Molecular and cellular determinants of mouse corticothalamic giant terminals

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Aard- en Levenswetenschappen
op dinsdag 29 september 2015 om 11.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Min Seol

geboren te Seoul, Korea
Promotor: Prof. dr. M. Verhage

opromotor: Prof.dr. T. Kuner
# Table of Contents

**GENERAL INTRODUCTION** ............................................................................................................ 1

1-1. SYNAPSE AND SYNAPTIC TRANSMISSION ................................................................................. 2
1-1-1. SIZE AND TYPES OF SYNAPSES ............................................................................................. 2
1-1-2. TRANSMITTER RELEASE AND POSTSYNAPTIC RESPONSE .................................................. 2
1-1-3. SYNAPTIC PLASTICITY .............................................................................................................. 4

1-2. TECHNICAL APPROACHES TO DISSECT SYNAPTIC CONNECTIONS ....................................... 5
1-2-1. SELECTIVE TARGET GENE PERTURBATION .............................................................. 5
1-2-2. DEFINED SINGLE PRESYNAPTIC STIMULATION ....................................................................... 5

1-3. SENSORIMOTOR INTEGRATION IN THE RODENT WHISKER SYSTEM ...................................... 6
1-3-1. INVOLVEMENT OF POM IN WHISKER BEHAVIOR .............................................................. 6
1-3-2. POM ENGAGES IN CTC LOOPS .............................................................................................. 7
1-3-3. CORTICOThALAMIC PROJECTIONS ....................................................................................... 9

1-4. POSTSYNAPTIC DETERMINANTS OF THALAMIC TRANSMISSION .............................................. 11
1-4-1. AMPA RECEPTORS .................................................................................................................. 11
1-4-2. HIGH AFFINITY NICOTINIC RECEPTORS .............................................................................. 11
1-4-3. VOLTAGE-GATED T-TYPE CALCIUM CHANNELS .................................................................... 12

1-5. GOALS OF STUDY ........................................................................................................................ 12

1-6. OBJECTIVES ................................................................................................................................ 13

**PROPERTIES OF MOUSE ROSEBUD SYNAPSES** ........................................................................... 14

2-1. INTRODUCTION .......................................................................................................................... 14

2-2. RESULTS ....................................................................................................................................... 15
2-2-1. LABELING OF CORTICOThALAMIC GIANT TERMINALS ....................................................... 15
2-2-2. SELECTIVE STIMULATION OF EVOKED POSTSYNAPTIC CURRENTS .................................. 16
2-2-3. MATURATIONAL CHANGE OF AMPAR REDISTRIBUTION .................................................... 17
2-2-4. PROPERTIES OF EVOKED EPSCS ......................................................................................... 18
2-2-5. SYNAPTIC SHORT-TERM DEPRESSION AND RECOVERY OF EPSCS ............................... 20
2-2-6. QUANTAL SIZE AND CONTENT ............................................................................................... 22
2-2-7. AMPLIFIED POSTSYNAPTIC POTENTIAL WITH A DELAY .................................................... 23

2-3. DISCUSSION ............................................................................................................................... 27
2-3-1. TEMPORAL ASPECT OF THALAMIC RESPONSE ................................................................. 27
2-3-2. RELATIONSHIP OF SYNAPSE SIZE AND EPSC AMPLITUDE ............................................. 28
2-3-3. MATURATIONAL CHANGES IN SYNAPTIC TRANSMISSION FROM L5B TO POM .............. 29

**AMPA RECEPTORS DETERMINE SYNAPTIC TRANSMISSION AT ROSEBUD SYNAPSES** ............. 31

3-1. INTRODUCTION .......................................................................................................................... 31

3-2. RESULTS ....................................................................................................................................... 32
3-2-1. EXPRESSION AND DELETION OF SYNAPTIC AMPARs ......................................................... 32
3-2-2. DELIVERY OF AAV-MORANGE WITHOUT CRE ............................................................... 34
3-2-3. PHYSIOLOGICAL CONSEQUENCES OF AMPA RECEPTOR DELETION ............................... 35

3-3. DISCUSSION ............................................................................................................................... 47
3-3-1. REMOVAL OF SYNAPTIC Ca\(^{2+}\)-PERMEABLE AMPARs AT A DEFINED SYNAPSE ........... 47
3-3-2. STD CHANGE IN THE ABSENCE OF POSTSYNAPTIC AMPARs ........................................ 48
CONTRIBUTION OF HIGH-AFFINITY NICOTINIC RECEPTORS TO
CORTICOTHALAMIC GIANT SYNAPSES ...................................................... 50

4-1. INTRODUCTION ......................................................................................... 50
4-2. RESULTS ................................................................................................. 51
  4-2-1. NO CHANGE IN SPONTANEOUS ACTIVITY BY DHβE ......................... 51
  4-2-2. SLIGHTLY SHARPER Evoked ACTIVITY BY nicOTINic INHIBITION ........ 52
  4-2-3. CHAnGE IN STD BY DHβE ................................................................. 53
  4-2-4. DHβE TRIGgers FASTER SPIKE RATES ............................................. 54
  4-4. NeURONAL EXCITABILITY IS CHANGED BY DHβE ......................... 55
4-3. DISCUSSION .......................................................................................... 57

ROLE OF THE T-TYPE CALCIUM CHANNEL CA3.1 IN POSTSYNAPTIC
INTERGRATION ............................................................................................ 59

5-1. INTRODUCTION ......................................................................................... 59
5-2. RESULTS .................................................................................................. 60
  5-2-1. EXPRESSION AND LOSS OF CA3.1 .................................................. 60
  5-2-2. NO BURSTING OF POM NEURONS IN THE ABSENCE OF CA3.1 .......... 61
  5-2-3. KNOCKDOWN OF CA3.1 CHANNEL AT A DEFINED SYNAPSE .......... 62
  5-2-3. DESIGN OF THE CONTROL GROUPS FOR CA3.1 KNOCKDOWN ........ 64
  5-2-4. PHARMACOLOGICAL SILENCING T-TYPE CHANNELS ................... 67
5-3. DISCUSSION .......................................................................................... 68
  5-3-1. NO BOOSTING POM OUTPUT WITHOUT CA3.1-MEDIATED LTS ....... 68
  5-3-2. OTHER POTENTIAL SOURCES FOR REBOUND BURST FIRING ........ 69

MATERIALS AND METHODS ........................................................................... 70

6-1. ANIMAL MODEL SYSTEM .................................................................... 70
  6-1-1. ALL EXPERIMENTS WAS CARRIED OUT IN MICE (Mus musculus) .. 70
  6-1-2. MICE LACKING AMPA RECEPTOR SUBUNITS ................................ 70
6-2. RNAi CONSTRUCTS ............................................................................... 71
6-3. PLASMID CLONING ............................................................................... 71
6-4. RECOMBINANT ADENO-ASSOCIATED VIRUS PREPARATION ............... 72
6-5. STEREOTAXIC INJECTION .................................................................... 73
6-6. PREPARATION OF ACUTE BRAIN SLICES .......................................... 74
6-7. ELECTROPHYSIOLOGY ......................................................................... 74
6-8. JUXTASYNAPTIC STIMULATION ............................................................ 75
6-9. IMMUNOHISTOCHEMISTRY .................................................................. 75
6-10. IMAGE ACQUISITION .......................................................................... 76
6-11. QUANTIFICATION OF TERMINALS AND SOMA SIZE ....................... 76
6-12. DATA ANALYSIS ................................................................................. 76

GENERAL DISCUSSION .................................................................................. 78

7-1. KEY FEATURES OF THE MOUSE ROSEBUD SYNAPSE .......................... 79
  7-1-1. STRUCTURE-FUNCTION RELATIONSHIP AT THE L5B-POM SYNAPSE AND COMPARISON TO OTHER GIANT SYNAPSES .................................................. 79
  7-1-2. COMPARISON OF ROSEBUDS BETWEEN MICE AND RATS .......... 80
  7-1-3. PHYSIOLOGICAL IMPACT OF STRONG SHORT-TERM DEPRESSION AT THE L5B-POM SYNAPSE ................................................................. 81
  7-1-4. VOLTAGE-DEPENDENT SWITCH OF SYNAPTIC TRANSFER MODE ...... 82
7-2. GLUa4 IS THE DOMINANT AMPAR SUBUNIT IN POM RELAY NEURONS .... 82
7-3. CAV3.1 ACTIVATES SYNAPTIC AMPLIFIER AT HYPERPOLARIZED MEMBRANE POTENTIALS .............................................................. 84
7-4. METHODOLOGICAL CONSIDERATIONS .............................................. 85
Chapter 1.

General introduction

Referring the Greek word *thalamos*, a room connecting directly or via a passageway, sensory information has to pass thalamus to reach the cortex and there is no functional cortical area without thalamic input. Apart from the conventional view of thalamus as a gateway, the connectivity and systemic function between cortex and thalamus are still unclear. Moreover, thalamic involvement has been implicated not only in sensory gating but also in cognitive functions, sleep, and neurological disorders including absence epilepsy and neuropathic pain. Therefore, parallel flows in information transmission exist between cortex and thalamus, providing top-down and bottom-up controls. Much of our knowledge about thalamic function on the processing of sensory stimuli comes from studies in the visual system. But also the vibrissae-to-barrel cortex of rodent is an excellent system for the assessment of thalamic influence on structural and functional properties.

The purpose of this study is to explore the underlying complex molecular and cellular processes that control the precisely timed generation and transmission of action potentials between synaptically coupled cortical and thalamic regions in the rodent whisker system. It is fundamental to gain insight into the synaptic properties of defined single synapses at a properly matured stage, contributing neuronal circuit network and systemic function *in vivo*. The advent of recording and imaging techniques combined with the development of genetically encoded fluorescent proteins allowed us to zoom into an individual corticothalamic synapse. We performed electrophysiological recordings with high temporal and spatial resolution from the identified neurons. The focus was set to characterize structural and functional properties of corticothalamic synapse in mouse and to determine the effect of genetic perturbations leading to altered levels of postsynaptic proteins on spike timing and pattern. Since the spike is the primary message by which information is transmitted in the brain, the molecular determinants underlying this neural code, which is not only rate-based but also contributed by spike-timing or synchronous activity, are of great importance.
1-1. Synapse and synaptic transmission

1-1-1. Size and types of synapses

Synapses are specialized structures for neuronal cell communication and are composed of pre- and postsynaptic compartments, which are separated by synaptic cleft in chemical synapses. The asymmetric structure between pre- and postsynaptic components with the pronounced postsynaptic density is a feature of excitatory synapses. The presynaptic elements are characterized by the arrangement of synaptic vesicles and by the specialized patches of the plasma membrane called active zones. The active zone is recognized by an electron-dense matrix of proteins for release of synaptic vesicles including docking, priming and fusion. The strength of a synaptic connection is established by the size and number of active zones formed within the synapse. Small synapses typically have one active zone and measure a diameter of about 1 µm. Large synapses have multiple active zones, up to 800, and can be up to several tens of µm in diameter. The latter, so called giant terminals, have special properties in setting the speed and reliability of synaptic transmission. Giant synapses are abundant in the thalamus, auditory brain stem, cerebellum and hippocampus, but also occur in other regions of the brain. Their common feature is that they operate many active zones in parallel, allowing them to mediate a large postsynaptic response and in some cases sustained high-frequency transmission (Rollenhagen and Lubke, 2006). The corticothalamic giant terminals contain large and round vesicles and are characterized by glomeruli forming several synaptic junctions on the proximal dendrites of thalamic relay cells, offering the direct examination of its synaptic property and function (Hoogland et al., 1987; Hoogland et al., 1991; Jones, 2007).

1-1-2. Transmitter release and postsynaptic response

The arrival of presynaptic action potential (AP) triggers the opening of voltage-gated calcium channels, primarily P/Q and N-type, resulting in elevation of local and transient Ca\(^{2+}\) ions. Synaptic vesicles can be fused with the target membrane by increased intracellular Ca\(^{2+}\) concentration and diffused into synaptic cleft toward the postsynaptic cells. The postsynaptic site of synapse is specialized for receiving neurotransmitters such as glutamates that are packed in synaptic vesicles and released from the presynaptic terminals, thereby transducing electrical signal into biochemical
changes in postsynaptic neurons. The release of neurotransmitter is regulated by presynaptic activity. Binding of neurotransmitters to ionotropic receptor on target postsynaptic neurons mediates selective ion flux across the plasma membrane, which brings about membrane potential change, then elicits a postsynaptic response. Therefore, there is a conversion of chemical signal, a series of conformational changes from ligand-bound receptors, into an electrical signal by opening the transmembrane pore. At excitatory synapse, membrane depolarization of postsynaptic cell is driven by activation of receptors such as AMPA receptors, as illustrated in Figure 1-1 (Lisman et al., 2007; Pereda, 2014).

1-1-2-1. Evoked response

Synchronous release occurs through exocytosis of synaptic vesicles triggered by invasion of presynaptic AP and it is temporally precise, leading to fast release of neurotransmitters. Upon elevated internal Ca\textsuperscript{2+} level, the fusion of vesicle at the active zone of target membrane happens within hundreds of microseconds in a probabilistic way, which, in turn, generates a postsynaptic response with a delay of approximately 1 ms. Although it is still debated whether both evoked and spontaneous release share the same vesicle pool, the evoked response is the consequence of release from parallel multiple active zones and proportional to the size of the vesicle pool. Thus, stimulating the giant presynaptic terminal containing several active zones likely generates a large postsynaptic response. On the other hand, there is also temporally desynchronized, asynchronous neurotransmitter release in response to the presynaptic AP (Kaeser and Regehr, 2014).
1-1-2-2. Spontaneous response

In the absence of AP, neurotransmitters can be released spontaneously in a stochastic and largely Ca\(^{2+}\)-independent manner. Without nervous activity, fusion events are ensued and evoke small synaptic responses, referred to as miniature postsynaptic currents or potentials (Fatt and Katz, 1952). These low release probability events represent the release of a single quantum that presumably arose from the fusion of a single vesicle with the target membrane (Katz and Miledi, 1969). The quantal analysis provides information about unitary properties of synaptic transmission, the probability of release (quantal content), the average number of receptors in postsynaptic site or the size of postsynaptic response (quantal size) and the number of release site (quantal event frequency).

1-1-3. Synaptic plasticity

The effect of synaptic signal transmission varies depending on the preceding activity at either or both sides of the synapse, release and receptivity of neurotransmitters. There is synaptic enhancement and depression, of which can be divided into short-term and long-term plasticity according to temporal aspect. At synapse where short-term depression (STD) occurs, the following stimulation at short time intervals evokes a response that is smaller compared to the first one and this reduction lasts for milliseconds to seconds. Various both pre- and postsynaptic mechanisms have been attributed to this synaptic short-term plasticity (STP) (Regehr, 2012). When subsequent stimuli are delivered faster than replenishment of the pool, leading to release fewer vesicles accompanied with smaller postsynaptic response. If the initial release probability is high, during which a large fraction of the readily releasable pool (RRP) is released by an action potential, this synaptic depression will be increased. Recovery from depression needs vesicle replenishment of RRP, to which vesicles either from newly endocytosed or from the reserve pool and occurs typically within seconds. In addition, ligand-gated ion channels such as glutamate receptors undergo desensitization in which the neurotransmitter remains bound but ion channel is closed. Receptor desensitization does not affect STP at most synapse in the central nervous system but postsynaptic mechanism for STD allows synapses to enhance the strength in a frequency-dependent manner (Chen et al., 2002).
CHAPTER 1

1-2. Technical approaches to dissect synaptic connections

1-2-1. Selective target gene perturbation

Targeting molecular perturbations precisely in space and time is an ultimate goal for contemporary neuroscience to map neuronal circuits and explore the function of target genes (Luo et al., 2008). The mouse is the primary model for manipulating gene function by knockouts, knock-in and transgenic methods. To avoid development and nonspecific abnormalities in mice with global gene manipulations, spatially and temporally restricted approaches have been developed. Cre/loxP-mediated conditional knockouts are to induce recombination between two loxP sites by bacteriophage recombinase Cre. Using RNA interference (RNAi) technology such as short hairpin RNA (shRNA), the expression of target gene can be selectively and specifically knocked down. Delivery of viral vectors has been widely employed as a transgenic strategy combined with transgenic animals, permitting for a novel way to selectively and rapidly activate and inactivate genes of interest at the injection site. Recombinant vectors including adeno-associated virus (AAV) are used for the long-term gene expression without evident toxicity. Expression of reporter genes encoding fluorescent proteins on infected cells allowed us to record genetically defined target postsynaptic neurons.

