HT (Rostene et al., 1983). There is also some indication that some

CR+ cells in hippocampus express corticotropin-releasing hormone (Gunn et al., 2019). In RNA sequencing datasets obtained from human middle temporal gyrus, VIP+ cells possess the richest diversity in transcriptomically-defined types when compared to non-VIP+ cells (Hodge et al., 2019). Specifically, in this study VIP+ cells were grouped into 21 different clusters. Notably, homologies in expression patterns were found in mouse visual cortex and lateral motor cortex, suggesting similar richness in VIP+ cell diversity across species and brain areas (Hodge et al., 2019). However, this may depend on the clustering method that one uses and the region being studied, since in a different RNA sequencing study conducted in the CA1 hippocampus, a smaller number of VIP+ cell clusters was found (Harris et al., 2018).

Importantly, some VIP+ cells, such as cholecystokinin co-expressing (CCK+) basket cells, primarily target excitatory cells, though they make up an apparently smaller fraction of VIP+ cells compared to I-S cells (Hájos et al., 1996; Kawaguchi and Kubota, 1996; Somogyi et al., 2004; Wang et al., 2004; Bezaire and Soltesz, 2013; Harris et al., 2018). Therefore, here we describe I-S cells generally as cells that preferentially inhibit other GABAergic interneurons. I-S cells, by this definition, have been described using imaging and electrophysiology techniques in several areas of the brain, including hippocampus (Gulyás et al., 1992, 1996; Acsády et al., 1996a,b; Tyan et al., 2014), frontal areas (Kawaguchi and Kubota, 1996, 1997; Pi et al., 2013), somatosensory cortex (Caputi et al., 2009; Lee et al., 2013; Prönneke et al., 2015), and visual cortex (Hajós et al., 1988; Zilles et al., 1991; Pfeffer et al., 2013). In this review we will highlight similarities in I-S cells that exist across these cortical areas.

In recent years, I-S cells with similar properties across several cortical areas have been characterized at multiple levels experimentally and inspired computational modeling work. Most commonly, I-S cells in various cortical areas have been studied *in vivo* through calcium imaging of VIP+ cells. This method of study alone however does not sufficiently distinguish VIP+ I-S cells from VIP+ non-I-S cells (i.e., such as VIP+ basket cells). As such, these studies can often lump together cells that inhibit pyramidal cells with those that disinhibit pyramidal cells. On the other hand, there has been much *in vitro* characterization work to complement the data obtained *in vivo*. In this review, we summarize previous work that has investigated and characterized the recruitment and impact of I-S cells found in various cortical areas of the brain. Ultimately, obtaining common principles in how disinhibitory I-S cells across cortical regions are involved in network computations will help inform disinhibitory strategies through which information is processed across the cortex.

HIPPOCAMPAL FORMATION

Characterization of interneurons in the hippocampal CA1 area (Freund and Buzsáki, 1996; Klausberger and Somogyi, 2008; Bezaire and Soltesz, 2013; Pelkey et al., 2017) has highlighted nuances in different forms of inhibitory control over inhibitory neurons beyond I-S cells (Chamberland and Topolnik, 2012). Based on synaptic density analysis, interneurons receive a substantial amount of inhibition relative to excitation (Gulyás

et al., 1999), similar to pyramidal cells (Megias et al., 2001). As such, inhibition of inhibition can arise through several types of circuits, one of which is simply from the inhibitory neurons that target other inhibitory interneurons in addition to targeting pyramidal cells (Chamberland and Topolnik, 2012). Some examples include connections of neurogliaform cells to other inhibitory interneurons (Armstrong et al., 2012), basket cell connections to other basket cells (Cobb et al., 1997; Karson et al., 2009), recurrent connections between oriens lacunosum moleculare (OLM) and bistratified cells (Leão et al., 2012), and various OLM cell connections to interneuron dendrites in stratum lacunosum moleculare (SLM) (Katona et al., 1999). Inhibitory neurons in CA1 also receive long-range projecting GABAergic inputs (Chamberland and Topolnik, 2012). These include GABAergic projections from medial septum onto inhibitory interneurons in stratum oriens/alveus (SO/A) (Freund and Antal, 1988) and GABAergic projections from entorhinal cortex onto SLM interneurons (Melzer et al., 2012). In particular, axonal boutons from GABAergic medial septal projections form close appositions to PV+, CR+, calbindin-expressing (CB+), and CCK+ somata and dendrites, among others (Unal et al., 2015). Together with excitatory projections from entorhinal cortex and medial septum, this unveils a feedforward disinhibitory circuitry of specific CA1 pyramidal cell morphological compartments.

In the hippocampus, most I-S cells (19.4% of the interneuron population) express VIP, although some CCK+ basket cells (9.4% of the interneuron population) also express VIP—calculated in Bezaire and Soltesz (2013) according to Fuentealba et al. (2010) [though see Lorén et al. (1979), Köhler (1982), Léránth et al. (1984), Miettinen et al. (1992), Hájos et al. (1996), Tricoire et al. (2011)] for additional immunohistochemistry work investigating distribution densities of VIP+ cell types in hippocampus]. In the hippocampal CA1 area, I-S cells are divided into three types [I-S1, I-S2, and I-S3; Acsády et al. (1996a,b), Gulyás et al. (1996), Chamberland and Topolnik (2012), Tyan et al. (2014), Francavilla et al. (2015); **Table 1**], as well as a long-range projecting cell type that innervates the subiculum in addition to the hippocampal area CA1 (Francavilla et al., 2018). Because CCK+ basket cells also express VIP, isolation and manipulation of I-S cells is not straightforward, since results can possibly be contaminated with cells that input directly to pyramidal cells [e.g., see Pi et al. (2013), Fu et al. (2014), Pfeiffer and Foster (2015), Karnani et al. (2016a), Garcia-Junco-Clemente et al. (2017), Turi et al. (2019)]. Along these lines, combinatorial genetic-viral targeting methods could help further disambiguate I-S from non-I-S cell types (He et al., 2016).

