Group II metabotropic glutamate receptors modulate sound evoked and spontaneous activity in the mouse inferior colliculus
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CHAPTER I
Introduction

Neuromodulation in auditory processing

Neuromodulation is a relatively broad term. In the broadest sense, it is defined as “a field of science, medicine, and bioengineering that encompasses implantable and non-implantable technologies, electrical or chemical, for the purpose of improving the life of humanity” (Krames et al., 2018). In this dissertation, the focus is on neuromodulation that naturally occurs in the brain and regulates neuronal properties. In the hearing research field, neuromodulation can be viewed as a multitude of mechanisms that change sound processing. Acoustic signals are primarily transmitted by rapid ionotropic receptors that are ligand-gated channels and open upon ligand binding to allow for immediate ion movement and action potential initiation. This fast sound processing—neuronal responses elicited by ionotropic receptors—can be modulated by slower G protein-coupled receptors (GPCRs). GPCRs are slower and exert longer lasting physiological effects because they do not form a pore to allow for direct ion movement, but instead act via G-protein coupled second messenger cascades of biochemical events within the cell. These second messengers can then activate a variety of ion channels and modulate sound processing. Many substances are considered as neuromodulators (Descarries and Mechawar, 2008; Marder et al., 2012), although in the auditory research field “conventional” neuromodulators—acetylcholine, serotonin, noradrenaline (or norepinephrine), and dopamine—received the most attention (Schofield and Hurley, 2018; Hurley, 2019; Schofield and Beebe, 2019).

Additionally, sound processing is subject to neuromodulation by glutamate—the major excitatory neurotransmitter—via its actions on G protein-coupled metabotropic glutamate receptors (mGluRs), and neuromodulation by the major inhibitory neurotransmitter GABA—via G protein-coupled GABA_{$\text{B}$}
receptors (Lu, 2014; Sanchez and Lu, 2017; Shi and Lu, 2017; Tang and Lu, 2018). Thus, glutamate and GABA can be viewed as both neurotransmitters and neuromodulators and such functional diversity is achieved because of their actions on both ionotropic and metabotropic receptors (Reiner and Levitz, 2018).

Building on previous research in our lab, the work described in this dissertation aims to expand the knowledge on modulation by group II mGluRs in the mouse inferior colliculus (IC). In the following Chapter I subsections, I will first provide a general overview of mGluRs, followed by a research summary about group II mGluRs in the auditory structures outside the IC. Then, I will describe in more detail current knowledge about group II mGluRs specifically in the IC and provide a rationale for our interest in these receptors. The Chapter will end with an overview of the specific aims of the dissertation.

**Metabotropic glutamate receptors**

mGluRs belong to the GPCR superfamily, which is the largest superfamily of membrane proteins in mammalian genomes (Katritch et al., 2013). In humans, over 800 GPCRs have been discovered so far, all of which share a structural similarity—the seven-transmembrane domain. Based on amino acid sequence similarity, GPCRs are typically divided into five families, with mGluRs belonging to family C (Hauser et al., 2017; De Oliveira et al., 2019).

mGluRs are further classified into three distinct groups according to amino acid sequence homology, second messenger coupling, and agonist selectivity (Nakanishi, 1992; Niswender and Conn, 2010). Group I includes receptor subtypes 1 and 5, group II – subtypes 2 and 3 (mGluRs2/3), and group III – subtypes 4, 6, 7, and 8. Within each group, amino acid sequence homologies are more than 70%, while between groups, homology is on average 45% (Willard and Koochekpour, 2013).

All mGluRs are stable dimers, meaning that a receptor consists of two mGluR proteins. All mGluRs can form homodimers in which the proteins are identical. Additionally, certain combinations where the two proteins differ form heterodimers (Doumazane et al., 2011; Lee at al., 2020). For example,
group II mGluRs, which are the focus of this dissertation, occur in several heterodimers, such as mGluR2/4 and mGluR2/7 (Habrian et al., 2019; Lee et al., 2020). Additionally, group II mGluR subtypes can heterodimerize with each other (Levitz et al., 2016; Lee et al., 2020). Dimeric structures are necessary for receptor activation by glutamate and are related to complex regulation of glutamate affinity, dynamic range of receptor sensitivity, and strength of receptor activation (Kniazeff et al., 2004; El Moustaine et al., 2012; Levitz et al., 2016; Habrian et al., 2019).

mGluRs are widely expressed in both rodent and human brains (Ribeiro et al., 2017). These receptors also have widespread distribution outside the central nervous system, playing roles in the gastrointestinal tract, endocrine, immune, musculoskeletal, and other systems (Julio-Pieper et al., 2011). mGluRs are thoroughly investigated because ligands targeting them have a potential for clinical development in several psychiatric and neurological disorders (Li et al., 2015; Nicoletti et al., 2015; Chaki, 2017; Ribeiro et al., 2017; Yang et al., 2017; Mazzitelli et al., 2018; Crupi et al., 2019). However, currently there are no FDA approved mGluR targeting drugs (Habrian et al., 2019).

mGluRs are also expressed throughout the auditory system and, based mostly on *in vitro* electrophysiological studies, are involved in modulation of several processes such as synaptic transmission, plasticity, and maintenance of excitation-inhibition balance (Lu, 2014; Tang and Lu, 2018). mGluRs might be important targets for pharmacological treatment of auditory disorders. However, our knowledge regarding mGluR roles in hearing is currently very limited.

**Group II metabotropic glutamate receptors in the auditory system**

With a focus on findings from mammalian animal models, in this subsection I will summarize our knowledge on group II mGluRs in the auditory brain regions outside the IC. In a subsequent section I will focus on the IC and provide a rationale for our interest in group II mGluRs in the IC.

Outside the IC, group II mGluR expression has been found in several central and peripheral auditory structures. mGluR2 (but not mGluR3) expression was demonstrated at the post-synaptic side of
the inner hair cell ribbon synapses in 6- to 12-weeks-old mice (Klotz et al., 2019). mGluR2/3 staining was shown throughout the granule cell domain of the cochlear nuclei, as well as in Golgi and unipolar brush cells of the dorsal cochlear nucleus (Petalia et al., 1996; 2000). In the medial nucleus of the trapezoid body, mGluRs2/3 were preferentially localized in astrocytes surrounding the calyces of Held during the early post-natal days, but were found in the pre-synaptic calyces of Held and post-synaptic principal cells in adult rats (Elezgarai et al., 2001). In the lateral superior olivary nucleus, mGluR2/3 labelling was dense in post-natal day 4 (P4) rats, but decreased in P18 animals (Nishimaki et al., 2007). Additionally, mGluR2/3 labelling was found in the medial geniculate body of young rats, especially in the marginal zone (Petalia et al., 1996). Finally, concentrated mGluR2 labelling was demonstrated in layer 4 of the primary auditory cortex in P10-19 mice, with weaker expression evident in layer 5; lower layer 5B was more heavily labeled than upper layer 5A (Covic and Sherman, 2011; Lee and Sherman, 2012; Venkatadri and Lee, 2014).

In vitro electrophysiological recordings from various auditory structures shed some light on the physiological mGluR2/3 roles. Group II mGluRs modulate both excitatory (Lee and Sherman, 2012) and inhibitory (Doleviczenyi et al., 2005; Nishimaki et al., 2007; Liu et al., 2014) synaptic transmission. Moreover, these receptors have both pre-synaptic (Doleviczenyi et al., 2005; Nishimaki et al., 2007; Lee and Sherman, 2012; Liu et al., 2014) and post-synaptic (Ene et al., 2003; Irie et al., 2006; Lee and Sherman, 2012) effects.

**Group II metabotropic glutamate receptors in the inferior colliculus**

To date, very few studies tested the role of group II mGluRs specifically in the IC. Our interest in group II mGluRs arises from recent results showing that behavioral signs of tinnitus in mice were suppressed by intraperitoneal administration of mGluR2/3 agonist LY354740 (Galazyuk et al., 2019). Furthermore, intravenous LY354740 injection reduced spontaneous activity in the IC. It is possible tinnitus was suppressed because of this reduced spontaneous firing. However, systemic mGluR2/3
activation does not inform us on the origin of the observed effect. As described above, group II mGluRs are expressed in many auditory structures. These receptors were found in other brain areas as well (Wright et al., 2001; McOmish et al., 2016). The IC is an integrative hub of the central auditory system: it receives multiple ascending and descending auditory projections, as well as some non-auditory inputs (Casseday et al., 2002; Gruters and Groh, 2012). Thus, spontaneous activity suppression in the IC following systemic mGluR2/3 activation could result from mGluR2/3 actions outside the IC itself. The research described in this dissertation utilized local mGluR2/3 activation and tested a hypothesis that receptors expressed within the IC exert modulatory actions in vivo.

Farazifard and Wu (2010) studied group II mGluR modulatory roles in vitro. Whole-cell patch-clamp recordings were obtained from the IC central nucleus (ICc) slices prepared from 8 to 17-days-old Long Evans rats. In voltage-clamp experiments, AMPA receptor-mediated excitatory post-synaptic currents (EPSCs) and GABA_{A} receptor-mediated inhibitory post-synaptic currents (IPSCs) were pharmacologically isolated and measured. EPSCs and IPSCs were evoked by an electrical stimulation of the lateral lemniscus (LL; a major afferent pathway to the ICc) before and after bath application of mGluR2/3 agonist LY379268. It was shown that 10-20 nM LY379268 reduced the amplitudes of both EPSCs and IPSCs to about 50% of the control (pre-drug) level. Such results indicate that mGluRs2/3 regulate both glutamatergic and GABAergic synaptic transmission in the ICc.

**Dissertation aims and summary**

The research described in this dissertation aims to expand our limited knowledge of the functions of group II mGluRs in the IC—a midbrain structure that is a major integration region of the central auditory system. We investigated how these receptors modulate sound-evoked and spontaneous firing in the mouse IC in vivo.
Specific Aim 1: Establish and validate normal-hearing transgenic mouse model for optogenetic cell type identification.

An in vitro study by Farazifard and Wu (2010) provides rationale that group II mGluRs modulate both glutamatergic and GABAergic synaptic transmission in the IC. To test whether mGluRs2/3 modulate both excitatory and inhibitory IC neurons in vivo, we needed a reliable method for cell type identification. As these cells cannot be distinguished based on their electrophysiological properties (Ono et al., 2017), we aimed to employ optogenetic cell type identification (Ono et al., 2016). To achieve this goal, we had to establish an optogenetics setup in the lab.

Even through transgenic mice suitable for optogenetic cell type identification were commercially available, C57BL/6J genetic background of these animals is known to cause hearing abnormalities (Zheng et al., 1999; Noben-Trauth et al., 2003; Ohlemiller et al., 2016). For our study, we aimed to test normal-hearing animals. Thus, we established a breeding scheme to avoid hearing abnormalities. We performed immunostaining and tested hearing thresholds to validate transgenic mice on a mixed genetic background.

Chapter II describes the research verifying that our newly established optogenetics setup works as expected and allows for cell type identification in normal-hearing transgenic mice.

Specific Aim 2: Determine the role of group II mGluRs in sound-evoked firing modulation in the mouse IC.

We hypothesized that group II mGluRs might be involved in modulation of sound level processing in IC neurons. Under physiological conditions, when sound level increases, glutamate release becomes more substantial. Clearance of glutamate by uptake mechanisms is delayed during high-rate synaptic activity (Scanziani et al., 1997). This results in increased glutamate concentration in and around the synapse and more mGluRs can be activated, possibly including receptors at the peri-synaptic and extra-synaptic membranes (Luján et al., 1997; Tamaru et al., 2001; Jin et al., 2017; 2018). Thus, mGluRs2/3 may modulate sound-level processing, especially at higher sound intensities. To test this
hypothesis, we performed in vivo single neuron extracellular recordings before and after pharmacological group II mGluR activation in the mouse IC. In vivo recordings allowed for sound level processing testing assessed by the rate-level functions. We optogenetically determined cell types to test a hypothesis that mGluRs2/3 modulate both inhibitory and excitatory IC neurons. Additionally, we labelled the recording sites to identify locations of recorded neurons within the IC.