1-2-2. Defined single presynaptic stimulation

To resolve how signals propagate from one neuron to another at the level of a single synapse, especially long-range connection between them, we applied the direct electrical stimulation of a presynaptic bouton while measuring the response in postsynaptic neuron intracellularly. The spatial and temporal resolution of this method is comparable with other techniques (Luo et al., 2008; Nauen, 2011). Both stimulation parameter and postsynaptic membrane change can be described as a measurable representation in a computational manner. In this study, the postsynaptic cell is filled with a dye to find contacts with genetically pre-labeled presynaptic terminals that originated from the barrel cortex. Therefore, we were able to visualize both pre- and postsynaptic compartments between identified connected neurons allowing us to reveal the synaptic properties of the synapse.
1-3. Sensorimotor integration in the rodent whisker system

Rodents heavily rely on tactile information from their whiskers (vibrissae) that are innervated by infraorbital branch of the trigeminal nerve in order to identify and locate objects, sensing and navigating environment. Vibrissa follicles, where each whisker is anchored to the skin of face, are innervated distinctly with deep and superficial nerve endings and terminated in particular area of trigeminal system. This structural feature is absent or sparse in non-whisking animals. Individual whisker is relayed to the corresponding neuronal modules in higher sensory areas such as thalamus and primary somatosensory (barrel) cortex (S1), thereby forming a topographic organization. Sensory information from whisker is used for driving goal-oriented movements that arise from interaction of neurons across multiple brain areas (Arvidsson and Rice, 1991; Knutsen et al., 2006; Diamond et al., 2008; Voigts et al., 2008; Kleinfeld and Deschênes, 2011). Therefore, there is interplay between sensory and motor system, so called sensorimotor integration. Whisker deprivation in neonatal rodent affects both structural and functional development of somatosensory system, and it even affects whisker-related behaviors such as decision-making, explorative activities, and social interaction later in life (Carvell and Simons, 1996; Celikel and Sakmann, 2007; Lee et al., 2009). Understanding where and how periphery sensory signal from whiskers is transformed into whisker-guided movements or other motor-related actions is one of fundamental question in neuroscience. Here, we will discuss sensorimotor integration at the level of thalamus, especially top-down control on the posteriomedial nucleus (POm).

1-3-1. Involvement of POm in whisker behavior

The POm has been known to convey mechanosensory signals from the paralemniscal pathway to the cerebral cortex, which is anatomically and functionally parallel to the lemniscal pathway via thalamic ventral posteromedial nucleus (VPM) (Ahissar et al., 2000; Yu et al., 2006). The axons from VPM receiving input from the principal sensory trigeminal nucleus in brain stem terminate in cortical barrels, dense neuronal clusters in layer 4, whereas those from POm of which afferents from interpolar part of the spinal trigeminal nucleus ascend ventral to the barrels in upper layer 5 (Fig. 1-2, gray line) (Diamond et al., 2008). Correlated to its topographic arrangement, the neurons in lemniscal pathway are sensitive to the somatotopically-connected whisker,
thereby representing as a barrelette in the principal trigeminal nucleus and a barreloid in VPM. In contrast, the neurons in the spinal trigeminal nucleus and POM have larger receptive fields that extend across multiple whiskers (Diamond et al., 1992a; Jacquin et al., 1993). Employing multiple whiskers, rodents discriminate textures, shapes, and locations during exploration and they have faster response times in decision-making than single-whisker animal (Celikel and Sakmann, 2007; Diamond et al., 2008). The POM carries temporal signals encoding whisker movement but not contact or touch information that is encoded in the lemniscal pathway (Yu et al., 2006). Moreover, the POM reaches the cortex with longer and more variable latencies that increased by repeated stimulation (Diamond et al., 1992a; Ahissar et al., 2000).

Besides its cortical projections, the POM also innervates the tectum, pons and striatum, which participate in control of motor function (Fig. 1-2, gray line) (Veinante et al., 2000; Smith et al., 2012).

1-3-2. POM engages in CTC loops
The thalamus is not just simply a relay center transmitting peripheral signals to the cortex, but also receives inputs from the cortex and sends integrated information to the cortex via the cortico-thalamo-cortical (CTC) loop. According to major input source, the thalamus can be divided into two types, first order and higher order. The former thalamic nuclei convey periphery information to the cortex but the latter, higher order relays serve to communicate between cortical areas via the CTC route (Sherman and Guillery, 2002; Sherman and Guillery, 2009). The CTC loop provides a substrate for synchronization of the global network (Steriade, 2000). Stroh and

Figure 1-2. Summary of synaptic connections in the POM.
(modified from Theyel, Llano et al. 2010).
colleagues showed that calcium transients are initially generated from the cortex and propagated throughout the cortex by recruitment of thalamus, highlighting corticothalamic inputs via CTC loops (Stroh et al., 2013). In the somatosensory system, the POm receives main inputs from the primary cortex and sends its output to a vast number of cortical regions, resulting in highly connected units (Jones, 2007; Sherman and Guillery, 2009). The POm has been shown to form the anatomical and functional link between S1 and motor cortex 1 (M1), both of which are essential for whisker-related sensorimotor integration (Urbain and Deschênes, 2007). Consistent with the global brain calcium wave, propagating activity from the somatosensory to motor cortex has been demonstrated in behaving animals (Ferezou et al., 2007). The essential role of whisker part of S1 has been examined in object localization, gap-crossing, aperture width dissemination and whisker retraction control (Hutson and Masterton, 1986; Krupa et al., 2001; Matyas et al., 2010; O’Connor et al., 2010; Bosman et al., 2011). When the S1 cortex is inactivated, POm cells are less responsive but not VPM neurons, showing the strong cortical control on the POm activity (Diamond et al., 1992b).

Instead of long-range of corticocortical communication streams, the POm is required to communicate interconnected cortical regions (Fig. 1-2) (Theyel et al., 2010). It has been suggested that activation threshold of corticocortical connections can be declined by fast thalamocortical parallel inputs to multiple cortical areas (Ullman, 1995). Additionally, the strength of thalamocortical synapses is greater than that of corticocortical contacts (Gil et al., 1999). In rodent whisker behavior, functionally distinct activation from S1 to the secondary somatosensory cortex (S2) and to M1 has been reported (Chen et al., 2013a; Yamashita et al., 2013). In addition, the afferents from layer 5 (L5) of S1 preferentially target to the anterior part of POm of which projections reach mainly to the superficial part of L5, layer 5A in S1 and send axonal collaterals to S2 and M1 (Veinante et al., 2000; Ohno et al., 2012).

On the other hand, there are no GABAergic interneurons within the POm and L5 originating axons do not innervate the reticular nucleus (nRT) (Barbaresi et al., 1986; Sherman and Guillery, 2009). Therefore, all GABAergic innervation are mediated by extrinsic afferents, and the membrane potential of POm is highly determined by driving inputs. GABAergic inhibition by the zona incerta (IZ) and the anterior pretectal nucleus (APT) have been shown to have large multisynaptic contacts on the POm (Fig.1- 2, blue dotted line). The inhibition from IZ onto POm
can be relieved by M1 activity, disinhibition. Hence, whisker-related sensorimotor information from POM can be transmitted to the cerebral cortex depending on the activity of M1. Moreover, the APT and the IZ have reciprocal connections, and both receive the L5 cortical input and innervate brainstem motor centers (Barthó et al., 2002; Bokor et al., 2005b; Lavallée et al., 2005; Urbain and Deschênes, 2007).

In conclusion, based on all these studies the ‘closed CTC loops’ model has been proposed, in which the thalamus and cortex act as a functional unit (Fig. 1-2). Indeed, dynamic information processing has been demonstrated in the POM of which relay cells are sensitive to the relative timing between peripheral and cortical inputs, thereby comparing intended and actual whisking frequency (Ahissar and Oram, 2013; Groh et al., 2013).

1-3-3. Corticothalamic projections
Despite numerical superiority of projections from cortex to thalamus over those from thalamus to cortex, the physiological function of corticothalamic synapse is not well investigated compared to that of thalamocortical pathway. There are morphological and physiological distinct two types of corticothalamic inputs, the driver and the modulator. All thalamic nuclei receive modulatory inputs from cortical layer 6 (L6), which form synapses with small synaptic boutons on the distal dendrites of the thalamic neuron. Activation of L6 evokes a small, graded, and facilitating postsynaptic potential that activates both ionotropic and metabotropic receptors (Fig. 1-3, red line). In contrast, driver inputs from L5 of cortex transmit information only to the higher-order thalamic nuclei, of which proximal dendrites making synaptic contacts with L5 projecting large boutons, and do not collaterally project to the reticular nuclei (nRT). Giant terminals have been described in different sensory afferents such as the somatosensory, visual, and auditory system from rodents, cats, and primates (Rouiller and Welker, 2000). These giant synapses from L5 generate a large, all-or-none, and paired-pulse depressing postsynaptic response by ionotropic receptor activation, so they function as a driver (Fig. 1-3, green line) (Sherman and Guillery, 2009, 2011).

L5 is a major output projection layer of the cortex and brief optogenetic stimulation on small cluster of L5 neurons can initiate calcium transients throughout the cortex (Stroh et al., 2013). Moreover, the global thalamic population activity is
reliant on the descending corticothalamic afferents (Contreras et al., 1996; Stroh et al., 2013). In the somatosensory system, the parent axon of cortical L5 continues past the thalamus into motor regions including the superior colliculus, pontine, and spinal cord (Veinante et al., 2000). These findings are in the line with Sherman and Guillery’s view proposing that the higher order thalamic nuclei such as POM carry a copy of motor instructions, efference copies, so may serve to monitor motor action (Sherman and Guillery, 2011). It has been recently reported that the anterior lateral motor cortex (ALM) is engaged for motor preparation in which sensory information from S1 is evaluated for decision prior to motor action (Guo et al., 2014). Moreover, the posterior area of thalamus is required for the communication from S1 to ALM (Fig. 1-2) (Mao et al., 2011; Guo et al., 2014).

The main excitatory input to POM arises from cortical layers 5B (L5B) neurons that are largely consisted of thick-tufted pyramidal cells receiving both whisker touch and motion signals from VPM and POM, respectively (Fig. 1-3, green line) (Petreanu et al., 2009; Oberlaender et al., 2011). L5B output from different barrels has been proposed to converge in the POM (Wright et al., 2000). Somatosensory cells in L5B have been shown to respond with short latencies and higher action potentials after whisker deflection (de Kock et al., 2007). Moreover, thick-tufted pyramidal neurons in L5 receive more densely converging thalamocortical inputs than L6 (Constantinople and Bruno, 2013).
1-4. Postsynaptic determinants of thalamic transmission

1-4-1. AMPA receptors

Ionotropic glutamate receptors form ligand-gated ion channels and are grouped into AMPA, kainate, NMDA receptors based on its specific agonist. AMPA receptors (AMPARs) mediate fast excitatory synaptic transmission and rapidly undergo desensitization by action of glutamate in the central nervous system. They are tetrameric assemblies, as a dimer of dimer, by various homo-and heteromeric combinations of four subunits, GluA 1-4 (Hollmann and Heinemann, 1994; Dingledine et al., 1999). Since each subunit act as a functional channel, occupancy of one or two of the four subunits suffices to activate gating (Traynelis et al., 2010). Upon binding of glutamate, the pore opening induces depolarization by the influx of $\text{Na}^+$, depending on subunit composition, and $\text{Ca}^{2+}$. After activation, AMPA receptors enter a desensitized state during which the dimer-dimer interaction is stabilized and agonist affinity is increased (Sun et al., 2002). AMPAR desensitization has been implicated in fidelity of synaptic transmission and plasticity (Chen et al., 2002; Heine et al., 2008). AMPARs-mediated synaptic currents display a range of different functional properties including ion conductance and gating channels depending on the composition of different subunits by alternative RNA splicing (Sommer et al., 1990; Mosbacher et al., 1994), RNA editing (Lomeli et al., 1994), post-translational regulation (Correia et al., 2003), and accessory subunits together with AMPAR-interacting proteins such as transmembrane AMPAR regulatory proteins TARPs (Tomita et al., 2005).

In this study, we made use of the mice with conditional alleles of AMPAR subunits, except $\text{Ca}^{2+}$-impermeable GluA2, to differentiate and relate different composition of AMPAR subunits to corticothalamic synaptic transmission at a mature state.

1-4-2. High affinity nicotinic receptors

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels and assemble as pentameric complex of $\alpha$ and $\beta$ subunits depending on the neuronal subtype (Dani and Bertrand, 2007). Owing to a wide variety of subtypes and their locations in both pre- and postsynaptic sites even in the nonsynaptic area, nAChRs play diverse roles like enhancing neurotransmitter release, evoking excitatory
postsynaptic response or synaptic plasticity in the central nervous system (Lena and Changeux, 1997). Activation of nAChRs depolarizes the membrane potential and causes the influx of varied ions including Ca$^{2+}$ (Gray et al., 1996). Not only at the cellular level, cholinergic projections are also broadly innervated in the brain, contributing various neuronal functions such as attention, learning and memory (Castro-Alamancos and Calcagnotto, 2001; Steriade, 2004).

1-4-3. Voltage-gated T-type calcium channels
Voltage-gated calcium channels fall into two categories: low voltage-activated (LVA) channels that require small membrane depolarization for opening and high voltage-activated (HVA) channels that are further classified according to biophysical and pharmacological properties (Simms and Zamponi, 2014). Low-voltage-activated (LVA) T-type calcium channels are inactivated at relatively depolarized membrane potentials near neuronal resting state, which allows thalamic relay cells to continuously fire following depolarizing input. On the other hand, a transient hyperpolarization de-inactivates T-type calcium channels that are activated once the membrane potential is repolarized, leading to generate low threshold spike (LTS). This T-type conductance can initiate a burst of APs (Llinas and Jahnsen, 1982; Jahnsen and Llinás, 1984).

There are three genes known to encode T-type calcium channels, Ca,3.1, Ca,3.2, and Ca,3.3 isoforms (or termed α1G, α1H, and α1I respectively) (Talley et al., 1999; Cain and Snutch, 2010). Due to lack of subtype specific blocker, we applied shRNA system to knockdown Ca,3.1 T-type calcium channel subunits.

1-5. Goals of study
It is technically challenging to assess the functional description of driver giant corticothalamic synapse because the connections between cortex and thalamus are long-range and reciprocal, and there are also small-modulatory corticothalamic terminals projecting from L6 cortical neurons (Sherman and Guillery, 2009). Here, we reveal the synaptic connection together with measure of synaptic strength of mouse corticothalamic giant synapses from L5B cortical neurons to POm relay cells, using electrophysiological recordings in combination with viral gene transfer to identify pre- and postsynaptic components (Chapter 2). With previously established
approach from our group, we visualized and distinguished L5B originating synaptic boutons that are large and located in the proximal dendrites of thalamic neurons, allowing the direct stimulation of individual corticothalamic giant terminals (Groh et al., 2008).

Furthermore, we sought to investigate how the presynaptic L5B input shapes the response dynamics of the POM postsynaptic neuron in the absence of Ca\(^{2+}\)-permeable AMPA receptors (Chapter 3), high-affinity nicotinic receptors (Chapter 4), and T-type calcium channels (Chapter 5) by spatiotemporally controlled genetic manipulations and pharmacological intervention.

1-6. Objectives

a) Label the giant terminals originating from L5B neurons of S1 using virus-mediated expression of fluorescent proteins

b) Characterize the synaptic properties of mouse POM relay cells by patch-clamp recordings simultaneously with the electrical stimulation of labeled presynaptic boutons

c) Examine the expression and distribution of AMPARs and Ca\(_{\text{v}}\)3.1 subunit of T-type calcium channel at POM neurons

d) Stereotaxic delivery of Cre-recombinase via adeno-associated viruses (AAV) to knockout GluA4 alone or GluA 1, 3, and 4 for triple knockout in POM relay neurons

e) Introduce AAV harboring shRNA for knockdown Ca\(_{\text{v}}\)3.1 T-type calcium channels and mismatch or scramble version of shRNA as controls into POM cells

f) Pharmacological blockers are used to verify the genetic manipulations and to inactivate nicotinic receptors

g) Physiological consequence of loss of AMPARs or T-type calcium channel or nicotinic receptor inactivation is determined by local electrical stimulation of presynaptic boutons combined with postsynaptic whole-cell recording
Chapter 2.

Properties of mouse Rosebud synapses

2-1. Introduction
The thalamus plays a role not only in processing information ascending from the periphery but also in integrating inputs descending from cortical brain areas. These cortical inputs are relayed back to higher order cortices, thereby forming cortico-thalamo-cortical (CTC) loops (Deschênes et al., 1998; Killackey and Sherman, 2003). The greater number of projections exists from cortex to thalamus (Sherman and Guillery, 2009), but these corticothalamic synapses are less well studied including the question how they contribute to higher brain functions such as sensorimotor integration.