The first type of I-S cells in the CA1, interneuron-specific 1 (I-S1) cells, have somata located in SO/A, pyramidale (SP), and radiatum (SR), express CR, and do not commonly express VIP (Klausberger and Somogyi, 2008; Chamberland and Topolnik, 2012; Harris et al., 2018) (**Table 1**). These primarily target CB+ cells, VIP+ basket cells, and CR+ cells, while at the same time avoiding PV+ basket and axo-axonic cells (Gulyás et al., 1996). Additionally, I-S1 cells are found to form dendro-dendritic and axo-dendritic connections with themselves with some indication of gap junction connections (Gulyás et al., 1996). The second

TABLE 1 | Hippocampal CA1 I-S cell types.

I-S cell type	Somatic location	Dendritic profile	Axonal profile	Expression profile	Synaptic targets
I-S1 cell	SO/A	SO/A	SO/A	VIP-/CR+	I-S1 cells
	SP	SP	SP		CB+ cells
	SR	SR	SR		VIP+ basket cells
		SLM	SLM		CR+ cells
I-S2 cell	SR	SR	SR	VIP+/CR-	CB+ cells in SR
	SLM	SLM	SLM		VIP+ cells in SR
					VIP+/CCK+ basket cells
I-S3 cell	SR	SR	SP	VIP+/CR+	OLM cells
	SP	SLM	SO/A	Penk+	Bistratified cells
	SO/A	SP		substance P receptor +	Basket cells
		SO/A		mGluR1α+	Axo-axonic cells
				COUP-TFII+	SO/A cells
				NOS+	
VIP-LRP cell	SO/A	SO/A	Subiculum	VIP+/CR+-	CA1: OLM cells
	SP	SP	SO/A	M2R+	Bistratified cells
	SR	SR	SP	CB+	CCK+ basket cells
	SLM	SLM	SR	CCK-	SC-associated cells
				NOS-	Subiculum:
				SOM-	Pyramidal cells
					Interneurons

SO/A, Stratum Oriens/Alveus; SP, Stratum Pyramidale; SR, Stratum Radiatum; SLM, Stratum Lacunosum Moleculare; SC, Schaffer-Collateral; (+), expressing; (−), non-expressing; (+−), conditionally-expressing.

type, interneuron-specific 2 (I-S2) cells, have somata present near the SR and SLM border, and express VIP, but not CR (Klausberger and Somogyi, 2008; Chamberland and Topolnik, 2012; Harris et al., 2018) (**Table 1**). These cells have different morphologies with axons that target CB+ and VIP+ cells in SR, including VIP+/CCK+ basket cells (Acsády et al., 1996a,b; Chamberland and Topolnik, 2012). Though I-S1 and I-S2 cells are thought to be VIP–/CR+ and VIP+/CR–, respectively, it is worth noting that more recently obtained transcriptomics data suggests a more nuanced expression profile (Harris et al., 2018). When clustering cell types according to transcriptomic expression profiles, it is observed that I-S1 cells (i.e., traditionally VIP-/CR+) show some expression of *VIP* and I-S2 cells (i.e., traditionally VIP+/CR-) show some expression of *Calb2* (i.e., the gene that codes for CR) genes relative to non-I-S cells (Harris et al., 2018). It is only when compared to each other, that these expression levels appear relatively low.

The third type (**Table 1**), are the interneuron-specific 3 (I-S3) cells, which co-express VIP and CR. These interneurons have cell bodies mostly within the SP and SR, with dendrites extending to SLM, and axons arborizing in the SO/A (Acsády et al., 1996a,b; Chamberland et al., 2010) (**Table 1**). Together with CR, I-S3 cells may co-express other neurochemical markers such as proenkephalin (Penk), substance P receptor, metabotropic glutamate receptor 1a (mGluR1α), COUP transcription factor 2 (COUP-TFII), and nitric oxide synthase (NOS) (Freund and Buzsáki, 1996; Blasco-Ibáñez et al., 1998; Ferraguti et al., 2004; Fuentealba et al., 2010; Tricoire et al., 2010). Electrophysiological characterization shows that I-S3 cells exhibit a high input

resistance with irregular or regular spiking firing pattern (Chamberland et al., 2010; Tyan et al., 2014; Guet-McCreight et al., 2016). Also, it is known from dendritic calcium imaging experiments in combination with computational modeling that voltage-gated channels can be present in proximal dendrites of I-S3 cells (Guet-McCreight et al., 2016). In particular, there are proximal dendritic distributions of kinetically fast Kv3.1 channel subunits, which was confirmed using immunohistochemical analysis (Guet-McCreight et al., 2016). Furthermore, I-S3 cell distal dendrites receive excitatory input from entorhinal cortex via the temporoammonic pathway, while the proximal dendrites receive excitatory input from CA3 via the Schaffer collateral pathway (Luo et al., 2020). As well, a proportion of inhibitory inputs onto I-S3 cells are from I-S1, I-S2, and other I-S3 cells (Luo et al., 2020). I-S3 cells primarily form synapses onto SOM+ and mGluR1α+ OLM cells in SO/A (Chamberland et al., 2010; Tyan et al., 2014; Francavilla et al., 2015), but also contact bistratified cells, basket cells, putative axo-axonic cells, and various other SO/A interneuron types (Tyan et al., 2014). Compared to medial septal input to OLM cells, inhibitory currents generated by I-S3 cell input are smaller amplitude and have a slower time course (Chamberland et al., 2010). Despite this, optogenetic activation of CR+ cells, which includes the I-S1 and I-S3 cell types, at 5 and 10 Hz frequencies is sufficient to control the spike timing of OLM cells and to pace their activity at theta frequency (Tyan et al., 2014). Calcium imaging of activity of putative I-S3 cells *in vivo* showed that these cells tend to spike toward the end of theta-run epochs (Luo et al., 2020). Putative I-S3 cells in this study were identified through expression of VIP, somata located near the SP

and SR border, and small somatic diameters (i.e., to distinguish them from VIP+/CCK+ basket cells, which have larger somatic diameters). Together with computational modeling and spike extraction analysis, it was found that I-S3 cells spike toward the rising to peak phases of theta waves, depending on the strengths of inputs from CA3 and entorhinal cortex (Luo et al., 2020). This capacity for phasic modulation suggests the involvement of I-S3 cells in the encoding and retrieval of information that occurs at distinct phases of theta waves (Siegle and Wilson, 2014). The VIP+/CR+ cells (i.e., putative I-S3 cells) that synapse onto SOM+ cells, including OLM cells, contain the $\alpha 5$ GABA$_A$ receptor ($\alpha 5$-GABA$_A$R). Interestingly, inhibiting $\alpha 5$-GABA$_A$R at these synapses can modulate anxiety-like behaviors, with a possible impact on memory representations in the ventral hippocampus (Magnin et al., 2019).