Chapter III describes the results showing that group II mGluRs modulate sound level processing in a subset of optogenetically identified GABAergic and non-GABAergic cell types. mGluR2/3 activation resulted in steeper rate-level function slopes, lower thresholds, and increased maximum firing rates. Neurons affected by pharmacological group II mGluR activation were distributed throughout the IC area tested, suggesting a widespread mGluR2/3 distribution in the mouse IC.

Specific Aim 3: Determine the role of group II mGluRs in spontaneous firing modulation in the mouse IC.

Based on previous work in the lab (Galazyuk et al., 2019), we hypothesized that group II mGluRs might be involved in spontaneous firing modulation. In contrast to previous research which utilized systemic group II mGluR activation, we aimed to test the effect of local receptor activation in the IC in order to avoid possible mGluR effects on other brain areas which provide inputs to the IC.

Chapter IV describes the results showing that spontaneous firing rates were elevated in IC neurons following group II mGluR pharmacological activation.

In the final Chapter V, I will provide concluding remarks summarizing how the findings described in this dissertation move the field forward by uncovering the role of group II mGluRs in sound processing. Additionally, this work has a broader value for the hearing research field outside mGluR-related projects. First, a wide variety of auditory research could benefit from our validation of the transgenic mouse model in normal hearing animals. Second, our fairly novel topical drug delivery method could be utilized to study the effect of various pharmacological agents on sound processing and spontaneous firing in the IC. Finally, Chapter V will summarize how our results suggest avenues for
future research directions. Better understanding of mGluR modulatory roles is crucial in opening pathways for mGluR-targeting drugs to be exploited to treat hearing disorders.
CHAPTER II

Validating normal-hearing transgenic mouse model and optogenetically identifying cell types

Introduction

Zhao et al. (2011) established the VGAT-ChR2-EYFP transgenic mouse line, which expresses channelrhodopsin-2 (ChR2) fused with an enhanced yellow fluorescent protein (EYFP) under the control of the vesicular γ-aminobutyric acid (GABA) transporter (VGAT) promoter. VGAT is expressed in GABAergic and glycinergic neurons and is responsible for loading GABA and glycine from neuronal cytoplasm into synaptic vesicles (Sagné et al., 1997; Gasnier, 2000). Thus, ChR2-EYFP fusion protein is targeted to inhibitory neurons. ChR2 is a naturally occurring algal protein, which is a light-gated cation channel (Nagel et al., 2003). When illuminated by ~470 nm blue light, it permits several monovalent (Na⁺, K⁺, H⁺) and some divalent cation (e.g. Ca²⁺) inward currents which last as long as the illumination. These currents cause depolarization large enough to stimulate action potentials (Boyden et al., 2005; Butler, 2012). VGAT-ChR2-EYFP mice express popular H134R variant of ChR2. This single point mutation at position H134 was developed to generate larger photocurrents than wild-type ChR2 (although it slows down channel kinetics; Nagel et al., 2005). EYFP fused to the C terminus of ChR2 allows for visual identification of ChR2-positive cells in certain experimental designs and enabled easy phenotyping of ChR2-EYFP-positive mice in our study (described below in the Methods).

We wanted to take advantage of this cell-type specific transgenic mouse line to optogenetically determine whether the recorded neurons are GABAergic or not. However, this mouse line was developed on the commonly used C57BL/6J strain background, which is not ideal for hearing research aimed at
understanding normal auditory function. These animals have early onset hearing deficits due to homozygosity for the Cadherin 23/Otocadherin (Cdh23) point mutation (G to A transition) at nucleotide 753 (also known as ahl mutation; Zheng et al., 1999; Noben-Trauth et al., 2003; Ohlemiller et al., 2016).

Functional cadherin 23 protein is essential for normal mechanotransduction, which is a process of converting mechanical forces arising from sound waves into electrochemical signals (Muller, 2008). Mechanotransduction is accomplished by the inner ear hair cells, specifically, highly organized stereocilia which move towards the tallest row of stereocilia in response to sound waves and increase a probability of opening cation-selective channels. Cadherin 23 is critical for both development and maintenance of several structures involved in this process (Siemens et al., 2004; Lagziel et al., 2005; Michel et al., 2005; Kazmierczak et al., 2007). Thus, it is not surprising that mutation of this important gene compromises hearing function.

To avoid hearing abnormalities found in C57BL/6J background, we crossed VGAT-ChR2-EYFP animals with CBA/CaJ mice and used F1 generation hybrid offspring for all experiments. CBA/CaJ mice are homozygous for the wild-type Cdh23 gene and maintain normal hearing through their lifespan. A single copy of the wild type Cdh23 allele is sufficient for normal auditory processing until at least one year of age (Frisina et al., 2011; Burghard et al., 2019; Lyngholm and Sakata, 2019).

Because our study is the first to cross VGAT-ChR2-EYFP mice with CBA/CaJ mice, we had to validate these animals to make sure that a mixed genetic background does not cause any unexpected issues. First, we performed immunostaining to verify that ChR2-EYFP transgene is correctly expressed in inhibitory neurons of mixed genetic background mice. Second, we compared auditory response (ABR) thresholds between ChR2-EYFP-positive and ChR2-EYFP-negative littermates to confirm that ChR2-EYFP transgene does not interfere with normal auditory processing. Finally, we performed pilot experiments to determine whether our lab’s newly established optogenetics setup produced the expected functional results in mixed genetic background VGAT-ChR2-EYFP mice.
Materials and methods

Throughout this dissertation, all procedures were conducted in accordance with the Northeast Ohio Medical University Institutional Animal Care and Use Committee and NIH guidelines. Efforts were made to minimize animal suffering as well as the number of animals used. All animals were housed on a 12-hour day/night cycle with ad libitum access to food and water.

All mice were bred in our animal facility, with breeding pairs purchased from the Jackson Laboratory (Bar Harbor, ME): two males hemizygous for channelrhodopsin-2 in inhibitory neurons (B6.Cg-Tg(Slc32a1-COP4*H134R/EYFP)8Gfng/J, also known as VGAT-ChR2-EYFP; stock # 014548) and six female CBA/CaJ mice (stock # 000656). Neonatal (P0-P3) F1 generation hybrid offspring were phenotyped by briefly placing them under Zeiss AxioImager.M2 microscope (Oberkochen, Germany) and illuminating with a blue ~470 nm light. The brains of the offspring carrying the transgene exhibited green fluorescence through the skull. These animals were used in all experiments.

Immunostaining and imaging

To test whether F1 hybrid offspring between VGAT-ChR2-EYFP and CBA/CaJ mice correctly expressed channelrhodopsin-2 in inhibitory neurons, two four-month-old mice (one male and one female) from different breeder pairs were sacrificed for immunostaining. Each animal was deeply anesthetized with an overdose of Fatal-Plus (>100 mg/kg, i.p., Vortech, Dearborn, MI) and perfused transcardially with 0.1 M PB to clear the blood, then with 50 ml of 4% paraformaldehyde in 0.1 M PB, followed by 50 ml of the same fixative containing 10% sucrose. Brains were removed and post-fixed in fixative containing 25% sucrose overnight. The following day, the midbrain was trimmed out, frozen, and coronal sections were collected from the IC on a sliding microtome at a thickness of 40 µm. Free-floating sections were rinsed in PBS, then permeabilized with 0.2% Triton X-100 in PBS for 30 minutes at room temperature. Nonspecific staining was blocked by incubating sections in a solution containing 10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for one hour at room temperature. A primary antibody solution containing 1% NGS and 0.2% Triton X-100 in PBS was applied overnight at 4°C.
Primary antibodies used were anti-GAD67 (Millipore Sigma, MAB5406; 1:250; to label GABAergic cells) and anti-GFP (Thermo Fisher Scientific, Waltham, MA; A10262; 1:400; to enhance EYFP fluorescence- the structure of GFP and EYFP are similar enough that anti-GFP antibodies can recognize EYFP). The next day, slides were rinsed in PBS, then a secondary solution containing Alexa Fluor 488-labeled goat anti-chicken (Molecular Probes, A11039; 1:100) and Alexa Fluor 546-labeled donkey anti-mouse (Molecular Probes, A10036; 1:100) in PBS was applied for one hour at room temperature. Sections were rinsed in PBS, then mounted from a 0.2% gelatin solution onto gelatin-coated slides. Slides were air dried then coverslipped with DPX mounting medium (Sigma-Aldrich). Photomicrographs were taken using a Zeiss AxioImager.Z2 microscope with a 63X oil-immersion objective (NA = 1.4) and an Apotome 2 to provide optical sectioning at 0.5 µm depth intervals. The high magnification images shown below in Results are maximum intensity projections of collected stacks. Adobe Photoshop was used to crop and colorize images, and globally adjust levels when necessary.

**Auditory brainstem response (ABR) testing**

To confirm that ChR2-EYFP transgene does not interfere with normal auditory processing, we compared ABR thresholds between three ChR2-EYFP-positive and three ChR2-EYFP-negative littermates from two different breeder pairs. At the time of ABR testing, the animals were about 7.5 months old. ABR recordings were obtained under ketamine/xylazine anesthesia (100 mg/kg and 10 mg/kg, respectively). Stainless-steel electrodes (LifeSync Neuro, Coral Springs, FL; product S02918-B) were placed subdermally, one at the ventral edge of each pinna, one along the vertex and the fourth one at the tail. The speaker (LCY-K100 Ribbon Tweeter; Ying Tai Trading, Hong Kong) was placed 10 cm from the animal’s head. Custom OpenEx software controlling the TDT RZ6 processor generated all stimuli and recorded responses. Tone pips were 5 ms duration (with 0.5 ms rise/fall time), at 4, 8, 12.5, 16, 20, 25, and 31.5 kHz, from 75 to 5 dB SPL (5 dB SPL steps, 300 repetitions at each sound level), and presented at 50 Hz. ABR threshold was considered the lowest sound level at which a waveform could be observed.
**Optogenetic cell type identification**

*In vivo* optogenetic cell type identification in the inferior colliculus (IC) was first described by Ono et al. in 2016, and then utilized in subsequent studies (Ono et al., 2017; 2018; Ma et al., 2020). The same transgenic animals were also used to determine the cell types *in vitro* in the IC (Naumov et al., 2019) and in the hippocampus *in vivo* (Xu et al., 2016). To accomplish optogenetic cell type identification in our laboratory, we established an optogenetics setup. 473 nm laser (Ready Lasers, Anaheim, CA; MBL-III-473 diode-pumped solid-state laser) was coupled to a fiber collimator and 400 µm fiber patch cable. Blue laser light was used to illuminate the craniotomy over the IC (the surgery is described in Chapter III Methods) via a 2 cm long, 400 µm diameter fiber optic cannula (Thorlabs, Newton, NJ; CFMC14L20). The tip of the cannula was positioned about 2 mm above the craniotomy. BrainWave software (DataWave Sciworks, Loveland, CO; model DW-USB6432-37-A) generated a TTL pulse to control the laser output timing. Laser intensity (at the tip of the cannula) was calibrated with a photodiode power sensor (Thorlabs, S140C) connected to a digital power and energy meter (Thorlabs, PM100D) and then manually adjusted for each experiment depending on the depth of the recording site (intensity varied from about 10 to 45 mW).

The specifics of acoustic stimulation and electrophysiological recordings will be discussed in detail in Chapter III Methods. Here, in Chapter II, I will focus on the protocol used for optogenetic cell type identification. When light pulses (30 ms duration, presented every 3 seconds, 15 repetitions) evoked firing, a neuron was classified as GABAergic. If light did not evoke firing, in some neurons further testing was completed to verify that light stimulation indeed reached the area of interest. First, responses to white noise (70 dB SPL, 200 ms duration, presented every 3 seconds, 15 repetitions) were recorded. If a neuron did not respond to white noise, instead a pure tone was used at the neuron’s characteristic frequency (defined as the frequency at which the lowest sound intensity evoked a response). Then, the same sound protocol was presented together with 30 ms duration light pulse. The light onset was adjusted to occur about 5-10 ms before the neuron’s response onset. The total number of spikes was compared between the
sound only and sound with light conditions. Light typically reduced the number of spikes by more than 50%, verifying light stimulation efficiency.