Barrel cortex L5B neurons form giant glomerular synapses harboring several synaptic junctions on dendrites of relay cells in the posteromedial (POm) nucleus (Hoogland et al., 1991; Jones, 2007). These giant L5B-POm synapses can switch in an activity-dependent manner between two functional modes: driver and coincidence detector (Hoogland et al., 1991; Veinante et al., 2000; Groh et al., 2008). The functional properties of these synapses will have a strong impact on the spiking activity in relay neurons, and thereby exert an extensive influence on many cortical regions contacted by the axons of these cells (Jones, 2007; Sherman and Guillery, 2009). The morphological and physiological properties of synapses from neurons in L5B to thalamic relay cells of the POm have been described in rats (Hoogland et al., 1987; Veinante et al., 2000; Groh et al., 2008), yet the equivalent synapse has not yet been characterized in mice.

Using stereotaxic viral gene delivery into the target areas, we visualized L5B-originating synaptic boutons and characterized them by electrical stimulation of individual presynaptic terminals in the mouse (Wimmer et al., 2004; Groh et al., 2008). Consistent with previous findings, the mouse L5B-POm synapse is formed by large functional driver terminals contacting the proximal dendrites of relay neurons. Here, we refer to this synapse as the rosebud synapse.
CHAPTER 2

2-2. Results

2-2-1. Labeling of corticothalamic giant terminals

Giant corticothalamic giant terminals (here referred to as ‘Rosebuds’) originating from L5B pyramidal neurons were labeled by stereotaxic delivery of adeno-associated virus (AAV) particles encoding enhanced green fluorescent protein (EGFP)-tagged synaptophysin, a presynaptic marker specific for vesicles in boutons, into the S1 region of 2 week old mice (Groh et al., 2008) (Fig. 2-1A). The labeled rosebuds had an average diameter of 3.34 ± 0.11 μm (n=55). They were typically situated on the proximal dendrites of the POm relay cells at an average distance from the soma of 18.59 ± 1.1 μm (n=34), suggesting close electrotonic coupling to the soma via the rather thick primary dendrite (Fig. 2-1B,C). Also, multiple L5B-originating giant terminals formed contacts with the same dendritic branch of the POm neuron (Fig. 2-1B).

![Figure 2-1. Visualization of L5B-POm terminals (‘L5B-POm’).](image)

(A) AAV-synaptophysin-EGFP was virally expressed in the S1 cortex to label the corticothalamic projections from L5B pyramidal neurons to the POm. Wide-field fluorescence image (width of image approximately 1 mm). The right panel is adapted from Paxinos & Franklin (2001)

(B) L5B-POm gaint terminals (green, here referred to as the Rosebud synapse), POm neuron (red). Scale bar see C.

(C) Multichannel confocal image showing cell morphology (gray), relay cell (red) and labeled synaptic boutons (green). Both patch and stimulation pipettes are filled with Alexa 594 to aid in identification. Scale bars: 10 μm.
2-2-2. Selective stimulation of evoked postsynaptic currents

To determine the synaptic properties of the mouse Rosebud, we electrically stimulated visually identified individual presynaptic terminals with a fine-tipped double-barreled the tip of which was advanced until it touched the terminal or was positioned within 1 µm of it, while recording from the synaptically connected postsynaptic thalamic relay neuron in the whole cell configuration (Fig. 2-1C) (Groh et al., 2008).

At a holding potential of -70 mV, the stimulation intensity was gradually increased until an excitatory postsynaptic current (EPSC) was evoked. Thereafter the EPSC did not further increase with larger stimulation intensities. This all-or-none behavior, tested for every single recording shown in this study, suggests that an action potential triggered in the presynaptic terminal caused glutamate release (Fig. 2-2A).

Figure 2-2. Postsynaptic current is evoked by stimulation of single corticothalamic terminals.
(A) Representative EPSC by a single presynaptic electrical stimulus in an all-or-none manner. The stimulus artifacts are blanked. The flat line shows subthreshold response.
(B) The EPSC is almost completely blocked by co-application of the AMPA receptor antagonist CNQX (10 µM, n=14) but not affected by the NMDA receptor antagonist APV (50 µM, n=4) at a holding potential of -70 mV.
2-2-3. Maturational change of AMPAR redistribution

A postnatal maturational change in the composition of AMPA receptor subunits has been observed at central mammalian synapses (Traynelis et al., 2010). We compared the spontaneous and evoked EPSCs between postnatal 4 week (wk) and 5-6 wk of wild type (WT) mice.

A larger amplitude and faster kinetics of spontaneous EPSCs were obtained from 5-6 wk old mice (n=80 cells) in comparison to 4 wk old mice (n=61 cells) (Fig. 2-3A). The increased amplitude (Fig. 2-3A,B) suggests an increased number of receptors at the postsynaptic site or a change in the geometrical distribution of synaptic AMPA receptors. A substantial increase of the spontaneous EPSC frequency was observed in 5-6 wk old mice (Fig. 2-3A) that is also reflected in the cumulative plot of inter-event interval, predicting a larger number of glutamatergic synapses connected to the neuron (Yang et al., 2011).

Figure 2-3. Maturational change of POm neurons in the spontaneous activity.
(A) Bar histograms represent mean value of EPSC amplitude, 20-80% rise time, decay time constant, and frequency ± SEM obtained for thalamic relay cells in slices from postnatal 4 wk (gray bars) and 5-6 wk (black bar) old mice. Amplitude: 4 wks 17.8 ± 0.6 pA versus 5-6 wks 19.3 ± 0.4 pA, p < 0.05; Frequency 4 wks 1.27 ± 0.12 versus 5-6 wks 1.57 ± 0.06, p < 0.05, t-test; rise time: 4 wks 0.4 ± 0.03 ms versus 5-6 wks 0.35 ± 0.01 ms, p < 0.05; decay time: 4 wks 1.1 ± 0.07 ms versus 5-6 wks 0.9 ± 0.03 ms, p < 0.05, t-test; n=61 in 4 wks, n=80 in 5-6 wks groups).
(B) Distribution of spontaneous events from postnatal 4 wk (left panel, n=61 cells) and 5-6 wk (right panel, n=80).
(C) Analysis of the cumulative probability of inter-event interval.
The evoked activity was also different between 4 wk (n=56 cells) and 5-6 wk old mice (n=79 cells), greater and faster EPSCs were evoked by L5B input in POm neurons from postnatal 5-6 wk old mice (Fig. 2-4).

These findings suggest that there may be a subunit switch to fast-gating AMPA receptors and led us to make patch-clamp recordings from more than 5 wk old animals that were assumed to reflect a mature stage.

**Figure 2-4. The evoked activity of rosebuds is changed between postnatal 4 wk and 5-6 wk.**

Bar histograms represent mean value of EPSC amplitude, 20-80% rise time, and decay time constant ± SEM obtained for thalamic relay cells in slices from postnatal 4 wk (gray bars, n=56) and 5-6 wk old mice (black bar, n=79). Amplitude: 4 wks 140.02 ± 15.87 pA versus 5-6 wks 270.88 ± 33.71 pA, p < 0.05, rise time: 4 wks 1.14 ± 0.07 ms versus 5-6 wks 0.84 ± 0.05 ms, p < 0.05, decay time: 4 wks 5.04 ± 0.38 ms versus 5-6 wks 3.48 ± 0.21 ms, p < 0.05, t-test).

### 2-2-4. Properties of evoked EPSCs

The average of EPSC amplitude was 256.67 ± 32.97 pA at -70 mV, the 20-80% rise time was 0.84 ± 0.05 ms, and the decay time constant was 3.48 ± 0.21 ms at room temperature (76 cells each). The rapid rise and decay kinetics of the evoked EPSCs suggest that most of the synaptic current was mediated by AMPA receptors. The NMDA receptor antagonist APV (17.3 ± 6.46% reduction, n=4) blocked the slower component of the EPSC but did not affect the early fast response and peak amplitude. In contrast, the fast component of the EPSC was nearly completely blocked by the AMPA receptor antagonist CNQX (61.64 ± 5.47% reduction, n=14) (Fig. 2-2B).
2-2-4-1. Variability in EPSC amplitudes

We found that the EPSC amplitudes were highly variable. To address whether this variability may reflect a difference in terminal size, we quantified the diameter of terminals from which postsynaptic responses were obtained from 5-6 week old mouse thalamocortical slice. Figure 2-5A shows the distribution of giant terminal diameters and its corresponding postsynaptic responses. Terminal size and EPSC amplitude did not correlate (Pearson correlation coefficient 0.22, P=0.11). Dispersed distribution of presynaptic terminal size related to the degree of postsynaptic response is consistent with the size heterogeneity of corticothalamic terminals (Fig. 2-5A,B). Less than 200 pA of EPSCs were evoked irrespective of the terminal size, up to 5 µm, predicting the diversity in activity of projection neurons from L5B (Slezia et al., 2011). Otherwise, the postsynaptic organization including AMPAR subunit composition may be variable in POm neurons receiving Rosebud inputs.

In addition, the average diameter of terminals was 3.34 ± 0.11 µm (n=55) in mice compared to an average diameter determined in rats of 5.1 ± 0.17 µm (n=8, Groh et al., 2008), suggesting that rat Rosebuds tend to be larger.

2-2-4-2. Heterogeneity in the same relay cell

To exclude cell-to-cell variability, we stimulated multiple rosebuds contacting the same thalamic relay cell and found that the EPSC amplitude was not correlated with the presynaptic bouton size (Fig. 2-5C, Pearson correlation coefficient 0.26, p= 0.1). Four cases out of 22 in Figure 2-5C show downward in the relationship between bouton size and EPSC amplitude, meaning relatively smaller terminals evoked greater EPSCs. On the other hand, similar EPSCs were obtained by stimulation of different size of presynaptic boutons, 6 out of 22 cases.

These results suggest that size and strength of synaptic transmission are not correlated and that the structure-function relationship of Rosebuds is highly variable.
2-2-5. Synaptic short-term depression and recovery of EPSCs

To describe the frequency-dependent properties of transmission, we repetitively stimulated presynaptic terminals 10 times at frequencies ranging from 10 to 100 Hz. At 10 Hz stimulation, the EPSC amplitude already dropped to 30% after the first EPSC and the amplitudes of the subsequent EPSCs were also reduced but less strongly, suggesting strong depression of the rosebud synapse upon repetitive stimulation. The extent of depression increased with stimulation frequency (Fig. 2-6A). The decline in EPSC amplitudes at different frequencies could be described with a single exponential function (time constants: 10 Hz 109 ± 18 ms (n=17), 20 Hz 35 ± 5 ms (n=17), 50 Hz 12 ± 2 ms (n=12), 100 Hz 4 ± 2 ms (n=9)).
Next, we determined the time course of recovery from STD using a double-pulse protocol with varying stimulation time intervals. Plotting the relationship between inter-stimulus interval and the EPSC magnitudes revealed a doubly exponential time course with a fast $\tau$ of $15 \pm 4$ ms and slow $\tau$ of $795 \pm 187$ ms ($n=7$). It took approximately 400 ms to recover 80% of the first EPSC amplitude (Fig. 2-6B).

To rule out a prominent postsynaptic contribution to STD, we reduced AMPA receptor desensitization and saturation by application of kynurenic acid (KY) (Partin et al., 1996; Dingledine et al., 1999). The EPSC's amplitude did not change in the presence of KY, showing a similar time constants at 50 Hz stimulation (Ctrl 22.614 ± 0.83 ms versus KY 25.27 ± 3.41 ms, $n=3$). These results suggest that STD is primarily presynaptic in the Rosebud terminals (Fig. 2-6C).

Figure 2-6. Frequency-dependent properties of transmission at L5B-POm synapses.
(A) Frequency-dependence of STD. Data fitted to double exponential function ($n=9$ to 17).
(B) Biphasic time course of recovery from STD determined using a double-pulse protocol ($n=7$). The insert shows a higher magnification until 550 ms.
(C) Normalized EPSC amplitudes in the absence (black) and presence (gray) of 1 mM kynurenic acid (KY) at 50 Hz stimulation ($n=3$).
In conclusion, our results predict that the mouse L5B-POm synapse will only work as a relay synapse up to a frequency of approximately 2 Hz (estimated from the recovery time course), but will increasingly fail with higher rates of presynaptic activity. Consistent with our previous work (Groh et al., 2008), the L5B-POm terminal functions as a low-pass frequency filter.

2-2-6. Quantal size and content
We next estimated quantal size and content by recording spontaneous EPSCs (sEPSCs) (Fig. 2-7A). The distribution of 25536 sEPSCs recorded from 80 cells was fit with a single Gaussian function revealing an average amplitude of 19.3 ± 0.4 pA (Fig. 2-7B). To further corroborate that the terminals we recorded from produced miniature EPSCs with the same amplitudes we stimulated corticothalamic giant terminals under conditions of low release probability (Jonas et al., 1993; Silver, 2003). In the presence of 1 mM Ca\(^{2+}\) and 5 mM Mg\(^{2+}\), the EPSC amplitudes fluctuated in a quantal manner and the failure rate was approximately 64% (n=5, Fig. 2-7C). We found an amplitude of 22 ± 8 pA, similar to the amplitude determined from analyzing spontaneous EPSCs.

The total pool size was 448 ± 46 pA (n=14) as determined by backextrapolation of EPSC amplitudes obtained from trains of stimuli at 50 Hz (Fig. 2-7D) (Groh et al., 2008). Hence, considering a quantal amplitude of approximately 20 pA, around 22 synaptic vesicles represent the readily releasable pool (RRP). This may reflect the presence of approximately 22 active zones within a single L5b-POm terminal assuming that multivesicular release does not occur at this synapse.

The release probability of a single L5B-POm synapse was 50.5 ± 6.8 % (n=14) as determined by dividing the total pool size by the EPSC amplitude generated by a single stimulus. Thus, approximately 11 synaptic vesicles can be released by a single presynaptic action potential at the L5B-POm synapse. The high release probability is consistent with the strong short-term depression found at this synapse (Fig. 2-6A).
Figure 2-7. Quantal estimations.
(A) Representative trace showing spontaneous EPSCs. Data filtered to 1 kHz for display.
(B) Distribution of spontaneous EPSCs (25536 events out of n=80 cells).
(C) A representative EPSCs under low calcium conditions.
(D) A representative cumulative plot of EPSCs during a 50Hz train, yielding a total quantal output. Data points between 100 and 200 ms were fitted with a linear function (gray dotted line).

2-2-7. Amplified postsynaptic potential with a delay

2-2-7-1. Voltage-dependent scaling of the input-output function in POM neurons

We next addressed how evoked postsynaptic currents at rosebuds in the relay neuron affect the input-output function of the POM neuron, we moved to current clamp recordings using the same stimulation protocol.

Only in 17% (13 out of 76) of the synapses recorded from, a single presynaptic stimulation triggered postsynaptic spikes (Fig. 2-8B). Interestingly, the EPSC amplitudes recorded earlier in the experiment were not correlated with the number of postsynaptic spikes generated (Pearson coefficient -0.34, P=0.1). A typical suprathreshold voltage trace recorded at a membrane potential of -70 mV is shown in Fig. 2-8A. Close inspection of the voltage trace reveals an initial steep slope corresponding to the rapid EPSC followed by a hump at -57 mV, and continued by a less steep slope leading to action potential initiation. This finding suggests that a voltage-dependent conductance gets activated that finally brings the cell to firing...
threshold. Low voltage-activated (LVA) T-type calcium channels are well suited for boost EPSP propagation (Fig. 2-8A) (Llinas and Jahnsen, 1982; Jahnsen and Llinás, 1984; Ulrich and Huguenard, 1997) and could underly that conductance. On the other hand, the size of EPSCs did not account for the number of excitatory postsynaptic potentials (EPSPs), suggesting a different composition of ion channels at individual rosebuds (Fig. 2-8B).

![Diagram A](image)

**Figure 2-8.** L5B-POm inputs produce different postsynaptic responses depending on postsynaptic membrane potential. 

(A) A single presynaptic action potential (AP) causes a long-lasting depolarization and a burst of APs. Note the delay from stimulus artifact to the first AP. Representative example taken from 13 recordings.

(B) Spike counts are not correlated with the size of EPSC.

(C) At a depolarized holding potentials of -60 mV, the POm neuron generates a single AP with no delay. Representative example taken from 5 recordings.

Conversely, POm neurons responded with only a single postsynaptic action potential (AP) to a single L5B input when the membrane potential was depolarized, near the resting membrane potential of -60 mV (n=5). At this depolarized condition, the EPSP amplitude suffices to reach the action potential threshold. Furthermore, due
to inactivation of the T-type conductance at depolarized potentials of -60 mV, these channels will not contribute much to the voltage trace after presynaptic stimulation (Fig. 2-8C) (Deleuze et al., 2012). A physiologically relevant 10 mV difference in membrane potential, e.g. from -70 to -60 mV within which cortical activity depolarizes thalamic neurons in vivo (Dossi et al., 1992) will suffice to shift between the two modes of transmission.