In addition to the three I-S cell types described above, it is also worth mentioning a recently characterized long-range projecting (LRP) VIP cell type found in CA1 SO/A, with axons projecting to subiculum as well as to local interneurons in CA1 (Francavilla et al., 2018; Luo et al., 2019). VIP-LRP cells can have soma located in different CA1 layers, express muscarinic receptor 2 (M2R), and CB, and test negative for CCK and SOM (**Table 1**). In some cases, VIP-LRP cells with somata located in SP, SR, or SLM tested positive for Penk or CR suggesting further diversity, though the co-expression profiles of these markers in this cell type remain unknown. In the subiculum, VIP-LRP cells target both pyramidal cells and interneurons indiscriminately, while in the CA1, they target OLM, bistratified, and CCK+ basket cells and Schaffer-Collateral-associated cells. They are also electrically coupled with each other, but this mostly generates asynchronous spiking between pairs when depolarized past threshold using sinusoidal current injections. Further analysis revealed that this was because of low pass filtering properties which favored the conduction of slow after-spike hyperpolarizations, while attenuating the transfer of fast action potentials between electrically coupled cells. *In vivo*, these cells have elevated activity outside of theta-run periods, which could correspond with theta-off cells (Buzsáki et al., 1983; Colom and Bland, 1987). As well, they do not show activation during sharp wave-associated ripples. Although the inputs to these cells have not yet been characterized electrophysiologically, it is possible that they receive inhibitory input from I-S3 cells, since the somata of VIP-LRP cells were often decorated by VIP+ and CR+ axonal boutons (Francavilla et al., 2018). Though previous studies of I-S cells in hippocampus have generally classified I-S cells into these four types, a more recent transcriptomics study has also revealed a larger diversity of I-S cells in the CA1 area, with at least 8 transcriptomically-defined clusters [Harris et al. (2018); I-S1: 3 clusters; I-S2: 2 clusters; I-S3: 3 clusters]. Altogether, the characterization of I-S cell types in hippocampal CA1 area has highlighted a vast array of different cell types that are likely to enrich the mechanisms dedicated to information processing in this cortical area.

Notably, there are not many studies to date that have examined different types of hippocampal CA1 I-S cells *in vivo* during active learning. However, there is a recent calcium imaging study looking at the activity of VIP+ cells in CA1 during head-restricted behavior (Turi et al., 2019), though this

does not disentangle I-S cell types (Acsády et al., 1996a) from VIP+/CCK+ basket cells (Klausberger and Somogyi, 2008; Tyan et al., 2014; Francavilla et al., 2018; Luo et al., 2020). In this study, CA1 VIP+ cells exhibit a state-dependent disinhibition of pyramidal cells, which supports learning of reward site locations (Turi et al., 2019). During head-fixed locomotion, VIP+ cells were divided based on their activity pattern into those that are positively modulated by velocity, and those that are negatively modulated by velocity. Negatively velocity-modulated VIP+ cells exhibited activity either before or after running epochs, or both. Introduction of a spatially-guided reward learning task modulated the numbers of cells which falls into these functionally-defined VIP+ cell types. Interestingly, optogenetically suppressing VIP+ cell activity during this task led to an impairment in learning of the reward location and a decrease in the localization of pyramidal cell place fields near to the reward site, suggesting a role for VIP+ cells in learning the reward location and sharpening the memory representation. A network model further predicted that this sharpening of memory representation was due to the contribution of VIP+/CR+ cells, and not of VIP+/CCK+ basket cells (Turi et al., 2019). In line with this finding, another study found that chemogenetic silencing of CA1 VIP+ cells impairs spatial learning (Magnin et al., 2019). Together, these studies suggest an important role of I-S cells in learning and memory (**Figure 1A**).

FRONTAL AREAS

The spatial localization of I-S cells in frontal cortical areas can be extrapolated from the immunohistochemical studies that focused on the location of VIP+ cells. In rats, VIP+/CR+ cells show spatial distributions in layers II/III (i.e., 71% of CR+ cells are also VIP+) and layers V/VI (i.e., 94% of CR+ cells are also VIP+) (Kawaguchi and Kubota, 1997; Kawaguchi and Kondo, 2002). Smaller CCK+ cells tend to also be VIP+ and appear to be distributed similarly to VIP+/CR+ cells (Kawaguchi and Kubota, 1997). As well, some cells in layers II/III are immunoreactive for either VIP (i.e., 43% of VIP+ cells) or CR (i.e., 29% of CR+ cells), but not both (Kawaguchi and Kubota, 1997), similar to what is seen for hippocampal I-S1 and I-S2 cells. Further, VIP+ cells in frontal cortex, as classified based on their morphology, include bipolar cells, double bouquet cells, small basket cells, and arcade cells, of which small basket cells have rounder somata and smaller somatic diameters (Kawaguchi and Kubota, 1996; Kawaguchi and Kondo, 2002; Wang et al., 2002). In human frontal and temporal cortices, VIP+ cells can be found across all layers (Ong and Garey, 1991). In particular, VIP+ cells with small somatic diameters are located mostly in layers I and IV, whereas VIP+ cells with moderate somatic diameters are more numerous and more densely packed in layers II/III (Ong and Garey, 1991).