Results

Channelrhodopsin-2 is correctly expressed in inhibitory neurons of mixed genetic background VGAT-ChR2-EYFP mice.

We examined IC neurons double-labelled with anti-GAD67 and anti-GFP antibodies, which are markers for GABAergic neurons and ChR2, respectively. Figure 1 shows representative images. Consistent with previous studies, we found that neurons that expressed EYFP routinely co-expressed GAD67 in cell bodies (Zhao et al., 2011; Ono et al., 2016; Naumov et al., 2019). We also observed punctate label, probably representative of EYFP-expressing axon terminals, which were also stained for GAD67. Our results confirmed that the ChR2-EYFP transgene is correctly expressed in mixed CBA/CaJ x C57BL/6J genetic background mice.
Figure 1. Channelrhodopsin-2 expression in inhibitory inferior colliculus neurons of mixed CBA/CaJ x C57BL/6J genetic background mice. (A) A low magnification image shows that the majority of inhibitory cells labeled with anti-GAD67 antibody (red) also express ChR2-EYFP labelled with anti-GFP antibody (green). White arrows show examples of double-labelled cells. The blue arrow shows a rare example of a cell that labeled with the anti-GAD antibody but did not express EYFP. Scale = 20 µm. (B) High magnification images of double-labelled cells. Scale = 20 µm.

Mixed genetic background VGAT-ChR2-EYFP mice have normal hearing.

In Figure 2 we plotted auditory brainstem response thresholds obtained from three ChR2-EYFP-positive and three ChR2-EYFP-negative littermates. For statistical analysis, hearing thresholds were transformed using the Box-Cox procedure to improve normality (Box and Cox, 1964). A mixed-effects ANOVA indicated a significant independent main effect for the stimulus frequency ($F_{6,66} = 115.6$, $p < .0001$), whereas the main effect of the genotype, as well as the interaction between the frequency and the genotype, were not significant ($F_{1,4} = .002$, $p = 0.97$, $F_{6,66} = .58$, $p = 0.75$, respectively). Post-hoc
pairwise comparisons of estimated marginal means (emmeans; also known as least squares means) showed no significant differences in hearing thresholds between ChR2-EYFP-positive and ChR2-EYFP-negative mice at all frequencies tested (all false discovery rate method (Benjamini and Hochberg, 1995) adjusted p-values > .22). These data demonstrate that at least until 7.5 months of age, F1 generation hybrid offspring from VGAT-ChR2-EYFP and CBA/CaJ mice have normal hearing thresholds.
Figure 2. Auditory brainstem response thresholds (expressed as back-transformed least squares means) were not significantly different between ChR2-EYFP-positive (ChR2-EYFP+) and ChR2-EYFP-negative (ChR2-EYFP-) littermates. Error bars represent 95% confidence intervals of means.

Cell type identification is feasible in mixed genetic background VGAT-ChR2-EYFP mice.

To evaluate a neuron’s firing activity in response to light presented alone or light combined with sound stimulus, we created peri-stimulus time histograms (PSTHs) for each cell. PSTH (also known as peri-event time histogram or post-stimulus time histogram) is a commonly used plot for easy visualization of neuronal spiking activity over time—before, during, and after stimulus presentation. The entire time period is divided into small time bins, e.g. 1 ms. The same stimulus is typically repeated over several trials and the plot represents total spikes which occur in each time bin for all trials.

Figures 3 and 4 show example data from two neurons which were identified as GABAergic and non-GABAergic using our newly established optogenetics setup. It is evident that a neuron shown in Figure 3 was activated by light: several spikes occurred at the beginning of the light pulse. As a side note, I would like to point out that spontaneous spikes were absent after light presentation. Suppression of spontaneous firing is known to commonly occur after sound presentation (Voytenko and Galazyuk, 2010; 2011; Galazyuk et al., 2017; 2019). In Figure 4 an example non-GABAergic neuron is demonstrated. Panel A shows that a neuron responded to sound: many spikes occurred at the onset of sound stimulus.
When a light was presented together with sound (Figure 4B), spikes were suppressed during light stimulus and instead occurred at about 15 ms after the light offset. Such spiking delay could be due to post-inhibitory rebound. This is a phenomenon where following a hyperpolarization (either intrinsic or from inhibitory inputs), a neuron’s membrane potential rebounds above the resting level and produces strong enough depolarization to result in action potentials. Post-inhibitory rebound spikes were described in several brain areas (Getting, 1989), including the IC (Kasai et al., 2012; Sun and Wu, 2008).

Overall, these results confirm that our newly established optogenetics setup works as expected and that F1 offspring from a cross between hemizygous VGAT-ChR2-EYFP male and wildtype CBA/CaJ female express functional ChR2.
**Figure 3.** A neuron activated by light; classified as GABAergic. The rectangular area shaded blue indicates light presentation. The y-axis indicates a total number of spikes in each 1 ms time bin over fifteen presentations of 30 ms duration light pulses.

**Figure 4.** A neuron suppressed by light; classified as non-GABAergic. Rectangular boxes shaded grey indicate sound presentation, rectangular boxes shaded blue indicate light. The y-axis indicates a total number of spikes in each 1 ms time bin over fifteen presentations of either sound alone (A) or sound and light (B) stimuli.
Discussion

Transgenic mice are often developed on C57BL/6J genetic background. In fact, this is the most widely used strain in biomedical research (Bryant, 2011). Though these animals may be well-suited for many research areas, they are not suitable for hearing projects aimed at studying normal hearing animals. C57BL/6J mice have abnormalities that occur early in life, in fact earlier than is often recognized. For example, the medial olivocochlear efferent feedback system’s activity is gone by eight weeks of age (Zhu et al., 2007). Sinclair et al. (2017) reported that the medial olivocochlear system functions abnormally just after hearing onset at about two weeks. Therefore, future studies aimed at studying normal-hearing animals and utilizing VGAT-ChR2 transgenic mice could rely on our validation of these animals on a mixed C57BL/6J x CBA/CaJ genetic background. We demonstrated that F1 generation offspring express ChR2-EYFP transgene in inhibitory neurons, have normal hearing thresholds, and that ChR2 is functional in these animals.

Instead of using F1 generation offspring, it could be possible to establish a mouse colony homozygous for the wild-type Cdh23 gene and hemizygous for the ChR2-EYFP transgene. Although that would require genotyping and would take several generations of mice, this might be a useful long-term solution. The breeding solution which we implemented requires phenotyping completion during the very first post-natal days (P0-P3), otherwise fluorescence is not clearly visible through the skull. This necessity might be viewed as inconvenient in some laboratories.

For optogenetic cell type identification, laser light was delivered from a fiber optic cannula positioned just above the IC surface. If most recordings were obtained from the deepest IC areas (which was not the case in the present study; described in Chapter III), such stimulation might be problematic due to diminished light penetration to the recording site. A possible solution could be a recently described custom-made glass optrode, which allows for light to be delivered through a recording glass electrode (Ono et al., 2018). In that setup, LED can be used as a light source. It is a cheaper option than a laser and might be attractive for some researchers. During our initial steps when establishing an optogenetics setup
in the lab, we also attempted to use LED as light source. However, as we delivered light to the brain surface, LED light power was insufficient to activate channelrhodopsin-2. As a result, we purchased the laser.
CHAPTER III

The role of group II mGluRs in sound-evoked firing modulation

Introduction

IC has a strategic position in the central auditory system: it receives multiple ascending and descending auditory projections, as well as some non-auditory inputs (Gruters & Groh, 2012; Casseday et al., 2002). Sound intensity coding is one of the tasks of IC neurons. This task is achieved by two main kinds of neurons—monotonic and non-monotonic (Rees et al., 1992). Monotonic neurons display a continuous increase in firing rate over a large intensity range. Non-monotonic neurons increase firing initially (with rising sound intensity) but eventually decrease firing. These neuronal properties are described with rate-level functions (RLFs), which are the curves showing the total number of spikes as a function of sound intensity. It is thought that monotonic neurons receive increasing excitatory signals as sound intensity rises. Non-monotonic neurons on the other hand, receive additional inhibitory signals at high sound levels (Sivaramakrishnan et al., 2004; Grimsley et al., 2013).

We investigated the effect of group II mGluR modulation on sound level processing in IC neurons. We hypothesized that sound level coding will be affected when higher sound levels result in more glutamate in/around the synapses and, consequently, an increased likelihood of extra- and peri-synaptic mGluR activation. The peri-synaptic region can be defined as a ring-shaped area of 100–200 nm surrounding the postsynaptic density that contains ionotropic receptors. The extra-synaptic area extends beyond the peri-synaptic region (Scheefhals and MacGillavry, 2018).
We tested whether mGluR2/3 activation impacts sound level processing in GABAergic and non-GABAergic cell types. As detailed in Chapter I, previous data suggest that group II mGluRs modulate both excitatory and inhibitory synaptic transmission (Farazifard and Wu, 2010). Thus, we hypothesized that both GABAergic and non-GABAergic cell types will be affected by mGluR2/3 agonist. We recorded single neuron responses and evaluated the RLFs to determine whether the shape of the curves changes following group II mGluR activation.

**Materials and methods**

*Experimental design*

The specifics of each experimental procedure will be discussed in detail in the following sections. Here, we provide a short overview of the experimental design. After locating a well-isolated single neuron (with a signal-to-noise ratio of at least about 5:1) and determining its characteristic frequency (CF), the cell type (GABAergic versus non-GABAergic) was then identified. Then, pre-drug rate-level functions (RLFs) were recorded three or four times. The drug (or the drug vehicle) was then applied topically on the craniotomy. In some experiments iontophoresis was used instead, in order to verify the results obtained using topical application. Post-drug RLFs were then recorded several times. Recording sessions typically continued for 1.5 - 2 h with three sessions conducted on each animal on separate days. At the end of each recording session, the recording site was labelled, then craniotomy rinsed with saline and covered with Kwik-Sil (World Precision Instruments, Sarasota, FL). After the end of the last recording session, mice were sacrificed for immunohistochemistry for the recording site and IC subdivision identification.

*Surgery*

Aseptic techniques were used for all surgical procedures. Surgical anesthesia was induced with isoflurane (3% induction, about 1.5% maintenance in air) and the mouse was placed in a stereotaxic...
alignment system (David Kopf Instruments, Tujunga, CA; model 1900). The eyes were covered with an ophthalmic lubricating ointment to avoid corneal drying (Puralube Vet Ointment, Dechra, Overland Park, KS). The skull surface was made clear of debris and dry. One scoop of C&B Metabond powder (Parkell, Edgewood, NY; product S399), 6 drops of C&B Metabond Quick Base (product S398) and one drop of C&B Universal catalyst (product S371) were mixed in a ceramic dish with a wooden stick and applied to the skull—with the exception of the area above the right inferior colliculus (IC)—to allow access for all subsequent electrophysiological recordings. A head-post was assembled from a hexagonal stainless-steel standoff (Unicorp, Orange, NJ; product P129M09F16256) and a screw (Bolt Depot, Hingham, MA; product 7662) threaded on one end. The head-post was held vertically and lowered onto the skull (~1.4 mm anterior from bregma) by a custom-made head-post holder, which was attached to the stereotaxic alignment system’s tool holder (David Kopf Instruments, product 1900-54-A). More Metabond mixture was applied around the head-post to secure it. A round craniotomy was made with 1.8 mm diameter trephine drill bit (Fine Science Tools, Foster City, CA; product 18004-18) over the right IC and covered with Kwik-Sil (World Precision Instruments). The surgical site was treated with an antibiotic cream and 0.5% bupivacaine, whereas ketoprofen (5 mg/kg) served as a systemic analgesic. The animal recovered from surgery for at least one day before the start of electrophysiological recordings.