Therefore, the resting membrane potential of POm relay neurons defines whether rosebud inputs get relayed 1:1 or whether the postsynaptic cell amplifies the input signal through voltage-dependent conductances.

2-2-7-2. L5B input temporally controls the neuronal spike on POm

To investigate further differences between the two thalamic firing modes, we analyzed additional features of the voltage responses at two different membrane potentials.

It is of great interest that there was a marked delay between the onset of the postsynaptic current and the first postsynaptic action potential. When the thalamic neuron was held at -70 mV, this delay took much longer than expected from a normal synaptic delay. While AMPA receptor-mediated currents subsided approximately 5 ms after stimulation (Fig. 2-2A, n=76) the postsynaptic cell continued to show a depolarizing response followed by action potentials after a delay (Fig. 2-8A, 16.34 ± 0.6 ms, n=13). In contrast, this synaptic delay was much shorter when the POm neuron fired in tonic mode at a depolarized membrane potential of -60 mV (Fig. 2-8C, 2.8 ± 0.8 ms n=5). In this depolarized condition, it is close enough to the threshold to generate spike so that postsynaptic potential was elicited instantly after stimulation. Consequently, the latency of AP initiation in POm relay neurons, defined as the time from stimulation to the first AP, depended on the membrane potential.

The low-threshold Ca\(^{2+}\) current (\(I_t\)) may contribute to this synaptic delay at Rosebud synapses. Sustained hyperpolarization is required to de-inactivate T-type channels and the subsequent depolarizing input generates low-threshold Ca\(^{2+}\) spike (LTS) overridden by bursts of Na\(^+\)/K\(^+\)-dependent APs. Thus, the period of hyperpolarization is critical to trigger LTS by recovery of T-type channels from the inactivation (Jahnsen and Llinás, 1984; Ulrich and Huguenard, 1997). Indeed,
preliminary experiments reveal that the postsynaptic potentials were depressed when the rosebud synapse was repetitively stimulated (Supplemental Fig. 1). The slow activation of T-type channels fits well to this long EPSP-AP delay and synaptic depression at Rosebud synapses for burst firing (Perez-Reyes, 2003).

We also found that the burst firing episode is determined by the preconditioning hyperpolarization. The POm neuron was unable to generate the rebound burst spikes by the same amount of hyperpolarizing current for less than 100 ms (Supplemental Fig. 2A). Moreover, further preliminary data may suggest that a subtle modification in holding potential from -70 mV to -75 mV increased spike number with prolonged delay in the synaptic transfer from L5B to the POm, in which more recovering T-type channels from inactivation may occupy (Supplemental Fig. 2C). However, we did not observe further latency changes in tonic firing with different depolarized membrane potentials, between -60 mV and -55 mV, where no LTS is generated (Supplemental Fig. 2B). Furthermore, slightly attenuated LTS amplitude gave rise to a reduced number of burst spikes at the same membrane potential (data not shown). A membrane potential shift from -70 mV to -65 mV decreased the amplitude of LTS and number of APs, while the latency at which APs were elicited was decreased (Supplemental Fig. 2D). Hence, the distance to spike threshold is larger with lower T-type channel-mediated charge ($I_t$), even at more depolarized membrane potential. These findings are in the line with the study presenting that amplitude of T-type conductance regulates the number and frequency of APs in burst mode (Tscherter et al., 2011). Therefore, the synaptic delay of APs at giant corticothalamic synapses was not only conditioned by the resting membrane potential of the relay cell but also by the time course of the stimulus and interval between presynaptic AP firings.

Taken together, POM relay cells displayed not only different firing patterns but also variable delay in AP initiation by voltage-dependent activation of T-type channels.
2-3. Discussion

2-3-1. Temporal aspect of thalamic response

The L5B-POm synapse exhibits strong frequency-dependent STD (Fig. 2-6A) and fast recovery from depression (Fig. 2-6B). Similarly, thalamic voltage responses were also depressed upon train of stimulations (Supplemental Fig. 1). Due to rapid and strong STD, the POm relay cell may respond most effectively at the onset of presynaptic activity and new stimuli, contributing adaptation and neuronal gain control (Chung et al., 2002; Abbott and Regehr, 2004; Rothman et al., 2009). Thalamic burst firing is well suitable to respond vigorously to novel stimuli but being dampened by repeated stimuli via synaptic depression (Sherman, 2001). Moreover, adaptation is believed to increase information transmission in sensory system in complex environment by enhancing thalamic neuronal sensitivity and synchrony with the respect of stimulus intensity (Wang et al., 2010b). Thus, the postsynaptic response of the POm neuron receiving rosebud synapses is dependent on the rate of firing and the temporal distribution of APs of L5B neurons. Moreover, POm cells respond to whisker stimulation with long latencies up to 22 ms (Diamond et al., 1992a) and the stimulus specificity of burst events has been reported from other sensory systems (Krahe and Gabbiani, 2004). Consequently, burst response in the POm can be preferentially triggered by low-frequency stimuli.

On the other hand, the activation of T-type calcium channels is dependent not only on voltage but also time, the periods of preceding hyperpolarization to de-inactivate these channels (Perez-Reyes, 2003). Our data showing the number and timing of postsynaptic APs is determined by the amplitude of LTS suggests that T-type conductance dynamically regulated the thalamic voltage response by the giant corticothalamic input (Supplemental Fig. 2) (Tscherter et al., 2011). It is conceivable that recovery of T-type calcium channels from inactivation could occur during the quiescent period that is caused by powerful STD at the rosebud synapse. Thereafter, the POm neuron responds with burst discharges by the subsequent excitatory L5B input, resulting in enhancement of postsynaptic outcome. Indeed, the required duration of silent period for L5B activity transmitting information to the POm (>100 ms) correlates well with the period of hyperpolarization that is needed for de-inactivation of T-type calcium channels, the refractoriness of these channels (Perez-Reyes, 2003; Groh et al., 2008). However, it still remains to determine the time course
to evoke bust of APs at rosebuds with varying stimulation time intervals, as we measured in EPSC (Fig. 2-6B). Moreover, inhibition and hyperpolarization from GABAergic and muscarinic cholinergic inputs, respectively, onto the POM may participate in not only adaptation but also boosting response to the subsequent depolarizing L5B input after silence by de-inactivation of T-type calcium channels (Fig. 1-2) (Bokor et al., 2005b; Ramcharan et al., 2005; Urbain and Deschênes, 2007).

In conclusion, voltage-dependent activation of T-type calcium channels with their distinct temporal kinetics finely tunes the POM outcome responding to L5B input in the spike number and the onset delay of AP with the graded amplitude of LTS, not simply determines two firing modes. Therefore, the delayer and amplifier properties of rosebud synapses are the key and common features in mice and rats (Groh et al., 2008).

2-3-2. Relationship of synapse size and EPSC amplitude

We show dispersed distribution of the size of corticothalamic terminals in relation to the amplitude of EPSCs (Fig. 2-5A, B). This finding is in good agreement with recent report of the diversity of driver terminal size and regional variability at an individual thalamic nucleus (Rovo et al., 2012). Or, the variability in the postsynaptic AMPAR content in the POM can also attribute to the heterogeneity in EPSC amplitudes. Furthermore, we demonstrate that the EPSC amplitude is not correlated to the size of L5B originating presynaptic bouton in the same POM neuron (Fig. 2-5C). The EPSCs in rosebud synapses are likely being variable according to the activities of L5B projecting neurons, this hypothesis is reinforced by the low connection probability between L5B neurons in S1 (Groh et al., 2010; Slezia et al., 2011). Although the absence of local GABAergic interneurons in the POM may render the connectivity of thalamic relay cells one another to be less tightly coupled or weak, it still remains to be determined whether individual L5B neurons can form divergent contacts with multiple POM neurons. (Barbaresi et al., 1986). However, synchronized multiple inputs from L5B neurons can enhance suprathreshold synaptic transmission and trigger postsynaptic action potentials on the POM neuron (Groh et al., 2008). Thus, neuronal response of the POM cell is regulated by not only the firing rate but also the state of S1 at given moment.
On the other hand, stimulating different size of presynaptic boutons, up to 2 fold larger than the other, generates approximately same amplitude of EPSCs in the same thalamic neuron (Fig. 2-5C). It is possible that a single L5B neuron make multiple contracts on the same POM cell, and activating one presynaptic bouton can trigger neighboring bouton by backpropagating activity (Nevian et al., 2007; de Kock and Sakmann, 2008). Otherwise, two boutons can have a different molecular composition (Craig and Boudin, 2001) and location (Micheva et al., 2010). Presynaptic patch recording may resolve this issue and answer the heterogeneity of postsynaptic responses. Alternatively, it may exist POM-targeting large terminals from unidentified cortical neurons, which may be resulted from the layer-unspecific deep cortical injection approach, and potential subpopulation of L5B neurons (Groh et al., 2008).

However, it should be noted that corticothalamic giant terminals were labeled by viral expression and we measured the signal of fluorescent EGFP in AAV particles at a single frame of image stack, which does not represent the exact size of synaptic terminals or the area, volume and the complexity of terminals either. However, our rationale of correlating bouton size and synaptic strength is that larger boutons are expected to contain a larger number of active zones (Taschenberger et al., 2002). Also, the distances between soma and terminal were estimated at 2D plane and the thickness of dendrites in which the presynaptic boutons are located was not considered in this study. The electrotonic location of the synapse and size of receptor populations are not taken into account either in this study.

2-3-3. Maturational changes in synaptic transmission from L5B to POM
Postnatal maturational change in synaptic rearrangements and refinement including subunits composition and localization of AMPA receptors is essential for establishment of precise neuronal circuits (Traynelis et al., 2010). To relate systemic function, it is necessary to study synaptic physiology at the matured state.

Rodents display an active whisker movement at P11 to P12 for mice and become more sensitivity to multi-whiskers in the thalamus with age (Shoykhet and Simons, 2008; Wang and Zhang, 2008). Sensory deprivation impairs anatomical and functional properties of somatosensory neurons, even whisker trimming on P12 gives rise to deficit in cortical integration of sensory information (Shoykhet et al., 2005; Li
and Crair, 2011). Most of mouse thalamic relay cells are born between embryonic day (E) 10 and E16, and corticothalamic projections reach the thalamus at E14–E15 (Auladell et al., 2000; Jones, 2007). Although physiological property change at the neurons of ventral posterior nucleus and reticular nucleus have been studied in the first postnatal two weeks, synaptic maturation in the POm has been not well described compared to other sensory thalamic nuclei (Warren and Jones, 1997; Wang and Zhang, 2008). Here, we reveal the postnatal development in synaptic activity at rosebuds between postnatal 4 and 5-6 wks (Fig. 2-4). Increased amplitude with faster kinetics of the EPSCs led us to conclude that there is a switch in AMPAR subunits. However AMPA/NMDA ratio change, switch from kainate receptor to AMPARs, silent/ few functional AMPARs, or AMPAR trafficking change could possibly take place during this time period, determining the size and shape of EPSCs in rosebuds. Nevertheless, the spontaneous activity change in the frequency suggests the presynaptic involvement in synaptic maturational process, probably quantal size and content change (Fig. 2-3). Otherwise, the activity-dependent synaptic rearrangement might occur between postnatal 4 and 5-6 wks. Hence, study in structural and functional maturation of rosebud synapses is needed to resolve these issues.
AMPAR receptors determine synaptic transmission at Rosebud synapses

3-1. Introduction
AMPAR receptors (AMPARs) mediate fast excitatory synaptic transmission and display a range of different functional properties depending on the composition of different subunits (Dingledine et al., 1999). For example, the GluA4 subunit gives rise to currents with the fastest kinetics among all subunit combinations (Mosbacher et al., 1994). In our previous work we found EPSCs with fast rise- and decay times in both rat (Groh et al., 2008) and mouse L5B-POm synapses (Fig. 2-2). Consistent with these considerations, GluA4 subunits are abundantly expressed in the thalamus (Mineff and Weinberg, 2000). Hence, we attempted to selectively delete the GluA4 subunit in relay neurons to study its role in synaptic transmission at the L5B-POm synapse.

Taking advantage of mouse models for targeted molecular perturbations, we used the Cre/loxP-mediated conditional knockout strategy to achieve POm relay cell-specific deletion of AMPAR subunits. We used stereotaxic delivery of AAV particles encoding Cre-recombinase and mOrange into the POm nucleus of mice harboring conditional GluA4 alleles (Fuchs et al., 2007). At the same time, we injected AAV-synaptophysin-EGFP into the S1 area to label the giant terminals. Then, electrophysiological recordings were established from mOrange-expressing GluA4-deficient POm cells making synaptic contacts with L5B neurons. To exclude the possible compensation by other AMPAR subunits in GluA4 knockout (KO), we made use of the conditional GluA1, GluA3, and GluA4 triple knockout (TKO) mice (Zamanillo et al., 1999; Sanchis-Segura et al., 2006; Fuchs et al., 2007). The choice of TKO, except GluA2, was based on the inward rectification of the isolated AMPAR-mediated EPSCs from the previous study (Groh et al., 2008).

We show the synaptic strength between L5B and POm is regulated by AMPAR subunits by tuning the spike counts and spike timing accompanied by changes in the size and kinetics of EPSCs.
CHAPTER 3

3-2. Results

3-2-1. Expression and deletion of synaptic AMPARs

To determine the consequence of selective regional ablation of synaptic AMPAR subunits at Rosebud synapses, we first assessed the distribution and relative density of each subunit of AMPAR in the POm using immunohistochemistry. Relay cells were labeled by injection of AAV-mOrange into the POm from postnatal 2-week (wk) old wild-type (WT) animals. After 3 to 4 weeks of expression, the region of interest was serially sectioned into 50 µm thick slices after transcardial perfusion with 4% paraformaldehyde. Immunostaining of the distribution of AMPAR subunits in the POm nucleus revealed a strong labeling by the GluA4 antibody in 20 sections obtained from 4 mice, out of which a representative example is shown in Fig. 3-1A. In contrast, there was only a moderate staining with anti-GluA2/3 antibody and the immunoactivity of GluA1 was weak (Fig. 3-1B, C), consistent with previously published results (Mineff and Weinberg, 2000).

![Figure 3-1. Expression and distribution of AMPA receptors at POm cells relate to giant terminals.](image-url)
(A) GluA4 signals on POm neurons labeled by mOrange is strong near EGFP-expressing corticothalamic terminals. Representative images (20 sections taken from 4 mice). Color code in merged panel: GluA4 (violet) on POm neurons (red), Rosebud terminals (green).
(B) Moderate immunoactivity of GluA2/3. Representative images (20 sections taken from 4 mice).
(C) Weak anti-GluA1 antibody staining. Representative images (20 sections taken from 4 mice).
Scale bars: 10 µm.

Consistent with previous work (Abraham et al., 2010), clearance of existing glutamate receptors from POm neurons required 3 to 4 weeks after viral gene transfer (data not shown). Immunohistochemistry was consistent with a reduction of AMPAR in Cre-expressing conditional KO mice. To exclude individual differences between mice, we injected AAV-iCre-mOrange into the POm of one hemisphere and AAV-mOrange without iCre into the contralateral POm of the same animal (n=5). The bilateral, focal injections into the POm are possible because thalamic projections did not cross the midline in mouse and because > 90% of these neurons can be specifically infected by targeted stereotaxic injections (Wimmer et al., 2010). Reduced anti-GluA4 antibody staining was observed in Cre-injected POm region compared to control side of POm in the same inducible GluA4 KO mouse (Fig. 3-2A,B). Moreover, mOrange-expressing thalamic neurons were positive to anti-Cre antibody after iCre-mOrange delivery (Fig. 3-2C). Hence, we established a basis to specifically record from cells devoid of or having a strongly reduced complement of GluA4-containing AMPARs and selectively patched mOrange-expressing AMPAR-deficient cells (Fig. 3-2D).
3-2-2. Delivery of AAV-mOrange without Cre

Although mOrange offers the high quantum yield with its relative photostability, fluorescent proteins can be toxic (Shaner et al., 2008). We introduced AAV-mOrange without Cre targeting the POm into our conditional AMPARs KO mice in attempting to assess potential change in neuronal properties and activity by mOrange expression. Patch-clamp recordings were performed at mOrange-infected thalamic relay cells and combined with the direct stimulation of synaptophysin-EGFP labeled giant terminals after 3 to 4 weeks of viral injection.

We observed that the size and shape of both spontaneous (amplitude: 21.97 ± 0.84, rise time: 0.46 ± 0.13 ms, decay time: 0.60 ± 0.02 ms, frequency: 1.62 ± 0.11 Hz, n=3) and evoked EPSCs (amplitude: 189.88 ± 60.73, rise time: 1.31 ± 0.27 ms, decay time: 2.99 ± 0.06 ms, n=4) for the mOrange-labeled neurons were indistinguishable to WT condition (p > 0.05, MW test). In addition, the expression of membrane-bound fluorescent proteins did not affect the intrinsic properties of neuron.