Notably, as far back as 2004, I-S cells were included in a network model of prefrontal cortex in order to make predictions on the neuronal operations that support working memory (Wang et al., 2004). Here, I-S cells were proposed to contribute to working memory by suppressing the dendrite-targeting
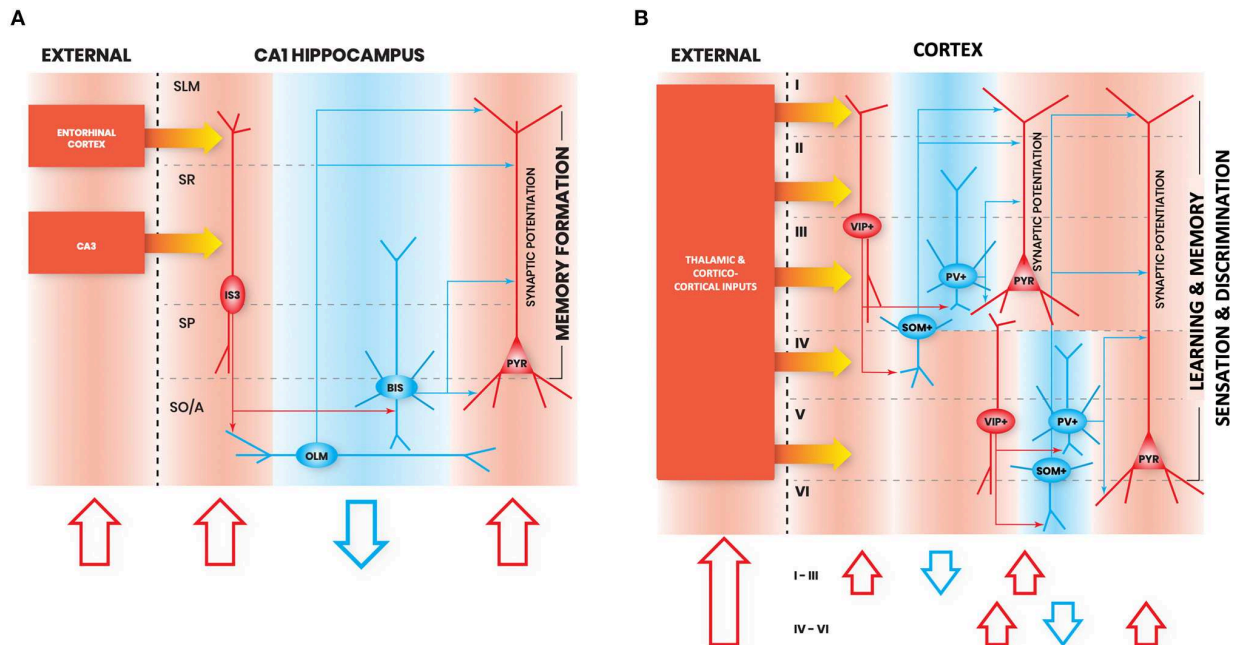
FIGURE 1 | Interneuron-specific cell circuitry across cortical areas allows disinhibition and synaptic potentiation of pyramidal cells via external inputs. In these schematic figures, we highlight the commonalities in I-S circuitry across **(A)** CA1 hippocampus, and **(B)** neocortex. We chose to provide illustrations for these brain regions because enough details on I-S cell circuitry in these areas have been reported in the literature. Red shapes denote increased neural activation, and blue shapes denote decreased neural activation. The flow of each schematic is the following: increased activation of excitation from external inputs (1st red arrow) increases activation of I-S cells (2nd red arrow), which decreases activity of other local SOM+ and PV+ cell types (blue arrow), and allows an increase in gain in pyramidal cells (3rd red arrow). In principle, this can be generalized as the basic mechanism through which activation of I-S cells in different brain areas can lead to enhancements in learning, memory, and sensory discrimination. SO/A, Stratum Oriens/Alveus; SP, Stratum Pyramidale; SR, Stratum Radiatum; SLM, Stratum Lacunosum Moleculare; PYR, Pyramidal neuron.

interneuron activity during sensory inputs, thus creating inverted sensory tuning curves in dendrite-targeting interneurons. Otherwise, dendrite-targeting interneurons provided resistance against distracting stimuli through elevated spontaneous activity. This pathway gating/ungating roles of I-S cells is further reviewed in Wang and Yang (2018).

Similar to I-S3 cells in CA1, VIP+ cells in auditory and medial prefrontal cortices primarily inhibit SOM+ cells, as well as a fraction of PV+ cells (Pi et al., 2013). A small percentage also contact pyramidal cells, which could represent an overlap with non-I-S VIP+ cells. *In vivo*, VIP+ cells in these areas are strongly but transiently activated following punishments in a go/no-go auditory discrimination task, and show a weaker but sustained response during rewards (Pi et al., 2013). Also, VIP+ cells are similar to pyramidal cells in that they exhibit responses during both go and no-go trials, while SOM+ and PV+ cells exhibit a response bias toward go trials (Kamigaki, 2019a).

In dorsomedial prefrontal cortex (also reviewed in Kamigaki, 2019a,b), activation of PV+ or SOM+ cells during this type of go/no-go task impairs performance, while activation of VIP+ cells enhances performance (Kamigaki and Dan, 2017). Similar findings were seen in a delayed two-alternative forced-choice task, which suggests an importance for VIP+ cell activation in enhancing short-term memory retention (Kamigaki and Dan, 2017). In addition, disinhibition through activation of VIP+

cells in prefrontal cortex enhances theta rhythm synchrony and communication from hippocampus to prefrontal cortex (Lagler et al., 2016; Lee et al., 2019). For example, in an elevated plus maze task, animals display avoidance of the open arm component of the maze, and this is accompanied with VIP+ cell activation and disinhibition of responses to hippocampal inputs, which can carry anxiety related-information (Lee et al., 2019). Correspondingly, inhibition of VIP+ cells leads to reduced open arm avoidance when there is theta synchrony between the two regions. During a different and more complex cue-matching-to-place task, the activity of PV+ basket cells in the prelimbic area of the prefrontal cortex forms neural ensembles that are either activated or suppressed at different stages of the task, thus allowing "multi-layered cognitive computations" (Lagler et al., 2016). More specifically, the activation or suppression of individual basket cells is correlated to the amount of VIP+ cell inputs that they receive.

In frontal association cortex *in vivo*, feedforward inhibition from VIP+ cells creates a pull-push circuitry, alternatively to the classical push-pull circuitry that can be generated through feedforward excitation (Garcia-Junco-Clemente et al., 2017). This is observed since VIP+ cells in this brain area make both direct connections to pyramidal cells in addition to connections to SOM+ cells. Without further characterization of VIP+ cell types in this brain area however, it is difficult to say whether this

arises from VIP+ cell uniqueness in frontal association cortex, or different I-S and non-I-S VIP+ cell types.