**In vivo extracellular recordings**

Electrophysiological recordings were conducted in a sound-insulated chamber (Industrial Acoustics Company, Bronx, NY). The mouse was briefly anesthetized with 3% isoflurane in air and then administered an intramuscular hind leg injection of a mixture of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg). The head-post was secured in a custom-made holder and served as the ground. The eyes were first covered with an ophthalmic lubricating ointment to prevent ocular dryness, and then with small pieces of black light-impermeable material to avoid laser light stimulation. The animal’s temperature was maintained at 37°C with a feedback loop-controlled blanket (Harvard Apparatus, Holliston, MA; model 50-7220F).
Single-unit extracellular spikes were recorded with micropipettes which were pulled from quartz glass tubes (Sutter Instrument, Novato, CA; product QF100-50-10) using a laser electrode puller (Sutter Instrument, model P-2000). Each micropipette tip was broken by a gentle hit against a hanging Kimwipe to create a tip diameter of 1-2 µm. The micropipettes were filled with a dye (see below ‘Recording site labeling’) and 1 M NaCl (electrode resistance was about 15-40 MΩ). Additional control experiments were performed using iontophoresis, which required manufacturing multi-barrel electrodes (see below ‘Manufacturing multi-barrel electrodes and iontophoresis’).

The craniotomy was viewed under a stereo microscope (Leica Microsystems, Buffalo Grove, IL; model M80) to remove the dura at the site of the electrode penetration and to position the electrode above the IC using a motorized micromanipulator (Sutter Instrument, model MP-285). The electrode was advanced into the IC in 2 - 4 µm steps by a micropositioner (David Kopf Instruments, model 2660). Extracellular action potentials were amplified (5000X) and bandpass filtered (1000 - 3000 Hz) with an extracellular preamplifier (Dagan Corporation, Minneapolis, MN; model 2400A), then digitized at 40 kHz sampling rate (DataWave Sciworks).

**Acoustic stimulation**

Sound intensities were calibrated using custom software written in MATLAB (TytoLogy by S.J. Shanbhag; [https://github.com/TytoLogy](https://github.com/TytoLogy)) and Brüel and Kjær (Duluth, GA) equipment: ¼ inch condenser microphone (model 4939), preamplifier (model 2670), and conditioning amplifier (Nexus model 2690). All sound stimuli were generated at 500 kHz sampling rate and 16-bit depth using BrainWave software (DataWave Sciworks), attenuated (Tucker-Davis Technologies, Alachua, FL; PA5 programmable attenuator), filtered (Krohn-Hite, Brockton, MA; model 3384), and amplified (Parasound Products, San Francisco, CA; model A23). Sounds were presented free-field, 45° to the left of the mouse mid-sagittal plane and contralateral to the recorded right IC. The distance between the animal and the speaker (LCY-K100 ribbon tweeter; Ying Tai Trading) was 10 cm.
All tones were 50 ms in duration (5 ms rise/fall times) and were presented at a rate of 4/s, except for the optogenetic cell type identification as described in Chapter II. Tones used as search stimuli were presented at 70 dB SPL from 6 to 63.3 kHz in 1/4-octave steps. The same tone frequencies were used to determine the CF; sound intensity was reduced in 5 or 10 dB SPL steps. For the RLFs, a tone was presented at the neuron’s CF from 0 to 80 dB SPL, in 5 dB SPL increments. Each intensity was repeated 10 times.

*Pharmacological group II mGluR activation*

To pharmacologically activate group II mGluRs, which include subtypes 2 and 3, we used a prototypical potent and specific agonist LY354740 (Tocris, Minneapolis, MN; product 3246; Monn et al., 1997; Schoepp et al., 1999). In order to verify the results obtained with LY354740, we utilized another widely used group II mGluR agonist LY379268 (Tocris, product 2453; Monn et al., 1999). Both compounds were prepared in sterile water and pH increased to 8.5 using 1 N NaOH. The drug vehicle was used in control experiments. One µl of drug (or vehicle) solution was applied directly to the craniotomy above the IC using a pipettor. 90 µM LY354740 was used for the initial experiments and later the concentration of the drug was lowered to 30 µM. LY379268 was used at a concentration of 30 µM. These concentrations were selected to avoid possible non-specific drug effects (Schoepp et al., 1999). More details regarding the choice of concentrations and agonist properties are provided in the Discussion. For iontophoretic application, the concentration of LY354740 was 5 mM as in previous studies (Copeland et al., 2012; 2013; 2017).

*Manufacturing multi-barrel electrodes and iontophoresis*

Single micropipettes were pulled from quartz glass tubes (Sutter Instrument, product QF100-50-10) and then bent (to an angle of about 40°, at approximately 5 mm away from the tip) using a laser electrode puller (Sutter Instrument, model P-2000; part FPS is a special P-2000 accessory for bending the tips). The
micropipette tips were gently struck against a hanging Kimwipe, breaking-off to create a tip diameter of about 1-2 µm.

3-barrel micropipettes were prepared by placing about 1 cm length, 4 mm diameter polyolefin heat shrink tubing (Innhome, Ontario, CA; product HS-532B) about 1 cm away from the ends of 3-barrel borosilicate glass capillary tubes (Sutter Instrument, product FG-G3BF100-75-10) and shrinking it with an infrared lamp. The 3-barrel micropipettes were then pulled on a Gravipull-3 micropipette puller (Kation Scientific, Minneapolis, MN) and broken to an overall tip diameter of about 5 µm using a previously described method (Dondzillo et al., 2013).

Single and 3-barrel micropipettes were aligned and then glued together in a piggyback configuration (Havey and Caspary, 1980) by first applying black super glue (StewMac, Athens, OH) and then 5 Minute Epoxy (Devcon, Hartford, CT) using a similar method as described by Dondzillo et al. (2013).

Iontophoretic micropipettes were connected to Dagan 6400 advanced micro-iontophoresis current generator which provided independent control of two iontophoresis channels (drug/vehicle) and a balancing channel. Current parameters were selected based on previous studies which utilized LY354740 iontophoresis (Copeland et al., 2012; 2013; 2017): positive 15 nA retaining current prevented spontaneous drug diffusion, whereas ejection current was negative 25 nA.

Recording site labeling

After neuronal activity was assessed, recording sites were labeled with either 1% Neurobiotin 350 in 1 M NaCl (Vector laboratories, Burlingame, CA; product SP-1155), 1% Neurobiotin 488 in 1 M NaCl (Vector laboratories, product SP-1125-2), or 0.1 % Fluoro-Gold in saline (Fluorochrome, Denver, CO). Different dye colors distinguished the recording sites labelled on different recording days in the same animal. When a single micropipette was used, a drop of dye solution was placed on its back end and allowed to move to the tip; the micropipette was then filled with 1 M NaCl solution. All dyes were ejected using Dagan 6400 advanced micro-iontophoresis current generator (the wire which connected the
micropipette to the extracellular preamplifier during recordings was switched to the wire connected to Dagan 6400). Positive 1000 nA current was used to eject Neurobiotin 350 and Neurobiotin 488 (2 and 4 min, respectively) and negative 1000 nA current for Fluoro-Gold (4 min). The magnitude of the current is at least 10 times smaller than those used to cause electrolytic lesions (10 – 50 μA; Townsend et al., 2002; Ayala and Malmierca, 2015; Harris et al., 2017; Yang et al., 2020). Indeed, our histological examination did not reveal any lesions at the recording sites. This labelling protocol was selected based on the results of extensive pilot experiments aimed at determining the most reliable technique. Troubleshooting for pilot experiments is detailed in the Discussion.

**Recording site identification**

After the end of the last recording session, mice were injected with an overdose of Fatal-Plus (>100 mg/kg, i.p., Vortech). Following loss of corneal and withdrawal reflexes, the animal was decapitated, the skull partially removed, and the head placed in 4% paraformaldehyde (PFA) at room temperature for 30-60 min. Then, the brain was removed and stored at 4°C in 4% PFA containing 25% sucrose overnight. The midbrain was trimmed out, frozen, and coronal sections were collected from the IC on a sliding microtome at a thickness of 40 or 50 μm. IC sections were mounted serially from a 0.2% gelatin solution onto gelatin-coated slides.

Slides were air-dried overnight, then sections were stained on-slide for GAD67 and GlyT2 to determine IC subdivisions (Buentello et al., 2015). More details about this method for IC subdivision identification, as well as rationale for selecting this technique, are provided in the Discussion. A hydrophobic barrier pen was used to outline all sections on the slide. Once the hydrophobic barrier was dry, slides were rinsed in phosphate-buffered saline solution (PBS, 0.9% NaCl in 0.01 M phosphate buffer (PB), pH 7.4), then permeabilized in 0.3% Triton X-100 in PBS for 30 minutes at room temperature. A blocking solution made up of 0.1% Triton X-100 and 10% normal goat serum (NGS) in PBS was applied for one hour at room temperature. The primary antibody solution containing 1% NGS, 0.1% Triton X-100, mouse anti-GAD67 (Millipore Sigma, St. Louis, MO; MAB5406; 1:250) and guinea
pig anti-GlyT2 (Synaptic Systems, Goettingen, Germany; 272-004; 1:2500) was applied to the slides, which were then placed in a sealed container overnight at 4°C. The next day, slides were rinsed in PBS, then a secondary solution containing Alexa Fluor 750-labeled goat anti-mouse (Molecular Probes, Eugene, OR; A21037; 1:100) and Alexa Fluor 647-labeled goat anti-guinea pig (Molecular Probes, A21450; 1:100) in PBS was applied for one hour at room temperature. Slides were rinsed in PBS, air dried, and coverslipped with DPX mounting medium (Sigma-Aldrich, St. Louis, MO).

Slides were examined on a Zeiss AxioImager.Z2 microscope attached to a Neurolucida system (MBF Bioscience, Williston, VT). Images were taken with a 5X objective. In sections where a labeled recording site could be identified, the section was outlined using the Neurolucida system, subdivision outlines were added based on the GAD67 and GlyT2 staining (Buentello et al., 2015), and the recording site was marked. The outline was exported, then outlines from all cases with identifiable recording sites were overlaid onto a representative series in Adobe Illustrator.

**Electrophysiological data analysis and statistics**

Electrophysiological data was first processed using BrainWave software (DataWave Seiworks). For all recordings obtained from an individual neuron, spikes were extracted using a single visually determined threshold. To confirm a well-isolated single neuron recording, all spike waveforms were superimposed for visual examination. Spike data were binned (1 ms bins) and exported for all further analyses using R (R Core Team, 2020).

Rate-level functions were fit using a non-linear five parameter logistic model (Watkins and Barbour, 2011a; Watkins and Barbour, 2011b). Three values were extracted from each fitted curve: maximum firing rate, threshold, and saturation. Threshold and saturation values corresponded to sound level at 20% and 80% of the maximum firing rate, respectively. The slope was calculated as a ratio between the firing rate’s dynamic range and the range of sound levels between the threshold and saturation points.
Previous electrophysiological studies typically observed that mGluR2/3 pharmacological manipulation had effect in only a subset of neurons tested (Sanes et al., 1998; Voytenko and Galazyuk, 2011; Galazyuk et al., 2019). This could be due to several reasons, such as lack of uniform receptor expression on all neurons or incomplete receptor activation because of limited drug concentration at the recording site. Thus, we used k-means cluster analysis to assign neurons to either drug effect or no drug effect groups.

When raw data was not normally distributed, we used the Box-Cox procedure, which provides an optimal transformation to normality for non-normally distributed samples (Box and Cox, 1964). In a case where transformed data were still not normally distributed (as evaluated semi-qualitatively using Q-Q plots; Sokal and Rohlf, 2011), statistical analyses were performed using rank-transformed data (Conover and Iman, 1981). The mixed-effects statistical approach (Pinheiro and Bates, 2000) was utilized to account for random variation among individual neurons for population analyses (neuron was treated as a random factor). Non-parametric two-sample Kolmogorov-Smirnov tests were used for comparing monotonicity index distributions before and after drug application. Fisher’s Exact Test was used to assess the difference in proportions between nominal variables (e.g. sex). Paired t-tests were used to assess within-neuron differences pre- and post-drug conditions. When multiple comparisons were performed, p-values were adjusted using the Benjamini and Hochberg’s false discovery rate procedure (Benjamini and Hochberg, 1995); to indicate those cases, p-values are noted as adj. p in the Results.