Figure 3-2. Spatiotemporally controlled deletion of GluA4 subunits.

(A) Reduced GluA4 immunoactivity from Cre-injected POm area compared to control side of the same conditional GluA4 KO mouse. Representative example taken from 25 sections collected from 5 mice.

(B) Anti-GluA4 antibody staining (green) on mOrange-labeled POm neurons (red) at the AAV-mOrange without Cre delivered animal. Representative example taken from 25 sections collected from 5 mice.

(C) mOrange (red) are positive to anti-Cre antibody (green).

(D) Overlay of three channels for electrophysiology; cell morphology (gray), Cre-infected POm neurons expressing mOrange (red), and EGFP-labeled corticothalamic giant terminals (green). Scale bars: 10 µm.
3-2-3. Physiological consequences of AMPA receptor deletion

3-2-3-1. Spontaneous activity changes

To address the contribution of AMPA receptors, spontaneous EPSCs in POm neurons were recorded at -70 mV. The mean amplitudes of spontaneous EPSCs for GluA4 KO and TKO were considerably different to WT (Fig. 3-3A). The amplitude histograms of spontaneous EPSCs were well fit by a single Gaussian in all three genotypes, suggesting that mainly one glutamatergic synaptic input exists on the POm neuron (Fig. 3-3B). There was no difference in kinetics and frequency of spontaneous EPSCs in GluA4 KO (p > 0.05, ANOVA), while these parameters were changed in TKO (p < 0.05. ANOVA) (rise time: WT 0.35 ± 0.01 ms versus GluA4+/− 0.39 ± 0.01 ms versus TKO 0.43 ± 0.03 ms, decay time: WT 0.91 ± 0.03 ms versus GluA4+/− 0.95 ± 0.04 ms versus TKO 1.03 ± 0.07 ms, frequency: WT 1.57 ± 0.06 Hz versus GluA4+/− 1.56 ± 0.1 Hz versus TKO 1.21 ± 0.07 Hz; WT n=80, A4 KO n=65, TKO n=39) (Fig. 3-3A).

However, there was a substantial decrease of sEPSC frequency in TKO, which is not reflected in the cumulative plot of the inter-event interval (p < 0.05, Kolmogorov-Smirnov, Fig. 3-3C). Rather, presumed multiquantal events reflected in the positive skew of the histogram were detected less frequently, but the smaller size of spontaneous events was more frequently observed in TKO (Fig. 3-3B). The smaller size of the sEPSCs in TKO may result in less reliable detection of these events and therefore pretend a decreased frequency.
To test if the removal of AMPAR was complete in the TKO, we compared TKO with pharmacological blockade of the receptors with CNQX. The extent of change in the spontaneous activity was comparable in both conditions (Fig. 3-4A). Beyond mean values, the cumulative probability of inter-event interval reflected between two genotypes was indistinguishable (p > 0.05, Kolmogorov-Smirnov, Fig. 3-4C). Also, the distribution of spontaneous events between TKO and CNQX were virtually overlapping (Fig. 3-4B). Furthermore, there was no measurable change in the size of spontaneous EPSCs from both GluA4 KO (GluA4−/− 19.64 ± 1.09 pA versus GluA4−/− with CNQX 17.18 ± 1.07 pA (n=4), p=0.069, MW test) and TKO by CNQX TKO 20.70 ± 1.81 pA versus TKO with CNQX 19.89 ± 1.45 pA (n=8), p=0.38, t-test) (Fig. 3-6A). These results suggest that AMPA receptors in the POm are likely to comprise of GluA1, GluA3 and GluA4 subunits, which are effectively removed by Cre-induced knockout approach.
Figure 3-4. Comparable change between TKO and CNQX (10 µM) in the spontaneous activity. 
(A) Bar histograms represent average values of EPSC amplitude, 20-80% rise time, decay time constant, and frequency ± SEM obtained for thalamic relay cells in slices from TKO (red, n=39) and CNQX (light red, n=14). CNQX amplitude: 12.49 ± 1.49 pA, rise time: 0.54 ± 0.06 ms, decay time: 6.64 ± 0.73 ms, frequency: 1.16 ± 0.18, n=14, p > 0.05, t-test. 
(B) Cumulative probability of inter-event interval curves. 
(C) Distribution of spontaneous EPSCs.

3-2-3-2. Altered evoked postsynaptic currents
To test whether thalamic neurons respond differently according to the composition of AMPAR subunits by corticothalamic stimulation, EPSCs between WT and AMPAR KO mice were compared. The response evoked by a single presynaptic stimulation was profoundly changed in both KO mice compared to WT (Fig. 3-5A). The EPSC amplitudes were decreased to about 35% at rosebud synapses without synaptic AMPARs (Fig. 3-5B). The spontaneous activity was only decreased by 10% reduction in the absence of GluA4 (Fig. 3-3A) (Paz et al., 2011). Therefore, AMPARs at corticothalamic synapses are predicted to dominantly comprise of homomeric GluA4, but they may contain a small fraction of GluA1 and GluA3 subunits (Mineff and Weinberg, 2000).

The decay time constant, but not rise time of EPSCs in GluA4 KO synapses (p > 0.05, ANOVA), was greatly increased compared to WT, while both rise time and decay kinetics for TKO were significantly different to WT (p < 0.05, ANOVA) (Fig. 3-5C).
D. Intrinsic properties

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GluA4 KO</th>
<th>TKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{m}$ (mV)</td>
<td>-58.4 ± 0.888</td>
<td>-58.6 ± 0.959</td>
<td>-57.6 ± 0.897</td>
</tr>
<tr>
<td>$R_{in}$ (MΩ)</td>
<td>122.63 ± 8.373</td>
<td>164.30 ± 10.003</td>
<td>151.86 ± 17.777</td>
</tr>
</tbody>
</table>

Figure 3-5. Strong reduction of AMPAR-mediated currents in AMPAR KO synapses.
(A) Postsynaptic currents in WT, GluA4 KO and TKO.
(B) Bar histograms represent mean values of EPSC amplitude ± SEM from WT (n=76 cells, black bars), GluA4-/- (blue), TKO (red), and CNQX (light red). WT 256.67 ± 32.97 pA, n=76 versus GluA4-/- 78.52 ± 5.1 pA, n=42 versus TKO 71.79 ± 8.51 pA, n=28, p < 0.05, ANOVA
(C) Decay time constant ± SEM obtained for thalamic relay cells in slices. Rise time: WT 0.84 ± 0.05 ms versus GluA4-/- 0.93 ± 0.07 ms versus TKO: 0.98 ± 0.13 ms, decay time: WT 3.48 ± 0.21 ms versus GluA4-/- 6.25 ± 0.53 ms versus TKO: 7.15 ± 0.56 ms.
(D) Resting membrane potential ($V_{m}$) and membrane input resistance ($R_{in}$) in three genotypes (WT (n=76 cells), GluA4-/- (n=42) and TKO (n=28), p >0.05, ANOVA).

On the other hand, we found no significant difference in the amplitude and kinetics of the evoked EPSCs between GluA4 KO (n=42) and TKO (n=28) (p > 0.05, t-test), leading us to postulate that GluA4 is preferentially targeted to synaptic sites at which L5B inputs connect. It is difficult to determine the relative contribution of AMPARs due to the lack of specific blockers to distinguish different subunits of AMPA receptors.

Furthermore, the reduced EPSC by CNQX was similar to GluA4 KO and TKO (CNQX amplitude: 69.76 ± 9.75 pA, decay time: 6.42 ± 0.65 ms, n=15, p > 0.05, ANOVA) (Fig. 3-5B). The proportion of remaining currents after CNQX application (38.36 ± 5.47%) resembled that of TKO (34.66%). Also, CNQX only marginally blocked EPSCs of both GluA4 KO (GluA4-/- 71.56 ± 16.71 pA versus GluA4-/- with CNQX 58.92 ± 15.8 pA, p = 0.3, MW test) and TKO synapses (TKO
79.98 ± 16.76 pA versus TKO with CNQX 58.71 ± 16.16 pA, p = 0.19, MW test) (Fig. 3-6B), suggesting that the EPSC is mediated almost exclusively by the GluA4 subunits. Additionally, there was no detectable change in the peak of EPSCs for both WT (Fig. 2-2-B) and TKO (TKO with APV 5.92%) when the NMDAR antagonist APV was present in the bath solution, consistent with the fact that NMDAR contribute only little to the EPSC at a membrane potential of -70 mV.

Collectively, GluA4-deletion effectively removed AMPAR from the POm relay neuron, yet a remaining current not blockable by the AMPAR blocker CNQX persisted.

3-2-3-3. Less prominent frequency-dependent STD in the absence of AMPARs
The longer decay rate of EPSCs in AMPAR KO mice addresses the question of whether the AMPA-mediated currents would remain longer after stimulation, increasing the duration of excitatory input from L5B (Fig. 3-5A). To test this hypothesis, we examined how short-term depression (STD) is changed by loss of Ca\(^{2+}\)-permeable glutamate receptor subunits upon repetitive stimulation.

AMPAR\(^{-/-}\) mice developed STD in a frequency-dependent manner and the reduction in EPSC amplitudes was described with a single exponential function (Fig. 3-7). However, the time constants of STD in the absence of synaptic AMPARs were slower than WT (10 Hz: WT 109 ± 18 ms (n=17) versus GluA4 \(^{-/-}\) 196.9 ± 5 ms (n=4)
CHAPTER 3

versus TKO 116 ± 2 ms (n=5), 20 Hz: WT 35 ± 5 ms (n=17) versus GluA4−/− 72.1 ± 13 ms (n=5) versus TKO 37.8 ± 5 ms (n=7), 50 Hz: WT 12 ± 2 ms (n=12) versus GluA4−/− 34.8 ± 9 ms (n=5) versus TKO 37 ± 4 ms (n=7), p < 0.05, ANOVA). Also, the amplitude of the second EPSC was less strongly reduced at all stimulation frequencies and less frequency-dependent in AMPAR−/− mice compared to WT (10 Hz: WT 70.18 ± 3.79 % (n=17) versus GluA4−/− 67.84 ± 10.18 % (n=4) versus TKO 66.71 ± 4.68 % (n=5), 20 Hz: WT 68.99 ± 16.1% (n=17) versus GluA4−/− 66.73 ± 8.56 % (n=5) versus TKO 66.71 ± 5.76 % (n=7), 50 Hz: WT 38.31 ± 4.55% (n=12) versus GluA4−/− 59.21 ± 8.03% (n=5) versus TKO 57.56 ± 3.49% (n=7), p < 0.05, ANOVA) (Fig. 3-8A). Both GluA4−/− and TKO failed to evoke the second response during the brief interval between synaptic events when stimulating at 100 Hz.

![Figure 3-7. STD in AMPAR KO mice.](image)

(A) STD developed in a frequency-dependent manner without GluA4 subunits (n=4 to 5). Normalized data taken from 5 neurons and fitted by a single exponential curve. (B) The initial depression is less frequency-dependent in TKO (n=5 to 7).

The plot obtained by the paired pulse ratio (PPR) with various intervals demonstrates that AMPAR−/− mice are particularly affected during high frequency neurotransmission (Fig. 3-8A). Hence, we compared STD at 50 Hz stimulation paradigms and very little depression for AMPAR KO synapses is illustrated in Figure 3-8B. Also, the cumulative normalized EPSC amplitude during a 50 Hz train from both GluA4−/− and TKO synapses was steeper than WT condition (p < 0.05, Kolmogorov-Smirnov test, Fig. 3-8C). Nevertheless, the curves plotted from TKO are almost overlapped to GluA4 KO (Fig. 3-8).
Figure 3-8. STD in the absence of AMPARs. 
(A) Paired-pulse ratio (PPR) is calculated by the amplitude of the second EPSC to that of the first. 
(B) Less pronounced STD is observed in KO mice than WT at 50 Hz stimulation. 
(C) Cumulative normalized and non-normalized EPSC amplitudes during 50 Hz train are constructed from WT (black, n=8), GluA4 (blue, n=5), and TKO (red, n=7).

Having established the efficiency of genetic manipulation (Fig. 3-1 to 6), we measured the degree and rate of STD in the presence of CNQX. The time constants over train stimuli was slower (10 Hz: Control 90.6 ± 2 ms versus CNQX 74.4 ± 1 ms, p < 0.05, 20 Hz: Control 24.9 ± 3 ms versus CNQX 17.6 ± 2 ms, p < 0.05, 50 Hz: Control 6.4 ± 1 ms versus CNQX 9.2 ± 0.2 ms, p < 0.05, n=3, ANOVA) (Fig. 3-9A, B) and the PPR was less powerful with CNQX (10 Hz: Control 68.14 ± 17.19% versus CNQX 71.99 ± 9.43%, 20 Hz: Control 54.45 ± 8.98% versus CNQX 64.73 ± 5.64%, 50 Hz: Control 38.59 ± 11% versus CNQX 49.65 ± 11%, p < 0.05, MW-test) (Fig. 3-9C). Also, the plot obtained from the normalized EPSCs over 50 Hz train was steeper by CNQX (p < 0.05, Kolmogorov-Smirnov test) (Fig. 3-9D). Altogether, L5B-POm synapses are less susceptible to depression without synaptic AMPARs.
3-2-3-4. Change in the input-output function in synapses lacking AMPARs

We next asked how the strongly decreased EPSC amplitudes in AMPAR-deficient POm neurons would affect the input-output function of the neuron. Current-clamp recordings from AMPAR-deficient POm neurons revealed that a single L5B input could still drive several postsynaptic action potentials (APs), yet the latency from the stimulus to the first action potential was markedly prolonged a hyperpolarized membrane potential of -70 mV (WT 16.3± 6 ms, n=13 versus GluA4⁻/⁻ 94 ± 11 ms, n=4 versus TKO 80.4 ± 19 ms, n=3, p < 0.05, ANOVA) (Fig. 3-10). Similar results were obtained when applying CNQX (CNQX 90 ± 2.7 ms, n=3) (Fig. 3-13A). However, the average number of spikes in AMPAR KO mice was slightly reduced compared to WT (WT 4 ± 0.5, n=13 versus GluA4⁻/⁻ 3.3 ± 0.3, n=4 versus TKO 3, n=2, p > 0.05, ANOVA).
Once thalamic neurons spiked, postsynaptic voltage responses were depressed like WT condition (n=7) but AMPAR KOs (GluA4 -/- n=5, TKO n=5) displayed a prolonged postsynaptic spike timing by high frequency presynaptic stimulations (Fig. 3-11).
When the membrane potential was depolarized to $-60$ mV, all three genotypes generated a single spike but the latency between stimulus and spike was again prolonged in AMPAR KO mice (WT $2.8 \pm 0.8$ ms, $n=5$ versus GluA4 $/-$ $5.9 \pm 0.8$ ms, $n=5$ versus TKO $6.1 \pm 0.6$ ms, $n=2$, $p < 0.05$, ANOVA) (Fig. 3-12).

![Figure 3-12](image.png)

Similar results were obtained when applying CNQX was applied at $-60$ mV (CNQX $3.8 \pm 0.3$ ms, $n=3$) (Fig. 3-13B). Surprisingly, the remaining, non-glutamatergic current of unknown origin was able to drive postsynaptic action potentials.

Together, the lack of AMPARs in POM relay neurons decreased temporal precision of signal transfer from L5B neurons to the POM relay neurons.

![Figure 3-13](image.png)
3.2-3.5. Intrinsic excitability of AMPAR KO mice

The slowed latency for AP firing is solely caused by dramatically reduced postsynaptic currents, but also changes in the excitability of the postsynaptic neuron by synaptic AMPAR deletion needs to be considered. To address this question, we compared the active properties of voltage-gated conductances between WT and AMPAR KO mice at different amplitudes of somatic current injection. Various amounts of depolarizing currents were injected for regular firing and bursting of APs that was induced when the membrane potential rebounded to the resting state following a hyperpolarizing prepulse for 300 ms. We plotted the number of spikes at any given current step that follows a sigmoid function in tonic mode (I_{50}: WT 152.33 ± 5.09, n=37 versus GluA4^{-/-} 97.56 ± 8.05, n=43 versus TKO 98.8 ± 5.07, n=46, rate: WT 66.16 ± 5 versus GluA4^{-/-} 56.34 ± 5.59 versus TKO 53.38 ± 3.61, the maximal number of spikes: WT 15.04 ± 0.64 versus GluA4^{-/-} 14 ± 0.91 versus TKO 13.88 ± 0.58; p < 0.05, two-way ANOVA). The curves for KO mice were shifted to leftward (Fig. 3-14A). Similarly left-shifted curves were obtained from KO groups in burst mode as shown in Figure 3-14B (I_{50}: WT 104.28 ± 8.06, n=37 versus GluA4^{-/-} 63.09 ± 4.63, n=43 versus TKO 72.83 ± 4.99, n=46, p < 0.05, rate: WT 46.48 ± 7.46 versus GluA4^{-/-} 32.22 ± 3.31 versus TKO 28.28 ± 3.87, p < 0.05, the maximal number of spikes: WT 3.77 ± 0.32 versus GluA4^{-/-} 3.2 ± 0.18 versus TKO 2.77 ± 0.17, p > 0.05, two-way ANOVA). Also, the incidence of APs for AMPAR KO mice at the same lowest current step was higher than that for WT (WT 18.91% versus GluA4^{-/-} 55.81% versus TKO 47.82%). On the other hand, two AMPAR KO mice displayed lower and wider potentials at any given current (Fig. 3-14C, D). Among all the parameters we examined in TKO, none were significantly different from GluA4 KO (p > 0.05, two-way ANOVA).