In summary, from work done in frontal areas, it is clear that I-S cells are involved in enhancing learning and memory discrimination, are recruited during theta rhythms, and primarily target SOM+ cells, but also PV+ cells (i.e., similar to hippocampal CA1 I-S3 cells; **Figures 1A,B**). Further characterization of VIP+ cell types in frontal areas is needed to distinguish how much I-S cell types in these areas contact pyramidal cells, compared to non-I-S VIP+ cells.

SOMATOSENSORY CORTEX

In the somatosensory barrel cortex, VIP+ cell dendrites often exhibit bipolar or tufted morphologies and span across cortical layers and columns (Bayraktar et al., 2000; Prönneke et al., 2015). Particularly, in layer IV, VIP+ and SOM+ cells, but not PV+ cells, show a higher density within intercolumnar septal areas (Bayraktar et al., 2000; Almási et al., 2019). Moreover, different VIP+ cell morphologies can be found across all layers in barrel cortex, though they exhibit a higher density in layers II/III (Prönneke et al., 2015; Almási et al., 2019). In a study of CR+ cells spanning across cortex, it was also found that CR is generally expressed in bipolar and multipolar cells (Caputi et al., 2009). While both preferentially target other interneurons, electrophysiological characterization of these cells in barrel cortex demonstrated key excitability and synaptic differences—most notably the existence of asymmetric electrical connections between multipolar CR+ cells and multipolar PV+ cells (Caputi et al., 2009).

Also in the somatosensory barrel cortex, VIP+ cells receive inputs from the primary vibrissal motor cortex pyramidal cells, and preferentially inhibit SOM+ cells (Lee et al., 2013). This highlights another cortical disinhibitory circuitry, where during whisking behavior *in vivo*, VIP+ cells show enhanced activation, while SOM+ cells reduce their activity (Gentet et al., 2012; Lee et al., 2013; Yu et al., 2019). This finding was both layer-specific (i.e., VIP+ cells in layers II/III) and behavior-dependent, where it was specifically observed during active wakefulness voluntary whisking (Yu et al., 2019). When tested in a goal-directed behavioral context where mice were actively attempting to localize an object, whisker touch events slightly suppressed VIP+ cells, with an immediate activation of fast spiking cells and a delayed activation of SOM+ and excitatory cells (Yu et al., 2019). VIP+ cells have also been shown to contact CB+ cells (Staiger et al., 2004) as well as PV+ cells (Dávid et al., 2007), where higher densities of VIP+ cell inputs to PV+ cells were associated with higher densities of excitatory inputs. Axon terminals from VIP+ cells in somatosensory cortex also highly express mGluR7a, a metabotropic glutamate receptor, which can be expressed in axon terminals that contact SOM+ and PV+ cells (Dalezios et al., 2002). As well, VIP+ cells can also target excitatory cells (Zhou et al., 2017), though it is unknown if this is due to different VIP+ cell types, or specialized layer-specific connections across the cortical layers of barrel cortex. In addition to inputs from the primary vibrissal motor cortex, VIP+ cells in somatosensory

barrel cortex generally also receive inputs from thalamus, and other cortical areas [i.e., proportionally more inputs than other inhibitory cell types receive; Wall et al. (2016)], in a spatially-specific way (Sohn et al., 2016; Almási et al., 2019). For example, when preferentially stimulating either the layer IV-projecting first-order ventral posterior medial thalamic nucleus or the layer I and V-projecting higher-order posterior thalamic nucleus, VIP+ cells are more broadly activated across layers when compared to the excitatory cells, PV+ cells, and SOM+ cells (Sermet et al., 2019). This is consistent with their bipolar dendritic arbors, which span across layers and can allow VIP+ cells to receive inputs that arrive to different cortical layers. In line with this, cortico-cortical and thalamo-cortical inputs in barrel cortex preferentially target the distal dendritic compartments of VIP+ cells (Sohn et al., 2016). Likewise, SOM+ cell inhibitory inputs also preferentially target distal dendritic compartments of VIP+ cells, while PV+ cell inhibitory inputs target the perisomatic compartments of VIP+ cells (Sohn et al., 2016). Higher-order thalamic inputs to VIP+ cells in particular are critical to the induction of LTP in disinhibited layers II/III pyramidal cells during whisker stimulation (Williams and Holtmaat, 2019).

Furthermore, following the study of Lee et al. (2013), it was found that the activity of SOM+ cells in barrel cortex during whisking depends highly on the cortical layer and the SOM+ cell morphology (Muñoz et al., 2017). In particular, some whisking-suppressed SOM+ cells presumably receive stronger inhibition from VIP+ cells during whisking, which, when removed, causes a reversal in their responses to whisking behavior. Blocking cholinergic receptors with bath application of atropine further highlights a state-dependent response, since all SOM+ cells become suppressed during whisking following this manipulation (Muñoz et al., 2017). In barrel cortex, there are also some data on the synaptic inputs by VIP+ and PV+ cells onto Martinotti cells, a SOM+ cell type that is analogous to OLM cells in the hippocampus (Walker et al., 2016). Specifically, Martinotti cells receive a stronger inhibition from PV+ cells. As well, PV+ cell inputs show a frequency-invariant short-term depression, while VIP+ cell inputs exhibit short-term facilitation. This suggests two temporally-specific modes of control over Martinotti cell spiking that can coordinate the disinhibition of pyramidal cells.

In barrel cortex and entorhinal cortex *in vitro*, VIP+ cells also show differential activation according to brain states (Neske et al., 2015; Neske and Connors, 2016). While PV+ cell activity is dominant during up states, there is also considerable spiking from SOM+ and VIP+ cells in barrel cortex, though spiking from these cells during up states is considerably smaller in the entorhinal cortex (Neske et al., 2015). Surprisingly, while optogenetic silencing of SOM+ cells enhances pyramidal cell excitability during up states, optogenetic activation or silencing of VIP+ cells in barrel cortex does not have any effect on pyramidal cell spiking (Neske and Connors, 2016). This suggests the existence of dynamically modulated states where VIP+ cells will not significantly impact network activity, despite their inhibitory connections to SOM+ cells.