To accomplish all analyses, several add-on R packages were required: car (Fox et al., 2011), lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), emmeans (Lenth, 2017), plyr (Wickham, 2016), nplr (Commo and Bot, 2016), minpack.lm (Elzhov et al., 2016), ggplot2 (Wickham, 2020), cluster (Maechler et al., 2019), factoextra (Kassambara and Mundt, 2019), knitr (Xie, 2020).
Results

MGlur2/3 activation enhances sound level processing in a subset of IC neurons.

The effect of group II mGluR activation on sound level processing was tested in 31 IC neurons from thirteen VGAT-ChR2-EYFP mixed genetic background mice. Extracellular single-cell recordings were obtained in response to 50 ms duration pure tones presented at the neuron’s characteristic frequency from 0 to 80 dB SPL. Each neuron was tested with the same sound stimulation protocol several times before and after topical mGluR2/3 agonist LY354740 application, resulting in multiple rate-level functions (RLFs). The same analysis window was used to assess all pre- and post-drug data from an individual neuron. The window was adjusted for each neuron to capture sound-evoked spikes based on visual inspection of peri-stimulus time histograms plotted for each RLF.

Each RLF was fit with a non-linear five parameter logistic model and the peak firing rate, slope, as well as threshold were extracted from the fitted curve (Figure 5). This RLF quantification approach has been previously used by several research groups (Watkins and Barbour, 2011a; Watkins and Barbour, 2011b; Moore and Wehr, 2013; Stefanescu et al., 2015; Chambers et al., 2016; Ono et al., 2017). To assess how well each curve fit the RLF data, all fits were visually examined. Additionally, we obtained the average error between the fitted curve and the data points, i.e. the residual standard error (RSE). In the final data set, the median RSE was 1.70 and the mode was 0.11. Several RLFs with RSE values larger than ten were excluded from further analyses; this cutoff point was determined by visual inspection of the fits.
Figure 5. Example rate-level function fitted with a curve. The maximum firing rates, slopes, and thresholds were extracted from the fitted curves for subsequent data analyses.

Each dependent variable (i.e., the peak firing rate, slope, and threshold) was first normalized using the Box-Cox procedure (Box and Cox, 1964). Then, a separate mixed-effects ANOVA test was used for each dependent variable to compare pre-drug and post-drug RLF data obtained from all 31 neurons. After mGluR2/3 agonist application the peak firing rates were significantly higher ($F_{1,255.1} = 47.9$, adj. $p < .0001$), the slopes steeper ($F_{1,255.4} = 13.8$, adj. $p < .001$), and thresholds became lower ($F_{1,255.2} = 38.0$, adj. $p < .0001$). In Figure 6 we plotted mean pre-drug and mean post-drug peak firing rate, slope, and threshold for each neuron, as well as population data visualized with boxplots (the plot presents Box-Cox transformed data).
Even though population-level analysis demonstrated significant RLF changes after mGluR2/3 activation, visual examination of RLFs indicated that the magnitude of drug effect slightly varied between neurons and that the effect was absent in some neurons. Previous studies also described that only a subset of neurons was affected by mGluR2/3 pharmacological manipulation (Sanes et al., 1998; Voytenko and Galazyuk, 2011; Galazyuk et al., 2019). Thus, we utilized cluster analysis to empirically sort the neurons into groups based on the overall similarity of LY354740 effect on their RLFs. Using the differences between pre-drug and post-drug mean peak firing rate and firing rate's dynamic range as variables for k-means clustering, we found three distinct clusters with eight, eleven, and twelve neurons in each. Empirically derived cluster group was considered a fixed factor in subsequent mixed-effects ANOVA analyses, which were conducted separately on all three dependent variables described in Figure 6, i.e. the peak firing rate, slope, and threshold. Detailed results are provided in Table 1. Post-hoc pairwise comparisons of estimated marginal means showed that each dependent variable was significantly affected by drug application in two clusters, but not in a third cluster. Therefore, for all subsequent analyses we
collapsed the first two cluster groups into a single group. *Figure 7* shows drug effect on the resulting two groups of neurons. In the first group (*Figure 7A*), mGluR2/3 agonist significantly increased the peak firing rates ($F_{1,169} = 99.0$, adj. $p < .0001$), steepened the slopes ($F_{1,169} = 25.6$, adj. $p < .0001$), and reduced the thresholds ($F_{1,169} = 41.1$, adj. $p < .0001$). No such significant changes were found in the second group [*Figure 7B*; peak firing rates ($F_{1,85} = 5.2$, adj. $p = .08$), slopes ($F_{1,85} = 0.1$, adj. $p = .71$), and thresholds ($F_{1,85} = 1.0$, adj. $p = .50$)]. Thus, we refer to these groups as *drug effect* versus *no drug effect* in subsequent descriptions.
| Term               | Statistic | P-value |
|--------------------|-----------|---------|
| **Peak firing rate** |           |         |
| **Overall ANOVA**   |           |         |
| Drug               | $F_{1,253} = 80.8$ | <0.0001 |
| Cluster            | $F_{2,28} = 2.8$  | 0.07    |
| Drug-cluster interaction | $F_{2,253} = 44.3$ | <0.0001 |
| **Pairwise comparisons with clusters** |           |         |
| Cluster #1: pre-drug – post-drug | $t_{253} = 10.7$  | <0.0001 |
| Cluster #2: pre-drug – post-drug | $t_{253} = 5.9$  | <0.0001 |
| Cluster #3: pre-drug – post-drug | $t_{253} = -1.8$ | 0.07    |
| **Slope**          |           |         |
| **Overall ANOVA**   |           |         |
| Drug               | $F_{1,253} = 13.9$ | <0.001  |
| Cluster            | $F_{2,28} = 0.9$  | 0.4     |
| Drug-cluster interaction | $F_{2,253} = 5.6$ | <0.01   |
| **Pairwise comparisons with clusters** |           |         |
| Cluster #1: pre-drug – post-drug | $t_{253} = 2.5$  | <0.05   |
| Cluster #2: pre-drug – post-drug | $t_{253} = 4.3$  | <0.0001 |
| Cluster #3: pre-drug – post-drug | $t_{253} = -0.4$ | 0.7     |
| **Threshold**      |           |         |
| **Overall ANOVA**   |           |         |
| Drug               | $F_{1,253} = 47.0$ | <0.0001 |
| Cluster            | $F_{2,28} = 0.8$  | 0.4     |
| Drug-cluster interaction | $F_{2,253} = 11.1$ | <0.0001 |
| **Pairwise comparisons with clusters** |           |         |
| Cluster #1: pre-drug – post-drug | $t_{253} = -6.6$  | <0.0001 |
| Cluster #2: pre-drug – post-drug | $t_{253} = -4.4$  | <0.0001 |
| Cluster #3: pre-drug – post-drug | $t_{253} = -0.5$ | 0.6     |

**Table 1.** The effect of topical LY354740 application on rate-level functions’ peak firing rates, slopes, and thresholds. The drug had a significant effect on all three dependent variables in cluster #1 and #2, but not in cluster #3 neurons.
Figure 7. Neurons were assigned to two groups: those which had rate-level functions affected by topical mGluR2/3 agonist LY354740 (A; n = 20), and those in which mGluR2/3 activation did not have a clear effect (B; n = 11). Box-Cox transformed rate-level functions’ peak firing rates (A1, B1), slopes (A2, B2), and thresholds (A3, B3). Each neuron’s (n = 31) pre-drug and post-drug means are connected by grey lines; population data is summarized with boxplots. ***p<0.0001, NS p>0.05.

We observed that RLFs became slightly more monotonic after drug application. To quantify that, for each RLF we calculated the monotonicity index (MI; Ono et al., 2017): firing rate at the maximum sound intensity level (80 dB SPL) was divided by the maximum firing rate. Neurons with more monotonic RLFs had MIs close to one, as their maximum firing rates occurred at/near the maximum
sound intensity level. In Figure 8 we plotted MIs separately for neurons assigned to the drug effect and no drug effect groups. Two-sample Kolmogorov-Smirnov test indicated statistically significant difference between pre-drug versus post-drug MI distributions in the drug effect group (D = 0.25, p < 0.05), whereas no such difference was found in the no drug effect group (D = 0.16, p = 0.62).
Figure 8. The effect of topical mGluR2/3 agonist LY354740 application on neurons’ monotonicity. All pre-drug and post-drug monotonicity indexes are plotted as cumulative distributions. (A) Neurons from the drug effect group (n = 20) became significantly more monotonic after drug application. (B) Monotonicity did not significantly change in neurons assigned to the no drug effect group (n = 11). *p<0.05, NS p>0.05.

MGluR2/3 activation has similar effect on GABAergic and non-GABAergic IC neurons.

As experiments were conducted on VGAT-ChR2-EYFP transgenic mice in which inhibitory neurons express channelrhodopsin-2, laser light pulses evoked firing only in inhibitory cells, allowing us to distinguish GABAergic from non-GABAergic cell types optogenetically. From a total of 31 neurons tested with topical LY354740 application, six were inhibitory. Five of them were from the group where mGluR2/3 activation resulted in significant RLF changes (Figure 7A). In Figure 9 we plotted example monotonic and non-monotonic RLFs from both cell types before and after topical LY354740 application. The effect typically occurred at 1 to 5 minutes after drug application and lasted throughout the recording, about 12 to 15 minutes after drug application.
Figure 9. Examples of rate-level functions before and after topical LY354740 application. (A, B) Non-GABAergic neurons. (C, D) GABAergic neurons. Pre-drug rate-level functions are plotted in solid black, blue, and grey lines in all panels (legend only shown in panel B). Post-drug rate-level functions are color coded yellow, green, pink and orange to demonstrate drug effect over time; time after drug application is indicated for each individual rate-level function. Error bars represent ± SEM of ten sound stimulus presentations at each sound level.
**Relationship between mGluR2/3 activation and neurons’ temporal response type.**

To gain some insight into which IC cell types might be modulated by group II mGluRs, we examined whether mGluR2/3 activation induced RLF changes more often in neurons with certain temporal response type. Also, we asked whether mGluR2/3 agonist changed those temporal response patterns. To answer these questions, for each neuron two peri-stimulus time histograms (PSTHs) were created: one from all pre-drug data, the other from post-drug data. All PSTHs were visually examined and assigned to either phasic/onset or tonic/sustained temporal response pattern. Whereas all neurons maintained their characteristic response pattern after drug application, tonic/sustained type was more common amongst the neurons assigned to the drug effect group. Fourteen of twenty (70%) neurons from this group had tonic/sustained firing. Neurons from no drug effect group displayed almost equal numbers of temporal response patterns: 45.5% had phasic/onset firing and 54.5% had tonic/sustained pattern. However, Fisher’s Exact Test did not result in statistically significant association between the temporal response type and drug effect group (p = .45).

**Age, sex, and drug dose do not mediate RLF changes in response to mGluR2/3 activation.**

It has been shown that mGluR expression is developmentally regulated in several brain regions (Catania et al., 1994; Defagot et al., 2002; Doherty et al., 2004; Frank et al., 2011; McOmish et al., 2016; Hernandez et al., 2018). Thus, we examined whether age differences between the animals could explain the variability in LY354740 effect (Figure 10A). Two sample t-test demonstrated that there was no significant age difference between the neuron group in which the drug significantly changed the RLFs (mean = 194 days, SD = 47.3 days) versus the group without such changes (mean = 197 days, SD = 43.0 days; t_{22} = 0.2, p = .86). Furthermore, the animals’ sex also did not explain drug effect variability (Figure 10B). Sixteen out of 31 neurons were recorded from female mice and 15 neurons from male mice. According to the Fisher’s Exact Test result, the proportion of male and female mice did not differ by drug
effect group \( (p = .14) \). Similarly, Fisher's Exact Test showed that proportion of neurons tested with lower (30 \( \mu \text{M} \)) and higher (90 \( \mu \text{M} \)) LY354740 concentration was not significantly different between drug effect groups \( (p = .72; \text{Figure 10C}) \). These data suggest that factors other than sex, age and drug dose determine whether RLF is affected by group II mGluR activation. However, it is possible that a larger sample size would reveal sex differences.
Figure 10. Association between the animals’ age, sex, drug dose and the effect of topical LY354740 application on rate-level functions. All neurons were split into two groups: those which had rate-level functions affected and unaffected by mGluR2/3 activation. No significant differences were found in age (A), sex (B), and drug dose (C) between these two neuron groups. NS p>0.05.