Collectively, POm neurons were more excitable when synaptic AMPARs were ablated, which is consistent with the greater membrane input resistance (R_{in}) for AMPAR KOs compared to WT (Fig. 3-5D) (Paz et al., 2011; Yang et al., 2011). This increased excitability of AMPAR deficiency does not account for the longer latency in AP firing at the rosebud synapse.

Until now, we demonstrate that aberrant AMPA receptor expression in POm relay neuron brings about impaired synaptic transmission at the L5B-POm synapse.
Figure 3.14. Intrinsic properties between WT and AMPAR KO mice.

Direct somatic currents from -300 pA to 400 pA (in 50 pA increments) were applied for 300 ms and measured the potentials. Hyperpolarizing currents up to 300 pA led to burst firing (A) and depolarizing currents resulted in tonic spikes (B) in POm neurons. The output gain of thalamic relay cells was described with the parameters spike number, amplitude (C), and half width (D) according to genotype, WT (black, n=37), GluA4 KO (blue, n=43), and TKO (red, n=46).
CHAPTER 3

3-3. Discussion

3-3-1. Removal of synaptic Ca$^{2+}$-permeable AMPARs at a defined synapse

GluA4 is the main AMPAR subunit expressed in the thalamic nuclei including the POm following GluA3, whereas GluA1 and GluA2 are most abundant subunits in the mature neocortex, suggesting different strength of connectivity between thalamocortical and corticothalamic projections depending on the differential expression of AMPAR subunits (Mineff and Weinberg, 2000). Selective regional elimination of GluA4 or combined with other Ca$^{2+}$-permeable synaptic AMPARs for TKO in POm neurons (Fig. 3-1,2) resulted in a strong reduction of the EPSC amplitude and slower kinetics, which brings about a change in postsynaptic spike timing at L5B-POm synapses (Fig. 3-10 to 12).

Each subunit-specific knockout of AMPA receptors or double KO of them has been shown in different forms of synaptic plasticity (Reisel et al., 2002; Abraham et al., 2010; Wang et al., 2011). This present study is, to the best our knowledge, for the first time to examine the phenotype of all three Ca$^{2+}$-permeable AMPA receptor subunits at the individual synapses. Gene deletions often result in compensatory changes in the expression levels of other proteins (e.g. upregulation of AMPAR). However, based on the lack of an effect of CNQX after deletion of AMPAR we conclude that such compensatory changes are unlikely (Fig. 3-6). On the other hand, the remaining currents in TKO and in the presence of CNQX prompted the question whether other ionotrophic glutamate receptors are involved in L5B-POm synaptic transmission (Fig. 3-5, 6). Kainate receptors are known to be blocked at CNQX concentrations of 10 µM such as used here, suggesting a minor contribution in agreement with previous work (Jones, 2007; Miyata and Imoto, 2009; Traynelis et al., 2010). Alternatively, CNQX-resistant kainate receptors may exist at the rosebud synapse. Consequently, a selective kainate receptor antagonist over AMPARs may resolve this issue.

NMDA receptors are equally unlikely to be involved because they are blocked by Mg$^{2+}$ at hyperpolarized potentials and a small residual current remained in the presence of APV and CNQX (Fig. 2-2B). However, NMDAR containing the NR3 subunit may contribute as these are insensitive to APV and Mg$^{2+}$ block (Pachernegg et al., 2012). The only other glutamate receptor subunits that could be considered are the delta subunits, although their functional properties have remained poorly
understood (Lomeli et al., 1993). In principle, also other synaptic transmitters targeting ionotropic receptors, such as acetylcholine, serotonin or ATP could be co-released with glutamate and generate an EPSC. Apart from these biological considerations, possible technical explanations need to be considered too. The stimulation with the double-barrel pipette may activate other synapses targeting the same postsynaptic neuron or may directly stimulate it. This is unlikely, because recordings showing EPSCs with smaller current amplitudes before reaching the threshold of the main EPSC occurred only in rare cases. Furthermore, recordings showing a postsynaptic response scaling with stimulus intensity were not included in the analysis, preventing the possibility that the EPSC component remaining after GluA4 deletion or CNQX application was generated by some direct postsynaptic stimulation. In conclusion, further studies may reveal the underpinnings of the remaining current and its contribution to synaptic transmission.

3-3-2. STD change in the absence of postsynaptic AMPARs

The rosebud synapse is characterized by a high release probability and strong frequency-dependent STD (Fig. 2-5, 7). Deletion of Ca\(^{2+}\)-permeable AMPA receptor subunits in POm neurons reduced the degree of depression (Fig. 3-7, 8), as did the addition of CNQX (Fig. 3-9).

Since most of the synaptic current was mediated by AMPA receptors in L5B-POm synapses (Fig. 2-2B and 3-5A,B), less number of synaptic AMPARs resulted in greatly reduced initial EPSC amplitude, which may bring about little change in the subsequent postsynaptic responses in the GluA4 KO and TKO synapses. Alternatively, ablation of AMPARs possibly influences both pre-and postsynaptic sites via crosstalk between them, and may affect the release probability in L5B-POm synapses. The PPR is commonly used for a presynaptic probe and inversely proportional to the initial release probability, here we found it was increased in the absence of synaptic AMPARs (Fig. 3-8A and 3-9C).

Compared to NMDARs, less well known about the contribution of Ca\(^{2+}\)-permeable AMPARs on short-term synaptic plasticity (Rozov and Burnashev, 1999). Even though it remains to uncover the underlying mechanism, less powerful and slower STD in AMPAR KO mice may delay the synaptic recovery after repetitive stimulation of the L5B giant synapse, in turn, affecting the firing pattern of thalamic
neuron, especially rebound burst after silence of this synapse. Furthermore, it is expected to cause a change in adaptation, by which reducing information flow in the sensory pathway (Chung et al., 2002; Abbott and Regehr, 2004; Wang et al., 2010b).
Chapter 4.

Contribution of high-affinity nicotinic receptors to corticothalamic giant synapses

4-1. Introduction

Cholinergic neuromodulation engages in setting the state of cortical networks and information flow between cortex and thalamus (Castro-Alamancos and Calcagnotto, 2001; Steriade, 2004). Acetylcholine (ACh) has been reported to be able to regulate GABAergic and glutamatergic synaptic transmissions, thereby shifting the balance between excitation and inhibition (McGehee et al., 1995; Lena and Changeux, 1997; Ren et al., 2011). The modulatory synapses of which relative proportion is greater in higher order thalamic relays, like POm and selectively target to these thalamic nuclei (Sánchez-González et al., 2005; Van Horn and Sherman, 2007). The cholinergic input from parabrachial region has been demonstrated to hyperpolarize thalamic neurons which in turn generate more spikes by action of muscarinic receptor 2 (M2), opening a voltage-independent K\(^+\) conductance (Mooney et al., 2004; Ramcharan et al., 2005).

In spite of abundant expression of M2 in higher-order nuclei, rapid decay time constants of the EPSCs at rosebuds led us to study the potential nicotinic contribution in corticothalamic giant synapses. The thalamus contains the highest density of high-affinity nicotinic receptors (nAChR) in the brain, in which \(\alpha_4\beta_2\) nicotinic receptor subunits predominate in the POm (Zoli et al., 1998; Barthó et al., 2002). Moreover, the calcium permeability of \(\alpha_4\beta_2\) nAChRs combined with \(\alpha_5\) is exceeded only by \(\alpha_7\) containing nicotinic receptors (Kuryatov et al., 2008).

Based on the rich expression of high-affinity \(\alpha_4\beta_2\) nicotinic receptor subunits, the specific antagonist for these nAChRs, dihydro-\(\beta\)-erythroidine hydrobromide (DH\(\beta\)E) was applied to investigate the effect of cholinergic receptors in the L5B-POm synapse. Surprisingly, DH\(\beta\)E increases the amplitude of the EPSCs and shortens the spike latency in the POm cell responding to L5B stimulation.
CHAPTER 4

4-2. Results

4-2-1. No change in spontaneous activity by DHβE

The high affinity nicotinic blocker DHβE did affect neither the amplitude nor the kinetics or frequency of spontaneous EPSCs in POm neurons (Fig. 4-1A). The absence of a significant change in frequency is supported by the plot of the cumulative probability of the inter-event intervals (p > 0.05, Kolmogorov-Smirnov test) (Fig. 4-1C). With DHβE present in the bath solution more multiquantal events appeared to be present, possibly resulting in the increase of amplitudes of spontaneous EPSCs (Fig. 4-1B).

![Figure 4-1](image-url)

**Figure 4-1.** The nicotinic blocker DHβE (5 µM) does not alter the spontaneous synaptic activity in the POm.

(A) Bar histograms represent mean values of EPSC amplitude, 20-80% rise time, decay time constant, and frequency ± SEM (n=9). Amplitude: Ctrl 15.91 ± 1.15 pA versus DHbE 18.32 ± 1.50 pA, rise time: Ctrl 0.53 ± 0.05 ms versus DHbE 0.53 ± 0.08 ms, decay time: Ctrl 1.14 ± 0.12 ms versus DHbE 1.28 ± 0.19 ms, frequency: Ctrl 1.87 ± 0.3 versus DHbE 2.14 ± 0.13, n=9, p > 0.05, t-test.

(B) Distribution of spontaneous events before and after treatment of DHβE (n=9).

(C) Analysis of the cumulative probability inter-event interval.
4-2-2. Slightly sharper evoked activity by nicotinic inhibition

Next, we examined whether the L5B corticothalamic input gives rise to different postsynaptic responses on the POM cell in the presence of DHβE. Unexpectedly, DHβE caused a sharpening of the EPSCs with accelerated kinetics in the postsynaptic currents of the POM neuron responding to the presynaptic L5B input (Fig. 4-2A,C). Although the mean EPSC amplitude did not statistically differ from that recorded in the presence of DHβE, the size of EPSCs was increased in all tested cases (Fig. 4-2B).

![Figure 4-2](image)

**Figure 4-2. The evoked EPSCs are changed by DHβE (5 μM)**

(A) Bar histograms represent average values of EPSC amplitude, 20-80% rise time, and decay time constant ± SEM. Amplitude: Ctrl 138.64 ± 32.25 pA versus DHβE 208.67 ± 44.04 pA, p = 0.1, rise time: Ctrl 0.80 ± 0.07 ms versus DHβE 0.47 ± 0.06 ms, p < 0.05, decay time: Ctrl 4.31 ± 0.81 ms versus DHβE 2.71 ± 0.47 ms, p = 0.05, t-test, n=12.

(B) DHβE enhanced the amplitude of EPSCs.

(C) Inward current at -70 mV was accelerated by DHβE. The stimulus artifacts are blanked. Representative trace taken out of 12 recordings.
A similar trend of DHβE was observed in GluA4 KO synapses (amplitude: GluA4\(^{-/-}\) 111.36 ± 39.49 pA versus GluA4\(^{-/-}\) with DHβE 143.28 ± 21.67 pA, rise time: GluA4 \(-/-\) 0.98 ± 0.41 ms versus GluA4 \(-/-\) with DHβE 0.86 ± 0.24 ms, decay time: GluA4 \(-/-\) 5.36 ± 1.75 ms versus GluA4 \(-/-\) with DHβE 5.16 ± 1.25 ms, n=2) (Supplemental Fig. 3, top panel) and triple knockout (TKO) synapses of AMPARs as well (amplitude: TKO 97.38 ± 32.13 pA versus TKO with DHβE 129.49 ± 48.45 pA, p > 0.05, rise time: TKO 0.96 ± 0.27 ms versus TKO with DHβE 0.80 ± 0.18 ms, p > 0.05, decay time: TKO 5.15 ± 0.36 ms versus TKO with DHβE 4.43± 0.51 ms, p > 0.05, MW test, n=4) (Supplemental Fig. 3, bottom panel). Despite DHβE not reaching statistical significance, all cases we examined here showed the same tendency. Moreover, enhanced EPSC amplitude by DHβE was decreased by co-application of CNQX in WT condition (data not shown).

4-2-3. Change in STD by DHβE
To test whether short-term synaptic plasticity is changed when the nicotinic receptors are inhibited by DHβE, we compared EPSC amplitudes under regimes of repetitive stimulation. Relatively faster short-term depression (STD) was evident in the presence of DHβE at Rosebud synapses (10 Hz: Control 62.2 ± 0.1 ms versus DHβE 124.3 ± 0.3 ms, p < 0.05, 20 Hz: Control 23. ± 0.7 ms versus DHβE 27.4 ± 1 ms, p > 0.05, 50 Hz: Control 57.6 ± 0.01 ms versus DHβE 73.1. ± 0.3 ms, p < 0.05, n=4, ANOVA) (Fig. 4-3A,B). The paired-pulse ratio (PPR) was reduced in the presence of DHβE at 10 Hz and 50 Hz, n=4, p < 0.05, MW test) (Fig. 4-3C). The cumulative plot of the normalized EPSCs during 50 Hz stimuli was slightly changed by DHβE as well (p < 0.05, Kolmogorov-Smirnov test, Fig. 4-3D).
CHAPTER 4

Figure 4-3 STD change by the nicotinic blocker DHβE (5 µM).
(A) Frequency-dependent STD in standard ringer solution (control, black solid line, n=4).
(B) STD is not changed by DHβE (gray dashed line).
(C) Paired-pulse ratio (PPR) between control (Ctrl) and DHβE. (10 Hz: Control 70.96 ± 4.07% versus DHβE 60.28 ± 2.48%, p < 0.05, 20 Hz: Control 54.79 ± 10.67% versus DHβE 54.73 ± 3.11%, p > 0.05, 50 Hz: Control 51.26% versus DHβE 32.46 ± 5.32%, p < 0.05, n=4, MW-test).
(D) Cumulative normalized EPSC amplitudes.

4-2-4. DHβE triggers faster spike rates
Furthermore, we investigated how enhanced postsynaptic currents modify the postsynaptic voltage response in the presence of DHβE, and found that the nicotinic blocker promoted the postsynaptic firing of the POm neuron by the L5B corticothalamic input. The latency to evoke the EPSPs became shorter in both WT (Control 23.4 ± 8.84 ms versus DHβE 7.13 ± 1.3 ms, n=10, p < 0.05, t-test) (Fig. 4-4) and TKO (TKO 90.6 ± 29.98 ms versus DHβE 8.9 ± 2.7 ms, p=0.06, MW test n=3) (Supplemental Fig. 4) following application of DHβE at a membrane potential of -70 mV. In other words, the excitatory L5B input drives the POm neuron to spike with a reduced temporal jitter in the presence of DHβE. Greater EPSCs by DHβE may lead to reduce the voltage distance to the spike threshold. A single presynaptic stimulation failed to trigger a postsynaptic AP and generated subthreshold EPSPs in TKO (Supplemental Fig. 4).
However, preliminary data may suggest that multiple APs are generated in the presence of DHβE (Supplemental Fig. 4D).

When membrane potential was shifted to -60 mV, a single spike was amplified up to 5 APs with no time difference in onset latency by the same stimulation parameter with use of DHβE (Supplemental Fig. 4B). By contrast, further preliminary data suggests that the shortened delay caused by DHβE can be reversed by the cholinergic agonist Carbachol. The onset delay for the AP was near control level without substantial changes in spike count (Fig. 4-4).

![Figure 4-4](image)

**Figure 4-4.** Spike timing is regulated by the nicotinic blocker DHβE (5 µM, gray dashed line) and the cholinergic agonist Carbachol (5 µM, gray line). Representative trace.