VIP+ cells in barrel cortex are also extensively characterized *in vitro* (Prönneke et al., 2015). This work demonstrates the existence of layer-specific distributions of VIP+ cell

morphologically-defined types as well as a variety of different electrophysiologically-defined types, which do not necessarily map onto each other (Prönneke et al., 2015). In fact, a large diversity of different neuronal types could be present in the VIP+ cell population in barrel cortex. Furthermore, certain VIP+ cells in barrel cortex are sensitive to depolarization-inducing neuromodulation from acetylcholine or serotonin, which can effectively switch their firing patterns (Prönneke et al., 2019). Similarly, in auditory cortex, VIP+ cells are very sensitive to nicotinic neuromodulation, where bath application of nicotine can induce large depolarizations in VIP+ cells, with consequential weak depolarizations in pyramidal cells (Askew et al., 2019).

In summary, similar to I-S cells in other areas, I-S cells in somatosensory cortex appear to also primarily target SOM+ cells. Particularly, they target Martinotti cells, which exhibit properties similar to OLM cells, the primary synaptic target of I-S3 cells in the hippocampus. As well, I-S cells in somatosensory cortex receive spatially organized inputs, which appears to be the case for I-S cells in other areas as well. Altogether, given the similarities in activation and circuitry between I-S cells across different cortical areas, it appears that they may provide similar contributions to network function, such as induction of synaptic plasticity in pyramidal cells during behavior (**Figure 1B**).

VISUAL CORTEX

In visual cortex, VIP+ cells have somata located in layers II/III, with dendrites distributed in layers I and II, and axons innervating layers I/II, IV, and VI (Hajós et al., 1988; Zilles et al., 1991; Ji et al., 2016). Notably, in both visual and auditory cortices, despite a higher somatic density in layers II/III, only VIP+ cells with somata in layer IV are likely innervated by thalamocortical inputs (Ji et al., 2016), which may be due to differences in laminar dendritic branching patterns between layers II/III and layer IV VIP+ cells or electrotonic distances from soma.

Furthermore, in visual cortex, the activity patterns of VIP+ cells *in vivo* are similar to PV+ cells in that they exhibit strong intra-population coupling (Knoblich et al., 2019). Similar to other areas, visual cortical VIP+ and SOM+ cells inhibit each other bidirectionally, and both SOM+ and VIP+ cells receive non-overlapping inputs from layers II/III pyramidal cells (Pfeffer et al., 2013; Karnani et al., 2016a,b). Moreover, ErbB4 (i.e., a tyrosine kinase receptor associated with signaling factor Neuregulin-1) in VIP+ cells regulates these connections throughout development (Batista-Brito et al., 2017). Specifically, ErbB4 regulates both VIP+ cell targeting of SOM+ cells, as well as excitatory synaptic targeting of VIP+ cells (Batista-Brito et al., 2017). In fact, abolishing ErbB4 in VIP+ cells alters network dynamics and leads to a loss in visual response selectivity and impaired sensory learning (Batista-Brito et al., 2017). *In vivo*, locomotion enhances VIP+ cell activation, which causes an increase in gain in visual responses (Fu et al., 2014), and modulation of cortical plasticity (Fu et al., 2015). This occurs through inputs from the basal forebrain to VIP+ cells, and

is independent of visual stimulation (Fu et al., 2014). This disinhibitory circuitry is context-dependent where during visual stimulation, all of VIP+, PV+, and SOM+ cells in layers II/III and IV show locomotion-associated activation. This indicates that SOM+ cells are activated by visual stimulation during movement, despite inhibition from VIP+ cells (Pakan et al., 2016). In darkness, VIP+ and PV+ cells remain locomotion-associated, while SOM+ cells become silent.

VIP+ cells in visual cortex *in vivo* are also active during non-locomotion, visual stimulation, and under anesthesia (Jackson et al., 2016). Specifically, two-photon calcium imaging has shown that VIP+ cell activity correlates most with the activity of pyramidal cells, and plays a causal role in generating states of high excitatory activity (Jackson et al., 2016). More specifically, suppression of VIP+ cells leads to a reduction of spontaneous excitatory network activity across different behavioral states (Jackson et al., 2016). This directly plays a role in visual spatial frequency tuning of pyramidal cells, where activation of VIP+ cells generates responses to higher visual spatial frequencies and inactivation of VIP+ cells generates responses to lower visual spatial frequencies (Ayzenshtat et al., 2016). During visual stimulation, VIP+ cells also respond differently to novel vs. familiar images (Garrett et al., 2020). While they become activated during novel images, they are suppressed during familiar images. As well, VIP+ cell activation ramps up when expected visual stimuli are omitted during sequences of visual stimuli (Garrett et al., 2020). Similarly, activation of VIP+ cells during visual stimulation (i.e., using a contrast drifting Gabor stimulus) enhances contrast detection, whereas activation of PV+ or SOM+ cells during the visual stimulation reduces contrast detection (Cone et al., 2019).

Interestingly, VIP+ cells in visual cortex following animal exposure to light exhibit a high expression of insulin like growth factor 1 (IGF1), an experience-regulated gene (Mardinly et al., 2016), which is in contrast to PV+ and SOM+ cells. Over-expression of IGF1 also promotes inhibitory inputs to VIP+ cells, thus reducing their activity, and, accordingly, the disinhibition of pyramidal cells. Additionally, when testing ocular dominance plasticity, knocking down IGF1 increases disinhibition and enhances visual acuity in an experience-dependent manner (Mardinly et al., 2016).

Visual acuity is also potentially modulated by inputs from the cingulate area in frontal cortex, which improves visual discrimination via strong connections to VIP+ cells in visual cortex, in addition to contacting PV+ cells, SOM+ cells, and pyramidal cells (Zhang et al., 2014). Particularly, focal layer-specific activation of cingulate area axons in visual cortex leads to SOM+ cell-mediated surround suppression and VIP+ cell-mediated center facilitation (i.e., disinhibition) (Zhang et al., 2014). Based on computational modeling work, these types of connectivity mechanisms can sharpen and enhance visual responses for optimal encoding of visual stimuli (Lee and Mihalas, 2017; Lee et al., 2017). Moreover, VIP+ cells *in vivo* possess vertically elongated axons with narrow spatial layouts that allow lateral disinihibition of pyramidal cells, which can generate local transient holes in the blanket of inhibition in visual cortex (Karnani et al., 2014, 2016a).