*The effect of mGluR2/3 activation on RLF changes is independent of agonist and drug application method used.*

The results described above were obtained using mGluR2/3 specific agonist LY354740 applied topically on the craniotomy above the IC. We performed experiments to further verify these results with two different positive control experiments. First, instead of topical drug application, we administered LY354740 using microiontophoresis (five neurons tested). This is a drug delivery method in which a small electric current is used to eject drugs from micropipettes attached to a recording electrode (Stone, 1985). This technique has been previously used to administer LY354740 (Copeland et al., 2012; 2013; 2017). Example RLFs recorded before and after iontophoretic LY354740 application are plotted in Figure 11A. For a second positive control experiment we used topical drug application but utilized another highly specific group II mGluR agonist LY379268 (three neurons tested; Monn et al., 1999). Figure 11B shows example RLFs obtained before and after topical 30 µM LY379268 application. In both positive control experiments, mGluR2/3 activation resulted in similar effect on RLFs’ peak firing rates,
slopes and thresholds as with our primary experimental protocol using topical LY354740 application. These data confirm that mGluR2/3 modulatory effect on sound processing in the mouse IC is robust and can be studied using different methods (further discussed in the Discussion). Negative control tests with topical and iontophoretic drug vehicle application did not result in RLFs’ modulation (six neurons tested).
**Figure 11.** Examples of rate-level functions before and after mGluR2/3 activation: iontophoretic LY354740 ejection (A) and topical LY379268 application (B). Pre-drug rate-level functions are plotted in solid black, blue, and grey lines in both panels; the legend is only shown in panel B. Post-drug rate-level functions are color coded yellow, green, pink and orange to demonstrate drug effect over time; time after drug application is indicated for each individual rate-level function Error bars represent ± SEM of ten sound stimulus presentations at each sound level.

**Neurons affected by mGluR2/3 activation were distributed throughout the IC area tested.**

At the end of the electrophysiological experiments, each recording site was labelled using iontophoretic dye ejection. From 31 neurons tested with topical LY354740 application, recording sites were successfully identified in twelve neurons. Fluoro-Gold labelling resulted in the largest proportion of identifiable sites (55% from 11 attempts), followed by Neurobiotin 350 (36% from 11 attempts) and Neurobiotin 488 (22% from 9 attempts). **Figure 12** shows examples of labelled recording sites. All dyes resulted in rather bright, well-confined labelling. This allowed for easy detection of recording sites in specific IC areas using a low (5X) magnification objective.
Figure 12. Example recording sites labelled with Fluoro-Gold (A), Neurobiotin 350 (B), and Neurobiotin 488 (C). Dashed lines indicate the boundaries of IC and its subregions: parts of the central IC nucleus - area 1 and area 2, as well as dorsal (d) and lateral (lc) cortex. Scale = 500 µm.

Figure 13 shows locations of successfully identified recording sites within the IC. Majority of recordings were from more dorsal IC parts due to concerns that the topically applied drug’s diffusion might be much lower in deeper regions. Indeed, drug effect was not observed in the two most deep successfully identified recording sites about 500 µm down from the IC surface. On the other hand, it is possible that drug effect occurred at such depths, but these recording sites were not recovered. In addition to histological recording site examination, all depths were noted during experiments based on how much the micromanipulator was vertically advanced into the IC. According to these data, the mean recording depth for neurons assigned to the drug effect group registered 251 µm (SD 107 µm), whereas the no effect group’s mean depth registered 333 µm (SD 134 µm). However, the mean difference was not statistically significant ($t_{17} = 1.7, p = .10$).

Successfully identified recording sites were somewhat equally distributed along the rostral-caudal IC axis: six neurons in the more anterior half of the IC, the other six in the more posterior half (Figure 13). Five of six rostrally located neurons were from the drug effect group, whereas only three of six caudal neurons were affected by topical LY354740 application. Such result suggests a possibility that more rostral IC parts might be subjected to larger modulation by group II mGluRs compared to its caudal regions.
Discussion

Our findings regarding the sound evoked activity—steeper RLF slopes, higher maximum firing rates, and lower thresholds—are partially congruent with the results from an in vitro study by Farazifard and Wu (2010). Both studies are in agreement that both excitatory and inhibitory synaptic transmission is modulated by group II mGluRs. However, modulation direction differs. Farazifard and Wu (2010) showed reduced excitatory and inhibitory post-synaptic currents after mGluR2/3 activation, whereas our in vivo results demonstrated increased firing. The in vitro experiments were conducted in 8- to 17-days-old Long Evans rats, whereas our data was collected in 4- to 9-months-old mice. Age and species differences might explain the observed discrepancy. Indeed, it has been demonstrated that mGluRs are developmentally regulated in several brain regions (Catania et al., 1994; Defagot et al., 2002; Colantuoni...
et al., 2008; Hernandez et al., 2018); unfortunately, no data are available regarding the IC. Thus, future comparative biochemical and immunohistochemistry studies evaluating the effects of age and species on IC mGluR2/3 expression would be very valuable.

In contrast to results reported here, an in vivo study by Voytenko and Galazyuk (2011) found no effect of iontophoretically delivered group II agonist on sound evoked firing in the IC. The animals in that study were 2.5 months younger than the youngest mice used in the present study. None of the recording sites were labelled by Voytenko and Galazyuk (2011). It is possible these data were obtained from deeper IC areas which might have lower/no mGluR2/3 expression. It is likely that only a specific subset of cells expresses these receptors. A small sample size in a report by Voytenko and Galazyuk (2011) might have contributed to the lack of observed effect. Therefore, it would be worthwhile to investigate possible differential receptor expression between IC subdivisions. Information about receptor density would also provide some clarification regarding the lack of drug effect in a subset of neurons described in the present study.

In our sample, 19% of recorded neurons were classified as GABAergic based on optogenetic cell type identification. These data are consistent with previous observations about the relative proportions of excitatory and inhibitory neurons within the IC. In the cortex, the majority of synapses are glutamatergic, and the remaining 20-30% are GABAergic (Markram et al., 2004; Buzsaki et al., 2007). Similarly, in the IC of various species, about 20-40% of neurons are inhibitory (Merchán et al., 2005; Mellott et al., 2014; Schofield and Beebe, 2019).

Traditionally, pre-synaptic group II mGluRs were the focus of most research. mGluRs2/3 are predominantly coupled to Gi/Go proteins, which are associated with classical inhibition of the adenylyl cyclase (AC)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway (Conn and Pin, 1997). More specifically, Gi/Go proteins directly inhibit AC which results in decreased production of downstream signaling molecules. It is widely suggested that reduced concentration of these signaling molecules is linked to reduced neurotransmitter release from the pre-synaptic neurons and subsequently decreased excitation and firing of the postsynaptic neurons. This is because those signaling molecules
regulate activity of various ion channels, such as potassium and calcium channels (Conn and Pin, 1997; Niswender and Conn, 2010; Muguruza et al., 2016). In such way, activated potassium channels and inhibited calcium channels reduce neuronal excitability and synaptic transmission.

More recently though, research has focused on the post-synaptic group II mGluRs as well. Jin et al. (2017) and Ster et al. (2011) examined subcellular mGluR2/3 localization and found evidence for both pre- and post-synaptic sites, confirming earlier findings (Petralia et al., 1996; Tamaru, 2001). Jin et al. (2017) also showed that in vivo iontophoretically applied mGluR2/3 agonist increased Delay cell firing in the primate layer III dorsolateral prefrontal cortex. The authors suggested a working model where post-synaptic mGluR2/3 activation closes potassium channels, resulting in enhanced cell firing. Several studies found that mGluRs2/3 mediate changes in neuronal excitability through altering post-synaptic NMDA and AMPA receptor function. Wang et al. (2013) showed that group II mGluR activation increased expression of GluA1 and GluA2 subunits on the post-synaptic membrane. Xi et al. (2011) reported that post-synaptic NMDA receptor expression and phosphorylation was increased following mGluR2/3 activation. Tyszkiewicz et al. (2004) demonstrated that group II mGluR activation increased NMDA receptor currents.

In light of these findings and the findings of the results described in this Chapter, it is possible that enhanced neuronal firing following group II mGluR activation may be exclusive to or more pronounced in brain regions containing primarily post-synaptic mGluRs2/3. As we learn more about receptor subcellular localization, and expand efforts to disambiguate subtype specific roles (further discussed in Chapter V subsection “Future directions”), it should be possible to better explain how group II mGluRs can exert both inhibitory and excitatory actions. It remains to be tested whether in the mouse IC post-synaptic receptors may be a major mGluR2/3 component. Also, it should be determined which intracellular signaling cascades are activated and what their targets are.

What is the biological relevance of enhanced sound-evoked firing in the IC following activation of group II mGluRs? Under physiological conditions, when sound level increases, elevated glutamate concentration in and around the synapse could activate more mGluRs, possibly including receptors at the
peri-synaptic and extra-synaptic membranes (Jin et al., 2017; 2018). Thus, mGluRs2/3 may enhance sound-level processing primarily at higher sound intensities. In our study, however, the exogenous agonist application must have been uniform across sound levels and increased firing irrespective of sound intensity.

**Technical considerations: drug delivery and concentration.**

As discussed in Chapter I, to the best of our knowledge, only one prior in vivo study was conducted to test the role of group II mGluRs in the IC (Voytenko and Galazyuk, 2011). Technical challenges related to recording the same neuron before and after receptor activation might discourage such projects. A more traditional approach is to deliver receptor agonists using iontophoresis, in which a small electrical current is used to eject a drug from a micropipette attached to the recording electrode. However, this method suffers from uncertainty regarding drug delivery per se, as well as unknown concentration of drug ejected into the tissue (Kirkpatrick and Wightman, 2016; Kirkpatrick et al., 2016). Drug concentration is important because even highly mGluR subtype-specific compounds can lose specificity at higher concentrations. Additionally, iontophoresis requires manufacturing multi-barrel electrodes and that is a complicated procedure. In our hands, commercially available multi-barrel electrodes (Kation Scientific) did not result in satisfactory signal-to-noise ratio. Furthermore, multi-barrel electrodes are significantly wider than single electrodes and inevitably cause more brain tissue damage, which might interfere with normal excitability. Considering these and other issues, as well as pressure injection as an alternative drug administration approach (Veith et al., 2016), we decided to utilize topical drug application on the IC surface as our primary drug delivery method and performed iontophoresis to only verify the results. Although topical drug delivery approach is more commonly used in cortex (Happel et al., 2010; Self et al., 2014; Zhou et al., 2014; Rasmussen et al., 2019), there are several studies in which a drug was applied to the surface of the IC (Ji et al., 2001; Ji and Suga, 2009; Scott et al., 2018). In mice, the IC, like cortex, is conveniently located on the brain surface.
Topical drug delivery has several advantages. First, controlled drug concentration can be applied on the brain surface, minimizing the risk of losing the compound’s specificity for the receptors of interest. Second, a single recording electrode causes minimal brain damage and allows successful recording over the course of several days testing in the same animal. Additionally, signal-to-noise ratio was typically larger when a single electrode was used compared to multi-barrel, which was important for our goal to accurately compare the response of well-isolated neurons before and after drug application. A disadvantage of this approach is that the exact drug concentration at the recording site cannot be known. As mentioned above, the maximum is determined by the solution applied to the brain surface, but diffusion into the tissue will lead to a decrease in concentration to an unknown level. Another disadvantage of topical drug delivery is that it only allows studying drug effects on a population of neurons. Nonetheless, we view topical drug administration as a valuable technique for testing the effects of pharmacological agents on IC physiology. Newly developing approaches that utilize light to activate and deactivate endogenous mGluRs may soon circumvent some of the problems of iontophoresis and topical application (Donthamsetti et al., 2019). Further details about the advantages of such new methods are provided in Chapter V subsection “Future directions”.