### 4-4. Neuronal excitability is changed by DHβE

To assess how the output gain is changed by DHβE at Rosebud synapses, we plotted the intrinsic thalamic neuronal properties against somatic current injections of different amplitude such as described in Figure 3-14. We found that the maximal number of spikes was affected by the nicotinic antagonist in both tonic (I_{50}: Control 48.03 ± 65 versus DHβE 32.33 ± 16.6, p < 0.05, rate: Control 47.45 ± 30.7 versus DHβE 123.13 ± 57.2, p < 0.05, the maximal number of spikes: Control 17.75 ± 10.8 versus DHβE 32.33 ± 16.6, p < 0.05, two-way ANOVA, n=7) (Fig. 4-5A) and burst mode firing (I_{50}: Control 48.03 ± 25.1 versus DHβE 10.31 ± 250, p < 0.05, rate: Control 46.13 ± 13.4 versus DHβE 112.23 ± 114, p < 0.05, the maximal number of spikes: Control 5.19 ± 1.33 versus DHβE 14.27 ± 19.7, p < 0.05, two-way ANOVA, n=7) (Fig. 4-5B). Also, greater potentials were evoked with DHβE at any given
current (Fig. 4-5C, p < 0.05, two-way ANOVA, n=7). These results show that the POm relay cell became more excitable in the presence of DHβE.

Collectively, we examined the involvement of nicotinic receptors at the individual L5B-POm glutamatergic synapse and found that the cholinergic regulation is considerable in Rosebud synapses, modulating the thalamic neuronal excitability and output timing.
4-3. Discussion

A growing number of evidence supports the crosstalk between the cholinergic and glutamatergic systems. Presynaptic nicotinic receptors facilitate excitatory synaptic transmission and glutamate is released synaptically from cholinergic neurons (McGehee et al., 1995; Ren et al., 2011). Due to the broad innervation and diverse effects of the cholinergic system, our data can be interpreted in several ways. The cholinergic modulation most strongly depolarizes L5 neurons (McCormick and Prince, 1986). Accordingly, less glutamate would be released from presynaptic L5B neurons and/or corticothalamic terminals when the nicotinic blocker DHβE was added to the bath solution. That is opposite to what we observed in our DHβE experiments showing a slight increase in the amplitude of EPSCs, suggesting that other mechanisms may account for this change. Besides, The presence of nicotinic receptors in corticothalamic terminals projecting from L6 has been shown in mice, whereas it has not been reported yet in the giant synapse originating from L5B (King et al., 2003).

Alternatively, blocking nicotinic activation may depolarize postsynaptic membrane potential of POm relay cells, consequently surpassing the spike threshold, which, in turn shortening the time course to trigger EPSPs. Indeed, we found the intrinsic and synaptic excitability of thalamic neurons are changed by DHβE (Fig. 4-4 to 5). However, nicotinic receptors are known as excitatory ligand-gated channels that allow the movement of Na⁺, K⁺, and Ca²⁺ ions, resulting in depolarization. On the other hand, the reversed onset latency by Carbachol that is an agonist for both nicotinic and muscarinic receptors has to be tested by co-application with the muscarinic antagonist atropine (Fig. 4-4).

Otherwise, there is the possibility of coexpression of nicotinic and AMPA receptors at postsynaptic sites of the thalamic relay cell and both receptors can be activated by the same L5B input, which is based on the assumption that glutamate and acetylcholine is coreleased from the same corticothalamic terminals. Regarding the much slower kinetics of nicotinic receptor activation, corticothalamic input to the POm may be largely, if not entirely, driven by glutamate when the nicotinic receptors are inactivated by DHβE. In the line with this model, DHβE speed up responses with accelerated kinetics in the EPSCs and facilitated spike timing (Fig. 4-2 to 4). Yet, this scenario does not explain our TKO experiment where the synaptic AMPAR-mediated
currents are largely absent but there was a moderate increase in the postsynaptic responses by administration of DHβE (Supplemental Fig. 3). Nevertheless, the possible cholinergic extrasynaptic location with long-range diffuse volume transmission can be considered (Dani and Bertrand, 2007).

The last hypothesis is that disinhibition under the nicotinic blocker influence by decreasing GABAergic transmission either from interneurons in L5B or inhibitory terminals originated from the zona incerta (IZ), the anterior pretectal nucleus (APT) or the reticular nucleus (nRT) onto the POm (Fig. 1-2). GABA can be released from by action of presynaptic nAChRs in the thalamus (Lena and Changeux, 1997). Moreover, ACh has the diverse effect on GABAergic interneurons (Lawrence, 2008). However, it requires determining whether, or if so, which types of nicotinic receptors are present at these systems in relation to rosebud synapses, the precise subcellular localization of presynaptic nicotinic receptors by immuno-EM. Recently, it has been shown that the postsynaptic response in the thalamic reticular neuron is mediated by \(\alpha 4\beta 2\) nicotinic receptors (Sun et al., 2013). Hence, blockade of \(\alpha 4\beta 2\) nicotinic activation in the nRT is likely to reduce the synaptic strength between nRT-POm, leading to hyperactivation of relay cells in the POm.
Chapter 5.

Role of the T-type calcium channel Ca\textsubscript{v}3.1 in postsynaptic integration

5-1. Introduction
The functional role of low voltage-activated (LVA) T-type calcium channels is well documented in both physiological and pathological conditions, sleep, absence seizures, pain and cognitive functions, but the contribution of T-type channel on sensorimotor behaviors has been poorly described (Kim et al., 2001; Cheong et al., 2011; Lee et al., 2012; Park et al., 2013). T-type calcium channels are shown to be concentrated at the proximal dendrites, sites are known to receive excitatory synaptic connections from corticothalamic feedforward projections (Williams and Stuart, 2000; McKay et al., 2006). Our previous work in the rat and mouse L5B-POm synapses revealed that a single presynaptic action potential can trigger several postsynaptic action potentials, hence functioning as a signal amplifier (Fig. 2-8A) (Groh et al., 2008). This feature may be related to the well-known switch between two modes of firing, tonic or burst (Llinas and Jahnsen, 1982; Jahnsen and Llináš, 1984), caused by the activation of T-type calcium channels (Perez-Reyes, 2003). This lead us to selectively interfere with the expression of the T-type calcium channel Ca\textsubscript{v}3.1, known to be highly expressed in thalamus (Talley et al., 1999), in POm relay neurons.

Viral shRNA expression was used to knockdown the T-type calcium channel Ca\textsubscript{v}3.1 in the POm. The physiological consequence of this perturbation was assessed by whole cell recordings from the genetically defined postsynaptic thalamic neuron while stimulating fluorescent protein-labeled synaptically coupled presynaptic terminals projecting from cortical L5B neurons. We found that T-type calcium channel is the crucial molecular switch for voltage-dependent thalamic firing pattern but also the molecular determinant for the onset spike latency at the synapse between L5B and POm, which will contribute to explore the causal link between two firing modes of thalamic neurons in sensorimotor coordination.
CHAPTER 5

5-2. Results

5-2-1. Expression and loss of Ca,3.1

To elucidate the molecular basis of voltage-dependent currents elicited by the EPSC in POM-neurons, we targeted a T-type calcium channel subtype prominently expressed in the POM (Talley et al., 1999). The Ca,3.1 gene of T-type calcium channels was selectively suppressed in the POM by viral expression of AAV harboring short hairpin RNA (shRNA) (Park et al., 2010). The somatic and dendritic expression of the Ca,3.1, especially the proximal location, on the thalamic neuron was shown by immunohistochemistry (Figure 5-1A) (Williams and Stuart, 2000; McKay et al., 2006). Successful loss of the T-type calcium channel subunit was indicated by reduced anti-Ca,3.1 antibody staining on the selectively Ca,3.1 knockdown POM neuron that was recognized by expression of mOrange, 3-4 weeks (wks) after viral injection (Fig. 5-1B). Thereafter, we characterized the corresponding response of Ca,3.1-deficient thalamic neurons to L5B presynaptic excitation using a fluorescence-guided local stimulation of the identified nerve terminals as described earlier (Fig 2-1C).

![Figure 5-1. Expression and deletion of Ca,3.1 T-type calcium channels in POM cells.](image-url)
(A) Expression of Ca\textsubscript{3.1} with Ca\textsubscript{3.1}-specific antibody within a mOrange-labeled POm cell in WT. Representative confocal images (20 sections taken from 4 mice). Color code in merged panel: Ca\textsubscript{3.1} (violet) on POm neurons (red).
(B) Loss of anti Ca\textsubscript{3.1} antibody signal from Ca\textsubscript{3.1} shRNA-infected POm neurons. Color code in merged panel: Ca\textsubscript{3.1} (green) on POm neurons (red).
Scale bars: 10 µm. Representative confocal images taken from 15 slices from 3 mice.

5-2-2. No bursting of POm neurons in the absence of Ca\textsubscript{3.1}

The functional loss of Ca\textsubscript{3.1} was examined by whole-cell voltage clamp analysis of POm neurons in acute brain slices of 5-6 wks old mice. Knockdown (KD) of Ca\textsubscript{3.1} substantially eliminated Ca\textsuperscript{2+}-mediated rebound depolarization in all 33 POm relay neurons examined by our standard current injection protocol described in Figure 3-14, which is consistent with the pioneering work in global Ca\textsubscript{3.1} null mice and Ca\textsubscript{3.1}-specific shRNA (Fig. 5-2A, gray line) (Kim et al., 2001; Park et al., 2010). In contrast, Ca\textsubscript{3.1}-lacking neurons displayed intact tonic spiking by injecting depolarizing currents (Fig. 5-2A, black line).

![Figure 5-2. Dual firing mode of thalamic neurons by somatic current injection in four different conditions.](image)

(A) No rebound burst firing in the absence of T-type channel Ca\textsubscript{3.1} but tonic spike is preserved by Ca\textsubscript{3.1}-specific shRNA. Representative trace selected from 33 recordings.
(B) The T-type blocker TTA-P2 (3 µM) abolished burst spikes (gray dotted line) but not tonic firing in wild type (WT). Representative trace selected from 5 recordings.
(C) Mismatch control (green, n=23).
(D) Scrambled control (orange, n=5).
5-2-3. Knockdown of Ca,3.1 channel at a defined synapse

5-2-3-1. Bursting failure in rosebud synapses

To examine the input-output function of the POm relay neurons in the absence of T-type channels, we stimulated Rosebuds while recording from the connected Ca,3.1-silenced postsynaptic relay neuron in the whole-cell mode. A single presynaptic electrical stimulus failed to evoke burst firing in the Ca,3.1-deficient POm neuron at a membrane potential of -70 mV. There was no difference in the action potential (AP) probability driven by the L5B corticothalamic input at different hyperpolarized holding membrane potentials in Ca,3.1 knockdown synapses (data not shown), indicating T-type calcium channels were effectively suppressed by our shRNA approach. In a few cases, a small-amplitude subthreshold glutamatergic EPSP (8.1 ± 2.4 mV) but not an AP was generated in Ca,3.1-silenced neurons (n=13) responding to the local presynaptic stimulation (Fig. 5-3A). Only repetitive stimulation resulted in propagation of a single postsynaptic AP, in which the burst of APs was prevented by knockdown of the T-type channel with 1st and 2nd stimuli at a resting potential of -70 mV (Fig. 5-3B). With every stimulus depolarization accumulated until a single spike is elicited by the following stimulus at the Ca,3.1 knockdown synapse. Notably, the delay between presynaptic stimulation and the postsynaptic action potential was very brief compared with wild-type (WT) responses, corresponding to the typical synaptic delay of approximately 1 ms.

Figure 5-3. Disrupted firing pattern in the absence of T-type channel Ca,3.1 by corticothalamic input.

(A) At hyperpolarized holding potential of -70 mV, the Ca,3.1-deficient neuron generates the subthreshold EPSP. Representative trace selected from 13 recordings.

(B) Upon repetitive stimulation induced membrane depolarization then triggered a single AP. Representative trace selected from 5 recordings.
5-2-3-2. Tonic firing of Ca\textsubscript{v}3.1-deficient thalamic relay cells

Consistent with the intact tonic discharge observed in Ca\textsubscript{v}3.1-lacking neurons upon depolarizing current injection (Fig. 5-2A, black line), we found that synaptic stimulation at a postsynaptic membrane potential of -60 mV produced a single postsynaptic AP with brief latency, similar to WT (WT 2.8 ± 0.8 ms, n=5 versus Ca\textsubscript{v}3.1 KD 2.0 ± 1.2 ms, n=4 p > 0.05, MW test) (Fig. 5-4A). Moreover, the duration (WT 2.16 ± 0.33 ms versus Ca\textsubscript{v}3.1 KD 0.74 ± 0.30 ms, p < 0.05, MW test) but not the amplitude of APs (WT 73.61 ± 8.90 mV versus Ca\textsubscript{v}3.1 KD 80.90 ± 17.59 mV, p > 0.05, MW test) were significantly changed when Ca\textsubscript{v}3.1 channels were not present in POhm relay neurons.

![Figure 5-4](image1)

**Figure 5-4. Altered tonic spike in Ca\textsubscript{v}3.1-lacking thalamic relay cells.**

(A) At a depolarized membrane potential, a single AP is elicited with shorter onset latency in Ca\textsubscript{v}3.1 KD neurons. Representative trace taken from 4 recordings in 13 mice.

(B) Repetitive stimulation in Ca\textsubscript{v}3.1 KD cells. Representative trace taken from 8 recordings in 13 mice.

Following high frequency stimulations, the spike onset of Ca\textsubscript{v}3.1-lacking neurons (n=8) was also faster in comparison with WT (WT 46.9 ± 14.8 ms, n=9 versus Ca\textsubscript{v}3.1 KD 31.4 ± 12.4 ms, n=8, p > 0.05, t-test). Interestingly, the after-hyperpolarization dropped the membrane potential to a level below threshold, so that the subsequent stimulation failed to spike in Ca\textsubscript{v}3.1 KD neurons in which no T-type calcium channels are recruited by the L5B corticothalamic input (Fig. 5-4B). However, Ca\textsubscript{v}3.1 KD cells generated an AP once the subsequent stimulation reached a membrane potential below -60 mV (Fig. 5-4B).
Considering the presence of functionally relevant T-type currents even at depolarized membrane potentials, a small LTS may account for the different firing at -60 mV between WT and Ca,3.1 KD (Deleuze et al., 2012). Indeed, we also obtained LTS-induced bursting from POM neurons at a relatively depolarized potential between -62 and -65 mV responding to the L5B input in WT groups but not in Ca,3.1 KD. Thus, our KD approach is efficiently blocking the contribution of T-type channels to LTS-induced burst spike at the rosebud synapse even at depolarized potentials.

Therefore, the T-type calcium channels-mediated LTS engages not only in firing pattern but also in pairing between pre- and postsynaptic neurons in both hyperpolarized and depolarized membrane potentials.

5-2-3. Design of the control groups for Ca,3.1 knockdown
To control for non-specific effects of shRNAs, we defined two control groups the mismatch (MM) and the scrambled (SC) versions of Ca,3.1 shRNA in our AAV system to carry different information in the same chemical composition (Fig. 6-2) (Park et al., 2010). Using the same experimental strategy of dual targeted viral expression of fluorescent proteins, we labeled L5B giant corticothalamic terminals by injection of recombinant AAV-synaptophysin-EGFP into the S1 area and introduced the control of Ca,3.1 shRNA either MM or SC into the POm from postnatal (P) 2-week-old mice. 3 to 4 wks after viral transfer, patch-clamp recordings were performed at control virus-infected thalamic relay cells that expressed the reporter gene, mOrange.

5-2-3-1. Preserved bursting in control paradigms
We first tested whether the ability to initiate a LTS subsequent to a hyperpolarizing prepulse was preserved in both control groups. POm neurons of both groups exhibited the rebound burst spike (Fig. 5-2C, D). We then examined synaptic response of single L5B-POm synapses. In both MM (n=5) and SC (n=4) treated cases, relay neurons responded with AP burst by L5B stimulation at -70 mV (Fig. 5-5A, B).
Figure 5-5. Single presynaptic stimulation evoked burst of APs in control groups for Cav3.1 KD shRNA.
(A) Mismatch (green) and (B) scramble controls (orange) exhibited rebound burst spikes at -70 mV. Both are representative traces selected from 5 and 4 recordings, respectively.

5-2-3-2. Postsynaptic currents between Ca,3.1 KD and control groups

It is possible that distinct postsynaptic firing in Ca,3.1 KD neurons was resulted from the difference in the evoked postsynaptic currents between Ca,3.1 KD and two control groups. To resolve this issue, we compared the compound EPSCs.