In visual cortex, there is also some indication that VIP+ cells may be involved in associative learning through long-range top-down projections from the retrosplenial cortex (Makino and Komiyama, 2015). Here it was shown that retrosplenial inputs to layers II/III pyramidal cells become enhanced during a visually-guided active avoidance task, where mice are trained to run on a treadmill when they see a drifting grating stimulus with particular orientation. Comparatively, bottom-up pyramidal cell projections from layer IV and SOM+ cell projections from layers II/III (i.e., which gate retrosplenial inputs) become weaker over the course of this task. Both retrosplenial inactivation and SOM+ cell activation were sufficient to reverse the learning-related changes in layers II/III. Although no changes in VIP+ cell activation were observed throughout this task, it is nonetheless possible that VIP+ cells are entrained by retrosplenial inputs to suppress SOM+ cells, which would ungate retrosplenial inputs to layers II/III pyramidal cells. This, however, remains to be tested directly.

Once again, like I-S cells in the hippocampus, frontal areas, and somatosensory cortex, I-S cells in visual cortex exhibit similar morphological properties and primarily target SOM+ cells. Similar to I-S cells in other areas, visual cortical I-S cell activation contributes through enhancement of sensory discrimination and by promoting plasticity mechanisms, which, again, suggests similar roles for I-S cells across cortical areas (**Figure 1B**).

MOTOR CORTEX

VIP+ cell morphological and electrophysiological characterization has also been performed in the motor cortex. Here I-S cells also tend to have bipolar morphologies with irregular spiking activity and local intracortical glutamatergic inputs from pyramidal cells (Cauli et al., 1997; Porter et al., 1998). They are further characterized as two distinct populations, based on differences in burst duration, and expression of CR and choline acetyltransferase.

In motor cortex, VIP+ cells are also involved in motor skill learning (Adler et al., 2019). In this study, mice were trained to run forwards or backwards at fixed speeds on a treadmill, effectively changing their gait patterns such that they run with a structured pattern. During this motor learning, layers II/III pyramidal cells exhibited sequential activation patterns across the population that are shifted compared to normal treadmill running—a finding that was dependent on the presence of CaMKII-mediated synaptic plasticity (Adler et al., 2019). SOM+ cells, on the other hand, exhibited diverse responses where they would become enhanced, suppressed, or unchanged at the onset of forward and backward running. As well, their responses to either forward or backward running was not indicative of what their response would be in the opposite case. Interestingly, activation of SOM+ cells suppressed both the temporal shift in the sequential activation of pyramidal cells as well as motor learning. Conversely, suppressing SOM+ cells during new learning de-stabilized previously learned motor skills (i.e., when switching from a forward running to backward running learning paradigm). Further tests revealed that VIP+ cells, which become activated during both forward and backward running, are necessary for the shifted sequential pyramidal cell activation patterns and motor learning to occur, but not necessary for preserving previously learned motor skills. Altogether, Adler et al. (2019) provides a clear demonstration of how VIP+ cells can inhibit SOM+ cells, causing disinhibition in pyramidal cells and allowing synaptic plasticity and learning to occur.

BASOLATERAL AMYGDALA

Although it is not a part of cerebral cortex, certain commonalities also exist with VIP+ cells located in the basolateral amygdala. In this area, VIP+ cells have been classified as either I-S or cannabinoid-expressing basket cells (Rhomberg et al., 2018). Additionally, three types of activity patterns are observed when recording from I-S cells, based on the number of spikes observed upon depolarization. Here, I-S cells primarily target other I-S cells, CCK+ basket cells, and neurogliaform cells. I-S cells themselves receive a dense inhibition, of which only a small proportion is from other I-S cells (Rhomberg et al., 2018). Though connections to SOM+ or PV+ cells were not tested in this study, other studies showed that VIP+ cells target CB+ cells, which include SOM+, PV+, and CCK+ cells (Muller et al., 2003; Krabbe et al., 2018). More recently, deep-brain calcium imaging and optogenetic experiments have demonstrated the involvement of the basolateral amygdala VIP+ cells in associative learning (Krabbe et al., 2019). More specifically, these cells were activated by aversive stimuli, were modulated by expectations, and were necessary for the induction of synaptic plasticity and learning to occur. Moreover, VIP+ cell contribution to circuit function is achieved through innervation of SOM+ and PV+ cells, which allows disinhibition of projecting neurons in basolateral amygdala (Krabbe et al., 2019). Overall, this circuitry motif and functional role are similar to the VIP+ cell circuitry and function seen in other brain areas.

I-S CELLS AS A CLINICAL TARGET?

Given their specialized connectivity, disinhibitory control, and potentially beneficial effects on learning and memory, it is worth considering I-S cells as potential targets in clinical studies. As such in this section we will give an overview of some studies that have already investigated I-S cells in various neuropathologies and clinical contexts.

In the CA1 hippocampus, I-S3 cells have been studied in the context of status epilepticus using a mouse model of temporal lobe epilepsy, where these cells showed altered dendritic morphologies and passive membrane properties, and provided a lower inhibition to their postsynaptic targets (David and Topolnik, 2017). Although I-S3 cell densities were preserved in this study, it has been shown in human dentate gyrus that densities of CR+ cells are reduced in epilepsy (Maglóczky et al., 2000). Furthermore, hippocampal CR+ cells have altered morphologies and connectivity to a degree which depends on the severity of the temporal lobe epilepsy (Tóth et al.,

2010; Thom et al., 2012). In another mouse model of epilepsy desynchronization of interneuron firing between regions CA1 and dentate gyrus lead to destabilization of CA1 place cell Shuman et al. (2020). As well, in an optogenetically-induced epilepsy model, it is observed that different motor cortex neuron types follow different trajectories throughout a seizure (Khoshkhoo et al., 2017). While PV+, SOM+, and VIP+ cells all become activated at the onset of a seizure, pyramidal cells exhibit a delay in activation. As well, PV+ and SOM+ cells exhibit a gradual increase in activation throughout the seizure, while VIP+ cell activation peaks near the mid-point of seizures. Interestingly, optogenetic inhibition of VIP+ cells consistently disrupts seizure onset, and reduces the duration of seizures, which highlights VIP+ cells as a potential neuromodulatory target for seizure control (Khoshkhoo et al., 2017). Along these lines, VIP+ cells, alongside PV+ and SOM+ cells in motor cortex, are known to be manipulable through brain machine interfacing (Mitani et al., 2018).