The two compounds that we used to pharmacologically activate group II mGluRs act at the orthosteric receptor site (Monn et al., 1999). This means that the drugs bind to the natural binding site of the endogenous ligand—neurotransmitter glutamate in this case. Glutamate is a flexible molecule that acquires various structural conformations when binding to its several targets—ionotropic and metabotropic receptors (Gonzalez and Barreto, 2013). LY354740—the mGluR2/3 agonist that we used for most experiments—was designed to serve as a conformationally restrained analog of glutamate that closely approximates glutamate conformation when it acts at the orthosteric site on group II mGluRs (Monn et al., 1997). LY379268—the mGluR2/3 agonist that we used to verify the results obtained with LY354740—is structurally similar to LY354740 and has similar potency and specificity (Monn et al., 1999). LY354740 has been characterized in cells expressing human mGluR2/3 receptors and in rat brain slices (Schoepp et al., 1999). It was concluded that LY354740 maintains its specificity even at
micromolar concentrations—in some tests at >100 µM. On the other hand, LY354740 had weak but measurable agonist activity at human mGlu8 receptors with EC50 value of 36 ± 5 µM. Although mGluR8 mRNA was detected in rat IC (Yip et al., 2001), it is currently not clear whether functional mGluR8 is expressed in the mouse IC (further discussed in Chapter V subsection “Other mGluRs, in addition to group II, might contribute to IC neural activity modulation”). Thus, considering the EC50 value of 36 ± 5 µM for mGluR8, in some of our experiments topically applied LY354740 concentration was 30 µM. In other experiments we used 90 µM concentration taking into account above mentioned Schoepp et al. (1999) data regarding the drug’s specificity. Our results showed that these two concentrations were not related to a neuron’s assignment to drug effect versus no drug effect groups (Figure 10). This is not surprising because at both these concentrations mGluR2/3 binding sites may have been saturated. Only a small amount of the drug is needed to activate the receptors because LY354740 is a very potent compound with EC50 values for mGluRs2/3 in the low nanomolar range (about 10 - 50 nM; Monn et al., 1999; Schoepp et al., 1999). On the other hand, as detailed in Chapter I, mGluRs are dimers and full receptor activation requires a cooperative process during which the binding sites on both mGluR proteins are occupied (Kniazeff et al., 2004; Levitz et al., 2016). Thus, sufficient drug concentration is important, although due to circulation and diffusion in some experiments it might have been too low for the observable drug effect.

**Technical considerations: recording site labelling and identification.**

As mentioned above, recording sites were successfully identified in twelve out of 31 neurons. It is disappointing that not all recording sites were recovered. Recording site labelling was a new method in the lab and we conducted extensive pilot tests to establish a reliable labeling protocol. It is not clear why Neurobiotin ejection protocol described by Ono et al. (2016; 2017)—200 nA, 50% duty cycle of 500 ms, 5 min—did not work for us. One possibility is the difference in electrode tip diameters between Ono et al. (2016; 2017) and our studies. Our tip diameters were about 1-2 µm. It is likely that the tips were larger in the previous studies, although such details were not described. Instead, it was stated that the electrode
impedance was 4–7 MΩ. In our study electrode impedance was at least twice as high because lower impedance did not produce desirable signal-to-noise ratio for reliable identification of well-isolated neuron spikes during all pre- and post-drug tests. It is also possible that subtle differences in electrode shapes between Ono et al. (2016; 2017) and our studies could explain the difference in Neurobiotin ejection efficiency.

In addition to Neurobiotin, we varied current ejection parameters and used different available equipment to test several other dyes: two fluorophore-conjugated dextrans, cholera toxin subunit B, and FluoSpheres. Overall, it seemed that the recording sites were more likely to be labelled with constant current ejection (as opposed to current pulses), more diluted dye solutions, and smaller molecular weight compounds. Additionally, survival time after dye ejection should be no longer than about 4 days. The question remains what could be done in the future studies to achieve a more reliable recording site labelling. As detailed in the Results, Fluoro-Gold labelling resulted in more successfully recovered recording sites compared to Neurobiotin 350 and Neurobiotin 488. The Fluoro-Gold solution was more diluted compared to the other dyes. It might be worth testing whether higher Neurobiotin dilutions produce more reliable labelling. It is possible that Neurobiotin concentrations in our study were too high and thus clogged the micropipette preventing reliable dye ejection.

To determine which IC subdivision the recording came from, we stained the brain sections for the glutamic acid decarboxylase 67 (GAD67) and neuronal glycine transporter 2 (GlyT2). GAD67 is the major enzyme converting glutamate into GABA and it is a popular marker for GABAergic neurons, whereas GlyT2 is a membrane-bound protein which reuptakes glycine from the synaptic cleft back into cells—a marker for glycinergic terminals. IC subdivisions can be defined using the relative density of GAD67 and GlyT2 labelling—a method described in a detailed immunohistochemistry study by Buentello et al. (2015). This work demonstrates a consistent pattern of two separate regions within the IC central nucleus (ICc). The two regions identified are the ventrolateral glycine rich region (also known as Area 1) and dorsomedial GABA dominant zone (known as Area 2). Such dominance of GAD67 and GlyT2 labeling in different ICc regions is not surprising. According to the synaptic domain hypothesis, IC
has several functional zones which are formed by the segregation of inputs from different sources (Oliver, 2005). Indeed, Area 1 receives glycinergic inputs from the lateral superior olive and a bit more widely distributed glycinergic terminals from the ventral nucleus of the lateral lemniscus. On the other hand, the most prominent GABA inputs are from the dorsal nucleus of the lateral lemniscus and superior periolivary nucleus. ICc generally has relatively more staining for GlyT2 compared to IC cortical subdivisions, which can be further divided into dorsal and lateral parts based on increased GAD67 staining in the latter.

As an alternative to GAD67/GlyT2 labelling, we considered Nissl staining, which has been a more traditional approach for IC subdivision identification (Meininger et al., 1986; Kádár et al., 2009). The advantage of Nissl staining is that the procedure is less time consuming and cheaper. However, GAD67/GlyT2 staining is currently considered a gold standard as it offers the most clear distinction of IC subdivisions. Thus, our choice of the method was guided by our aim to accurately determine IC subdivisions.
CHAPTER IV
The role of group II mGluRs in spontaneous firing modulation

Introduction

As mentioned in Chapter I, our interest in group II mGluRs arises from recent results showing that behavioral signs of tinnitus in mice were suppressed by intraperitoneal administration of mGluR2/3 agonist LY354740 (Galazyuk et al., 2019). In this study with another group of mice, intravenous LY354740 injection reduced spontaneous activity in the inferior colliculus (IC). Even though it was not directly tested whether reduction of behavioral evidence of tinnitus was linked to suppressed spontaneous firing, this is a possibility as spontaneous hyperactivity is thought to be one of the mechanisms underlying tinnitus (Shore et al., 2016; Shore and Wu, 2019).

We were intrigued that mGluRs2/3 might be targeted to reduce hyperactivity and alleviate tinnitus and aimed to examine how these receptors regulate spontaneous firing when activated specifically in the IC. Rapidly developing technology might allow for the delivery of drugs to specific brain areas, such as IC (Dagdeviren et al., 2018). This could be advantageous in severe tinnitus cases. Targeted drug delivery might be more effective overall and may also avoid a major limitation of systemic drug administration, which results in broader drug distribution than necessary and consequently increased risk for toxicity and side effects. Therefore, this is one of the other reasons—in addition to aspects of significance described in Chapters I and III—why it was crucial to investigate whether group II mGluR agonist could suppress spontaneous firing when delivered directly to the IC.
Materials and methods

To determine whether group II mGluR activation has effect on spontaneous firing, we studied the same 31 neurons described in Chapter III for the sound-evoked firing analyses. Spontaneous firing rates (spikes/second) were calculated from 50 milliseconds analysis window before the onset of each tone presented during rate-level function (RLF) data collection. For each RLF, a tone was presented 170 times: ten repetitions at seventeen sound intensity levels. Thus, spontaneous activity was assessed during the nine seconds period during each RLF test (170 trials * 50 ms = 8500 ms). As described in Chapter III, several RLFs were obtained before drug application and several after, all of which were included in the analyses. Post-drug data collection typically lasted for about fifteen minutes (Figure 9).

This type of spontaneous activity assessment method—when spontaneous firing rates are calculated from the silent intervals that separate tone presentations—is rather commonly used. However, it is known that spontaneous activity can be suppressed after sound presentation (Voytenko and Galazyuk, 2010; 2011; Galazyuk et al., 2017; 2019). This could potentially interfere with accurate spontaneous firing evaluation. Thus, we took this phenomenon into consideration and measured spontaneous activity during the 50 ms period before tone presentation. In such way, we excluded the period when spontaneous firing might have been suppressed by a preceding sound.

Results

Fifteen of 31 neurons were not spontaneously active before drug application, whereas after mGluR2/3 activation only six lacked spontaneous firing. In the entire sample, pre-drug spontaneous firing rates ranged from 0 to 5.5 spikes/second, while post-drug ranged 0 to 15.8 spikes/second. For statistical analyses, firing rates were rank-transformed because of non-normal distributions of raw data. Then, for each neuron, mean firing rate was calculated for all pre- and post-drug data and population-level analysis was performed using paired t-tests. In neurons assigned to the drug effect group, described in Chapter III (n = 20), there was a significant increase in spontaneous firing after drug application (t_{19} = 3.4, adj. p <
01; Figure 14A). In contrast, no significant spontaneous firing change was found in the no drug effect group (n = 11; t10 = 1.9, adj. p = .09; Figure 14B).
Figure 14. The effect of topical mGluR2/3 agonist LY354740 application on spontaneous firing rates. (A) Spontaneous activity significantly increased in neurons assigned to the drug effect group (n = 20). (B) Spontaneous activity did not significantly change in neurons assigned to the no drug effect group (n = 11). Each neuron’s pre-drug and post-drug means are connected by grey lines; population data is summarized with boxplots (the plot presents rank-transformed spontaneous firing rates). ** adj. p < 0.01, NS adj. p > 0.05

Discussion

We found that spontaneous firing rates increased after group II mGluR activation. This result contrasts with results obtained using systemic (intravenous) mGluR2/3 agonist application: spontaneous firing was reported to be decreased (Galazyuk et al., 2019). As mGluRs have not been thoroughly studied throughout the auditory system and their expression patterns and physiological roles have not been described in detail, at this time it is difficult to propose a much more in depth explanation of the discrepancy other than stating the obvious—systemic drug application is vastly different from local application used in the present study. IC is often referred to as a hub of the auditory system because of converging ascending and descending auditory and some non-auditory inputs (Casseday et al., 2002; Gruters and Groh, 2012); systemically delivered drugs can bind to receptors in many brain areas and the effect on spontaneous firing could be relayed to the IC. When receptor activation is limited to the IC, the effect might be different due to the lack of impact on inputs from other regions. Therefore, to achieve a
better understanding of how group II mGluRs might be utilized for possible treatment of tinnitus, which might be linked to elevated spontaneous activity (Sedley et al., 2019; Shore and Wu, 2019), it would be valuable to characterize the role these receptors play in spontaneous firing regulation not only in the IC but in other auditory structures as well.

Findings in line with those reported here—increased spontaneous firing after group II mGluR activation—were described in other brain areas. For example, increased spontaneous excitability was demonstrated in vitro in the hippocampal CA3 pyramidal cells (Ster et al., 2011) and in vivo in the cochlear nucleus (Sanes et al., 1998). In the hippocampus, increased spontaneous neuronal excitability resulted in synchronous network activity in the theta range (Ster et al., 2011). Although the exact function of hippocampal theta oscillations is not known, this might be vital for optimal memory encoding (Leung and Law, 2020).

In the auditory system, synchrony and increased spontaneous activity have been investigated as plausible mechanisms underlying tinnitus (Shore et al., 2016; Shore and Wu, 2019) and audiogenic seizures (Garcia-Cairasco, 2002; Faingold et al., 2005). Thus, it seems that elevation of spontaneous firing by activation of mGluR2/3 receptors in the IC could have negative consequences. Nevertheless, though our results may caution against group II mGluR activation directly in the IC, a possibility remains that systemic administration might be a valuable therapeutic approach to alleviate tinnitus.