Somatosensory cortex VIP+ cells have also been studied in a mouse model of neuropathic pain. Specifically, VIP+ cells are over-active during neuropathic pain, which parallels the activity of pyramidal cells, but is in contrast to a reduced activity of PV+ and SOM+ cells (Cichon et al., 2017). This combination of effects therefore leans in favor of pyramidal cell hyper-excitability through disinhibition. Moreover, activation of SOM+ cells was sufficient for suppressing pyramidal cell hyperactivity and reversing symptoms of neuropathic pain.

VIP+ cells can also contribute to the pathogenesis of Dravet syndrome, a neurodevelopmental disorder associated with a loss of functional variants of the gene SCN1A that encodes for the Nav1.1 channel subunits (Goff and Goldberg, 2019). More specifically, a mouse model of Dravet syndrome ($Scn1a^{+/-}$) had previously been associated with a loss of excitability in PV+ and SOM+ cells, which both express Nav1.1 (Yu et al., 2006; Ogiwara et al., 2007; Tai et al., 2014; De Stasi et al., 2016; Favero et al., 2018). In addition, in $Scn1^{+/-}$ mouse somatosensory and visual cortices, VIP+ cells that displayed irregular spiking, but not those that displayed continuous adapting spiking, were found to exhibit reductions in gain (Goff and Goldberg, 2019). Notably, the irregular spiking firing pattern did not coincide with whether VIP+ cells co-expressed CR or CCK, nor with whether the morphology was bipolar vs. multipolar. These irregular spiking VIP+ cells were also found to express Nav1.1, which explains their involvement in the pathogenesis, and the irregular spiking pattern was dependent on the activation of the M-type potassium channels (Goff and Goldberg, 2019).

CR+ cells have also been studied in the context of an Alzheimer's disease mouse model where decreased numbers were reported and, in parallel, increased amyloid beta deposits were observed within the axonal fields of CR+ cells (Baglietto-Vargas et al., 2010). These were distinguished from other Cajal-Retzius cells, which also express CR but did not show decreased numbers. However, reduced densities of CR+ cells have not been observed in human dentate gyrus or entorhinal cortex, and these do not appear to co-localize with neurofibrillary tangles, nor their axonal fields with amyloid beta plaques (Brion and Résibois, 1994). On the other hand, CR+ cells in this study did exhibit

reduced dendritic trees and dystrophic fibers in subjects with Alzheimer's disease (Brion and Résibois, 1994). During aging, there is also a down-regulation of genes associated with synaptic transmission in SOM+ and VIP+ cells in human frontal cortex, suggesting age-dependent changes in the functionality of this circuitry (French et al., 2017). As such, stimulating this circuitry (e.g., pharmacologically exciting VIP+ cells) could be a viable option for targeting age-related changes in cognition, such as learning and memory.

SUMMARY AND CONCLUSIONS

Overall, it is clear from this body of literature that I-S cells across cortex share many common principles in their organization, and I-S cell types may be particularly diverse. We highlight this with the illustrations shown in **Figure 1**. Of course, much more information on the detailed I-S circuitry organization is currently available for CA1 hippocampus when compared to other cortical areas, which often characterize cell types through neurochemical marker expression alone. Amongst commonalities we note the tendency of I-S cells to have vertical bipolar-shaped morphologies with dendrites that project across layers. This allows them to receive inputs from multiple layer-specific external projections. We also indicate commonalities in their preferential targeting of SOM+ cells (and PV+ cells to a lesser extent), as well as their ability to disinhibit pyramidal cells through this circuitry, which may create a window for synaptic plasticity to occur. Altogether, these features allow I-S cells across cortex to perform a wide array of different cortical computations during behavior. Because of their common ability to effectively gate synaptic plasticity in pyramidal cells across cortical structures, I-S cells in cortex offer an interesting therapeutic target for neuromodulation by using drugs that specifically modulate the excitability of I-S cells. For example, we can study how global enhancement or suppression of I-S cell activity across the cortex might impact behavior. Along these lines, since similar analogous circuitries exist across the cortex, it seems that I-S cells in general play a role in learning and sensory tuning of pyramidal cell receptive fields. As such, activation of I-S cells across cortical structures *in vivo* offers a target for globally enhancing performance on memory-related tasks.

It is important to note that VIP+ cells exhibit a large diversity in transcriptomic, morphological and physiological properties (Prönneke et al., 2015; Harris et al., 2018; Gouwens et al., 2019; Hodge et al., 2019), which so far made the definition of cell types largely impossible. While I-S cells exhibit commonalities across different cortical structures, these cells might thus still exhibit local differences in functional roles and contributions to network activity. Moreover, different balances of I-S vs. non-I-S VIP+ cells across different brain areas could give the appearance of different region-specific functional contributions to circuit function *in vivo*, when in fact it only appears this way because I-S cells are not sufficiently isolated from non-I-S cells when VIP+ cells are targeted (e.g., CCK+/VIP+ basket cells). Further classification studies based on transcriptomic and morphological characteristics in relation to synaptic connectivity (i.e., inputs and

outputs) across different cortical areas would help to elucidate these aspects of I-S cell diversity.

Another aspect to highlight from these studies is that I-S cells from any particular region appear to receive a mixture of inputs from different distant regions. It has already been postulated (Wang and Yang, 2018), and demonstrated in a model (Yang et al., 2016), that the reason for this is because I-S cells can gate particular external pathways and allow flexible switching between information arriving from different external structures. As such, without I-S cell contributions to circuit function throughout the brain, it is possible that information would not be properly routed during behavioral tasks where integration of different types of sensory stimuli are relevant. This is particularly relevant in Lagler et al. (2016) where basket cell activation during a complex behavioral task was dependent on the level of I-S cell activation, suggesting the existence of complex learned disinhibitory pathways.

To conclude, we present in this review compelling evidence indicating that I-S cells across different cortical areas share many morphological, physiological, and connectivity features, which allows them to contribute in similar ways to network function. As such, we highlight that I-S cells across cortex are a crucial network component that is necessary for supporting sensory discrimination, learning, and memory formation.

AUTHOR CONTRIBUTIONS

AG-M: conceptualization, visualization, writing—original draft preparation, and writing—review and editing. FS and LT: project administration, resources, supervision, and writing—review and editing.

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