**Technical considerations: anesthesia effect.**

Our results show that fifteen of 31 neurons were not spontaneously active before drug application. This was to be expected as the study was conducted on anesthetized animals. It is known that spontaneous firing in the IC of anesthetized animals can be reduced compared to recordings in awake animals, independent of species and anesthetic used (Torterolo et al., 2002; Duque and Malmierca, 2014; Shaheen and Liberman, 2018). A recent study (Ono et al., 2017) reported that about 74% of neurons recorded in anesthetized mice had no spontaneous activity at all, which is more than in our sample (48%). Even though anesthesia generally reduces spontaneous firing, it is possible that the magnitude of this
reduction varies depending on the specifics of the anesthesia method. We used the same dose of ketamine and xylazine as Ono et al. (2017). However, acepromazine dose was lower in our study (3 mg/kg compared to 10 mg/kg). In addition to ketamine/xylazine/acepromazine cocktail, Ono et al. (2017) also used isoflurane mixed with oxygen during the recordings. Different anesthesia methods might explain the discrepancy in proportions of spontaneously active neurons between the studies. There may be an optimal anesthesia approach allowing for larger numbers of spontaneously firing neurons in anesthetized animals.
CHAPTER V
Concluding remarks

The overall objective of this dissertation was to investigate the role of group II mGluRs in the mouse inferior colliculus (IC). To achieve this goal, we utilized several experimental approaches—single cell electrophysiology, optogenetics, pharmacological and anatomical techniques.

Chapter II details experiments conducted to achieve Specific Aim 1, which was to establish and validate normal-hearing transgenic mouse model for optogenetic cell type identification. The use of transgenic animals was necessary for testing of a hypothesis that group II mGluRs modulate both excitatory and inhibitory IC neurons. As VGAT-ChR2 transgenic mice were generated on C57BL/6J genetic background that is related to hearing abnormalities, we used a breeding scheme that allowed us to overcome this issue and to study normal-hearing animals. We first performed immunostaining and tested hearing thresholds to validate VGAT-ChR2 transgenic mice on a mixed C57BL/6J x CBA/CaJ genetic background. We confirmed that, as expected, hearing thresholds were normal and channelrhodopsin-2 was expressed in inhibitory cells. Further, Chapter II shows that our newly established optogenetics setup allowed for clear distinction of cell types, indicating that channelrhodopsin-2 is functional in mixed genetic background mice.

Chapter III describes experiments conducted to achieve Specific Aim 2, which was to determine the role of group II mGluRs in sound-evoked firing modulation in the mouse IC. Recordings in the same neuron before and after pharmacological mGluR2/3 activation allowed us to show that after agonist application sound level processing is enhanced in a subset of both GABAergic and non-GABAergic IC neurons: rate-level functions became steeper, thresholds lower, and maximum firing rates higher.
Chapter IV shows results obtained for Specific Aim 3, which was to determine the role of group II mGluRs in spontaneous firing modulation in the mouse IC. We found that spontaneous firing was elevated after mGluR2/3 activation.

In summary, this dissertation expands our knowledge of group II mGluRs in the mouse IC, providing strong evidence that these receptors modulate both GABAergic and non-GABAergic neurons. Future studies—namely those aimed at investigating animals without hearing abnormalities—may also benefit from our validation of normal-hearing VGAT-ChR2 transgenic mice.

Other mGluRs, in addition to group II, might contribute to IC neural activity modulation

In addition to group II mGluRs, some evidence suggests that other mGluRs might also modulate IC neural activity. Martinez-Galan et al. (2010; 2012) studied group I mGluRs in IC slices prepared from Wistar rats. They utilized optical imaging of the fluorescent Ca\textsuperscript{2+}-sensitive dye Fura-2 that serves as Ca\textsuperscript{2+} indicator. This widely used technique allows for fluorescence intensity conversion to intracellular Ca\textsuperscript{2+} concentration (Zanin et al., 2019). It was demonstrated that pharmacological group I mGluR activation elicited large Ca\textsuperscript{2+} responses at post-natal day (P) 6, but the responses were smaller by P13–P14. These electrophysiological findings are consistent with receptor mRNA data: the highest levels were found at P7, followed by decreases during post-natal development. Such results suggest that group I mGluRs might play an important role during the formation of the synaptic circuits early in development. The presence of group I mGluRs in the IC of older P35–55 Wistar rats was confirmed using real-time quantitative reverse transcription-polymerase chain reaction, Western blotting, and immunohistochemistry (Valero et al., 2015).

Voytenko and Galazyuk (2011) also studied group I in the IC. This \textit{in vivo} study showed that iontophoretically administered group I agonist increased sound-evoked firing in the IC of two months old mice. In contrast to these and above described findings, Farazifard and Wu (2010) did not observe
modulatory group I mGluR effect in the IC slices prepared from P8–17 Long Evans rats. As discussed in Chapter III, between species differences in receptor expression might account for the discrepancies. Various differences between the rat strains were reported in the literature (Luedtke et al., 1992; Webb et al., 2003; Porterfield et al., 2011; Coria et al., 2014). Nevertheless, the above described data provide some evidence that group I mGluRs are expressed in the IC and modulate its neuronal activity.

Group III mGluR mRNA and protein were both detected in the IC of adult rats (Yip et al., 2001). These results are supported by behavioral data showing that bilateral injection of group III mGluR agonist into the IC of sound sensitive genetically epilepsy-prone rats caused a short initial proconvulsant followed by a prolonged anticonvulsant effect against audiogenic seizures (Tang et al., 1997; Yip et al., 2001). Conversely, Voytenko and Galazyuk (2011) did not observe any effect on IC neural activity following group III agonist iontophoretic application in two-months old mice.

The question remains: how do different mGluR subtypes contribute to IC neural activity modulation? Future studies aimed at examining the distribution of different mGluR subtypes in the IC could provide significant insights into the roles of these receptors. It is possible mGluR subtypes are differentially expressed between IC subdivisions. Alternatively, several mGluR subtypes might be expressed in the same IC sub-regions or even on the same IC neurons and together modulate firing activity.

Future directions

As discussed in Chapter III, iontophoresis and topical drug application have several disadvantages. However, newly developing approaches that utilize light to activate and deactivate endogenous (i.e. naturally expressed) mGluRs may soon circumvent some of the problems of iontophoresis and topical application and offer substantial advantages. Donthamsetti et al. (2019) developed photoswitchable glutamate that can be genetically targeted to membranes of specific cell types. Using light, mGluRs can be activated and deactivated only on cells expressing this engineered ligand.
This is a crucial innovation that might help to disentangle mGluR roles in specific cell types. Additionally, unlike pharmacological receptor activation, light allows for rapid and reversible receptor manipulation. Thus, the neuron’s firing activity could be recorded in response to the same sound stimulation protocol several times when mGluRs are activated and deactivated, enabling clear demonstration of mGluR modulation. IC is a good candidate for such experiments because light can be delivered to the brain surface like in our optogenetic cell type identification tests.

It would be interesting to determine whether mGluR physiology in the IC is altered in hearing disorders. For example, we could test whether noise overexposure or aging relate to changes in receptor expression levels. Such knowledge might provide insights into receptor physiological roles and inform us when pharmacological activation is likely to be effective. So far, group III mGluR subtype 7 has received the most attention in relation to hearing. A study by Friedman et al. (2009) found that a single nucleotide polymorphism in GRM7 gene determines susceptibility to age-related hearing loss in humans. mGluR7 expression was found in both mouse and human inner and outer hair cells and spiral ganglion neurons. The authors hypothesized that normal expression of functional mGluR7 is necessary for glutamate release regulation to avoid toxicity. The results by Van Laer et al. (2010) and Newman et al. (2012) provide further evidence implicating GRM7 in age-related hearing loss in humans. Furthermore, mutant GRM7 allele was also linked to decreased susceptibility to noise-induced hearing loss (Yu et al., 2018).

Several discrepancies between studies investigating mGluRs were described in Chapters III, IV and V. These discrepancies pertain to both receptor expression and physiology, not only for group II mGluRs but other mGluR groups as well. Future comparative between-species studies could provide an explanation for differing results. Furthermore, the age of the animals should be taken into careful consideration when designing future experiments. As detailed in Chapters III, IV and V, the age of the animals varied between studies with conflicting results. Differences in receptor expression levels across ages have also been reported. It is possible that age variability is one of the contributing factors to the noted inconsistencies in published data.
Administration of selective mGluR2/3 antagonists, such as LY341495, could help determine whether the sound stimulation protocol used in our experiments results in receptor activation under physiological conditions (Kingston et al., 1998). If mGluRs2/3 are naturally activated by the sound stimuli that we used to construct the rate-level functions (RLFs), antagonist application should change those RLFs. More specifically, receptor blocking should result in the reverse effect observed when receptors were activated using the agonist. However, several studies using antagonists revealed no measurable effect, while an agonist had an effect (Adedoyin et al., 2010; Carlton et al., 2011; Jin et al., 2016; Dindler et al., 2018). This suggests that under the experimental conditions tested, receptor activation by endogenous ligand does not occur. Thus, if RLFs following antagonist application remained unchanged, mGluR2/3 activation by endogenous ligand glutamate is likely to occur under different sound stimulation conditions.

As described in Chapter I, group II mGluRs include mGluR2 and mGluR3 subtypes. The agonists used in our study activate both subtypes. It would be valuable to investigate which of the group II mGluRs mediates the actions of the mGluR2/3 agonists. It has been demonstrated that mGluR2 and mGluR3 have distinct regional distributions and in recent years research efforts have been expanding to study subtype specific roles (Gu et al., 2008; Ghose et al., 2009; Marek et al., 2010; Copeland et al., 2012; Li et al., 2015; Jin et al., 2018; Wood et al., 2018; Joffe et al., 2020).

Several approaches could be taken to disambiguate group II mGluR subtype contributions. First, dual immunolabeling against mGluR2 and mGluR3 could be performed to investigate differential receptor expression in the IC. Second, the same agonists and experimental approach used for the work described in this dissertation could be utilized in experiments on mGluR3 and mGluR2 knockout animals (Linden et al., 2009; Bernabucci et al., 2012; Olszewski et al., 2017; Yang et al., 2017). Our observed effects on spontaneous and sound-evoked firing should be lost in either mGluR2 or mGluR3 knock-out mice if one of these receptor subtypes mediated our results. Furthermore, the above described novel activation of endogenous mGluRs with light enables subtype-specific receptor manipulation. Finally, it may be possible to use subtype-specific agonists. However, most of the currently available orthosteric
compounds do not differentiate between mGluR2 and mGluR3 (Mazzitelli et al., 2018). It is challenging to develop subtype-specific drugs because of the high degree of protein sequence homology between the subtypes, with near 100% similarity occurring at the glutamate-binding site (Monn et al., 2015). This explains why orthosteric agonists, like the ones used in our research, activate both receptor subtypes. To overcome this issue, numerous research efforts are focused on the development of subtype-specific compounds that bind outside the glutamate-binding site. For example, a possible binding site within the seven-transmembrane domain has been examined as it offers a greater amino acid distinction between mGluR2 and mGluR3 than does the orthosteric binding site (Bollinger et al., 2017; Engers et al., 2017; O’Brien et al., 2018). Such compounds that bind outside the binding site of endogenous ligand are called positive and negative allosteric modulators (PAM and NAM). They increase or decrease glutamate-induced receptor activity. It is likely that subtype-specific PAMs and NAMs will become increasingly popular both as research tools and possibly therapeutic drugs. Selective manipulation of single mGluR subtypes, is necessary for fully understanding their roles and would allow for decreased undesirable side effects.

Many interesting questions remain to be answered regarding the role of group II mGluRs in the IC. The results described in this dissertation encourage further investigations by uncovering that group II mGluRs enhance sound-evoked and spontaneous firing in the mouse IC in both GABAergic and non-GABAergic cell types.
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