Even though Ca,3.1 KD exhibited drastically changed firing pattern at the LOm relay cell receiving rosebud synapses, the profile of evoked EPSCs were statistically indistinguishable from the mismatch control (amplitude: Ca,3.1 KD 123.73 ± 8.84 pA, n=37 versus MM 133.69 ± 26.54 pA, n=11 versus SC 87.83 ± 13.6 pA, n=4, rise time: Ca,3.1 KD 0.87 ± 0.06 ms versus MM 0.91 ± 0.12 ms versus SC 0.99 ± 0.23 ms, decay time: Ca,3.1 KD 5.52 ± 0.40 ms versus MM 5.50 ± 0.78 ms versus SC 2.41 ± 0.58 ms, p > 0.05, ANOVA) (Fig. 5-6B). Also, compound EPSCs between two control groups MM and SC were comparable (p > 0.05, ANOVA). These results reveal that the postsynaptic thalamic voltage response by the L5B corticothalamic input is strictly tuned by T-type channels, which is not dependent on the presence of enough AMPAR-mediated currents. In other words, the similar postsynaptic depolarizing current triggers different firing patterns according to the activation of T-type calcium channels.
Figure 5-6. Synaptic transmission is not affected by deletion of Ca,3.1.

(A) Bar histograms represent mean values of EPSC amplitude ± SEM. No difference in the spontaneous EPSC amplitudes among between Ca,3.1KD (black, n=39), mismatch (green, n=18), and scramble controls (orange, n=4).

(B) The evoked activity of Ca,3.1KD (n=49) is not different to mismatch (n=11) and scramble (n=4) controls (p > 0.05, ANOVA).

(C) Ca,3.1KD exhibit frequency-dependent STD (n=5).

(D) Intrinsic properties.

<table>
<thead>
<tr>
<th></th>
<th>Ca,3.1 KD</th>
<th>MM</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vm (mV)</td>
<td>-58.9 ± 1.404</td>
<td>-60.5 ± 1.825</td>
<td>-64.3 ± 2.728</td>
</tr>
<tr>
<td>Rn (MΩ)</td>
<td>134.30 ± 7.46</td>
<td>126.752 ± 11.181</td>
<td>106 ± 5</td>
</tr>
</tbody>
</table>

5-2-3-3. Difference in spontaneous EPSCs between Ca,3.1 KD and control groups

There was no difference in the size and shape of spontaneous EPSCs between SC and MM compared to Ca,3.1 KD (amplitude: Ca,3.1 KD 18.46 ± 0.6 pA, n=39 versus MM 17.67 ± 1.22 pA, n=18 versus SC 16.82 ± 1.31 pA, n=4, rise time: Ca,3.1 KD 0.42 ± 0.02 ms versus MM 0.34 ± 0.03 ms versus SC 0.25 ± 0.04 ms, decay time: Ca,3.1 KD 0.98 ± 0.05 ms versus MM 0.83 ± 0.07 ms versus SC 1.09 ± 0.13 ms, p > 0.05, ANOVA). In addition, the frequency of spontaneous activity for Ca,3.1 KD was not different to two control groups (frequency: Ca,3.1 KD 1.59 ± 0.13 Hz versus MM 1.61 ± 0.11 Hz versus SC 1.33 ± 0.13 Hz, p > 0.05, ANOVA) (Fig. 5-6A). Therefore, Ca,3.1 T-type channel involvement in synaptic release for both spontaneous and evoked EPSCs at rosebud synapses cannot be attributable (Huang et al., 2011).
5-2-4. Pharmacological silencing T-type channels

To determine whether the rescued LTS-mediated high-frequency firing in our control paradigms is specific to T-type channels, we applied the recently synthesized potent and specific T-type channel blocker TTA-P2 (Shipe et al., 2008).

TTA-P2 abolished the burst spike by depolarizing current application following the hyperpolarizing prepulse on the POm cell from WT and two control groups the MM and the SC as well, which resembles the Ca,3.1 deficiency (Fig. 5-2B). In the presence of TTA-P2, neither of the two controls MM (n=2) and SC (n=2) generated action potentials by L5B input, further supporting the specificity of the knockdown (data not shown). However, the evoked EPSC was not changed by the T-type channel blocker in terms of amplitude and kinetics. Similarly, we did not find any statistical difference in the profiles of EPSCs between Cav3.1 KD and two controls (Fig. 5-6A, B). These results suggest that a similar postsynaptic depolarizing current triggers different firing patterns according to the activation of T-type calcium channels (Deleuze et al., 2012).

In summary, the POm neuron has an intrinsic voltage-dependent responsiveness by L5B stimulation, which is critically regulated by T-type calcium channel Ca,3.1. This feature is proposed to be distinctly involved in thalamocortical synaptic communication.
5-3. Discussion

5-3-1. No boosting POm output without Ca,3.1-mediated LTS

The membrane potential-dependent synaptic amplifier function of L5B-POm synapses is the key feature of the rosebud synapse (Fig. 2-7). Our present study shows that T-type calcium channel Ca,3.1 is required for this function (Fig. 5-2, 3), which is consistent with results obtained from relay neurons in Cav3.1 global knockout mice (Kim et al., 2001). Local injection of AAV harboring Ca,3.1-specific shRNA into the POm neuron efficiently abolished burst firing by L5B input at hyperpolarized membrane potentials. In contrast, our mismatch and scramble control groups exhibited the post-inhibitory rebound burst spikes (Fig. 5-2, 5). Hence, the Ca,3.1 T-type channel is a molecular switch for two voltage-dependent firing modes of thalamic neurons and determines the amplifier function of the L5B-POm synapse. Injecting computer-stimulated T-type calcium conductance into thalamic neurons of Ca,3.1 KD mice has been reported to fully restore the LTS generation and burst firing, confirming the vital role of T-type calcium channels for AP bursting (Tscherter et al., 2011). Furthermore, loss of Ca,3.1 also displayed the shorter onset latency in tonic spike genesis at resting and depolarized potentials, triggering the change in coupling of excitatory presynaptic input to postsynaptic potentials in thalamic neurons (Fig. 5-4). Thus, T-type calcium channels may attribute to spike-timing-dependent plasticity by determining the timing between presynaptic and postsynaptic signals (Abbott and Nelson, 2000).

Bursts of action potentials in POm neurons have been implicated in neuronal oscillations, thereby possibly recruiting large-scale networks (Crunelli and Hughes, 2010; Poulet et al., 2012). Considering widespread outputs from the POm via CTC loops, temporally linked synchronization is predicted to be disrupted by the lack of T-type calcium channels, which may lead to reduced or impaired transmission from S1 to other cortical areas during sensorimotor processing (Wang et al., 2010a). Furthermore, the brief bursts generated by synaptic amplification could facilitate cortical synaptic terminals of relay neurons, thereby increasing the strength of synaptic transmission onto cortical neurons. This mechanism could be used to gate thalamocortical information transfer.
5-3-2. Other potential sources for rebound burst firing

With its dominant expression (Talley et al., 1999), knockdown of Ca,3.1 T-type channels completely abolished the LTS-induced burst firing in POM neurons, suggesting Ca,3.1 is responsible for most of the T-type current (I_t) in the POM region (Fig. 5-2, 3). Besides, we did not find any further change in postsynaptic current and voltage response either from Ca,3.1 KD by TTA-P2 that block all three subtypes of T-type channels. However, the potential contribution of other T-type channel subtypes for residual T-type Ca^{2+} currents in the L5B-POM synaptic transmission should be addressed in future studies (Cain and Snutch, 2010; Tschetter et al., 2011; Lee et al., 2014).

Nevertheless, it is possible that burst firing at the rosebud synapse can be modulated by other ion channels such as Ca^{2+}–dependent small and big K^{+} channels or hyperpolarization-activated HCN channels generating non-inactivating inward current, I_H (McCormick and Pape, 1990; Engbers et al., 2011). However, a recent report demonstrated the indispensable role of T-type calcium channels in burst firing but little influence of Ca^{2+}–activated K^{+} channels by Ca^{2+} entry on the thalamic burst firing pattern (Tschetter et al., 2011).
Chapter 6.

Materials and methods

6-1. Animal model system

6-1-1. All experiments was carried out in mice (*Mus musculus*)
Controls and Ca3.1 knockdown experiments were done in the C57BL6/J mouse strain. Experiments were conducted in accordance with the German animal welfare guidelines and were approved by the responsible authority (Regierungspräsidium Karlsruhe).

6-1-2. Mice lacking AMPA receptor subunits
The design of ‘floxed’ each GluA mouse line was described previously. All conditional knockout animals for GluA1, GluA3, and GluA4 targeted exon 11 by flanking loxP to each subunit from C57BL6, C57BL6/N, and C57BL6, respectively (Fig. 6-1) (Zamanillo et al., 1999; Sanchis-Segura et al., 2006; Fuchs et al., 2007). Gria1Rlox, Gria3Rlox, and Gria4Rlox were provided by Dr. Peter H. Seeburg, Dr. Rolf Sprengel, and Dr. Hannh Monyer from University of Heidelberg. All three strains have been backcrossed to C57BL6/J. Triple knockout (TKO) were generated by mating mice carrying a loxP-flanked of each GluA-allele.

![Diagram](image)

Figure 6-1. Generation of inducible GluA4, as noted GluRD, knockout mouse. (Fuchs et al., 2007).
6-2. RNAi constructs

The sequence information for short hairpin RNA (shRNA) to knockdown Ca$_{\text{v}}$3.1 T-type calcium channel and the scrambled version of Ca$_{\text{v}}$3.1 shRNA were obtained from Dr. Daesoo Kim, Korea Advanced Institute of Science and Technology (Park et al., 2010). Double-stranded oligonucleotide for Ca$_{\text{v}}$3.1 shRNA was synthesized following sequences

(5’-CGGAATTCGGGGAAGATCGTAGATAGGAAAAttcagagaTTTGCTATCTCTAC
GATCTTTTTTTGATATCTAGAC-3’).

A scrambled version (SC) of Ca$_{\text{v}}$3.1 shRNA oligonucleotide was like that

(5’-CGGAATTCGGGTAAGTGAACTGACAAGAAttcagagaTTCTTGTCAGTTC
ACTTACTTTTTGATATCTAGAC-3’).

Coding sequence of the mismatch (MM) control for Ca$_{\text{v}}$3.1 shRNA (gcagttgcacatggcaga) was cloned in here (provided by Michaela Kaiser) following sequences:

(5’-caccgcAGGTCGCACATGGGCAAGtggaagccacagatgTCTGCCATGTGCGA
ACTGCA
3’-AAAAGCAGTTGCACATGGGCAGAcatctgtggcttcacTCTGCCATGTGCGA
ACTGCA).

6-3. Plasmid cloning

All three conditions for Ca$_{\text{v}}$3.1 shRNA oligonucleotide were inserted into NotI/Eagl restriction enzyme sites of AAV vector containing the RNA polymerase III specific promoter U6. Expression of mOrange is under control of the RNA polymerase II promoter CAG, a combination of the cytomegalovirus enhancer element (CMVE) and chicken β-actin (CBA) promoter (Garg et al., 2004). The woodchuck posttranscriptional regulatory element (WPRE) and the bovine growth hormone poly-A (PA) were added to generate pAM-U6-shRNA Ca$_{\text{v}}$3.1–CAG–mOrange–WPRE–PA (Zufferey et al., 1999; Wimmer et al., 2004). pAM-CAG-iCre-2A-mOrange-WPRE-PA was obtained through the same procedure but utilizing codon-improved Cre (iCre) and 2A peptide that cleaves the two proteins posttranslationally, yielding two independent proteins (Shimshek et al., 2002). All constructs used in this study are listed in Figure 6-2 and were kindly provided by Michaela Kaiser.
6-4. Recombinant adeno-associated virus preparation

The production of AAV-synaptophysin-enhanced green fluorescent protein (EGFP) was described in elsewhere (Schwenger and Kuner, 2010). Neurons infected with AAV-iCre construct were filled with diffusible membrane bound Orange (mOrange), which were present throughout their cytoplasm, including axon fibers (Caputi et al., 2012). The recombinant adeno-associated virus particles of the chimeric 1/2 serotype consist of a vector plasmid (pAM) where AAV2 inverted terminal repeats flanked the expression cassette. Two helper plasmids, pDP1 and pDP2, carried all genes necessary for packaging the viral DNA into infectious particles. Recombinant AAV chimeric viruses contain 1:1 ratio of AAV1 and AAV2 and the foreign gene (During et al., 2003; Grimm et al., 2003).

The procedure is briefly described as follows: HEK293 cells were transfected with 4 x10^6 virus particles containing recombinant DNA from pAM and two helper plasmids, pDP1 and pDP2, using 125 mM CaCl_2, HBS (140 mM NaCl, 25 mM HEPES, 0.7 mM Na_2HPO_4, pH 7.05). The virus particles were harvested 3 days later using lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5) and benzonase endonuclease (Sigma). The AAV particles were purified by a heparin-agarose column and eluted with 15 ml elution buffer (500 mM NaCl, 50 mM Tris-HCl, pH 7.2) into a filter tube (Amicon Ultra Filter, Milliore). All viruses were kindly produced by Claudia Kocksch.

**Figure 6-2. List of AVV constructs used in this study.** (Note: Cav3.1 is written as α1G)
6-5. Stereotaxic injection

Stereotaxic setup: Model 1900 stereotaxic alignment system (David Kopf Instruments, California, USA) and an eLeVeLeR electronic leveling device (Wimmer et al., 2004). The coordinates were corrected depending on the distance between Bregma and Lambda (Paxinos and Franklin, 1997).

2 weeks old, postnatal 12 to 15 day, mice were placed on 35°C heating pad and sedated with 5% of mix isoflurane (Baxter, HDG 9623–vaporizer: Surgivet, Isotec4)/oxygen until animals were immobile and lower up to 1.5% mix isoflurane/oxygen and adjusted by checking the breathing rate during remaining procedures. Head of animals was fixed using ear bars and eyes were covered with eye cream (Bayer, Bepanthen Augensalbe) to prevent drying out. When animals did not respond to reflex tests, opened the skin by a scalpel blade with subcutaneous injection of 1% Lidocain. Bregma and Lambda were marked with pen and adjust tips of leveling device, eLeVeLeR, according the Bregma-Lambda distance. A hole at the desired position was drilled by dental driller (Osada EXL-40) and virus was loaded via the injection capillary (5 µl, BlauBrand, intraMARK, Wertheim, Germany) by controlled gentle pressure from 50 mL syringe that is connected by a silicon tube to the capillary. AAV-synaptophysin-EGFP (1.5 µl) was evenly distributed over 5 injection sites into S1 the following x,y coordinates relative to Bregma and middleline (in mm) (1) -3, 0 ; (2) -2.96, -0.2 ; (3) -2.92, -0.4 ; (4) -2.88, -0.6 ; (5) -2.84, -0.8. At each of these positions injection were done at depth (z) from -1.1 to 0.9 from dura. AAV-iCre-mOrange targeting AMPA receptors or AAV-shRNAs-mOrange targeted against Ca₃.1 T-type calcium channel subunits and its mismatch and scramble controls (0.4 µl) were injected into POM (1.7 mm posterior, 1.27 mm lateral, 3.27 mm below from Bregma). For immunohistochemistry, AAV-mOrange was introduced into the POM at one hemisphere and the other side of POM was infected by AAV-iCre-mOrange or AAV-Ca₃.1 shRNAs from the same animals, for AMPAR conditional or WT mice, respectively. The injection needle was retracted 90 seconds after injection and skin was sutured (BBraun, Dafilon DSMP11). After cessation of anesthesia, animals were recovered under pure medical oxygen gas until they were mobile. 20 minutes after recovery under infrared lamp, mice were returned to the home cage with mother.
6-6. Preparation of acute brain slices

After 3 to 4 weeks of virus incubation, mice were quickly decapitated after isoflurane anesthesia. Acute brain slices were prepared as described previously (Wimmer et al., 2004; Groh et al., 2008). 180 mm thickness coronal sections at the level of the thalamus were prepared on a Leica VT1200S vibratome in ice-cold artificial cerebrospinal fluid (ACSF) slicing solution containing (in mM) 125 NaCl, 2.5 KCl, 1.25 Na_2HPO_4, 25 NaCO_3, 25 glucose, 2 MgCl_2, and 0.1 CaCl_2. The slices were stored in ACSF bath solution. The slices were incubated for 30 minutes at 37°C and then kept at room temperature. To maintain the pH 7.4, slices were kept all the time in a bubble solution with a mix 95% O_2 and 5% CO_2.

6-7. Electrophysiology

Whole-cell recordings of POm relay cells were performed in acute brain slices from p28-p42 mice at room temperature using an EPC-10 plus amplifier controlled by PatchMaster software (HEKA Electronics). Patch pipettes had open tip resistances of 3-5 MΩ and were filled with solution containing (in mM) 130 K-gluconate, 20 KCl, 10 HEPES, 4 ATP-Mg^{2+}, 5 EGTA, 5 Na_2-phosphocreatine, and 0.03 Alexa 594 fluor hydrazide (Invitrogen). Liquid junction potentials were not corrected. Series resistance was compensated to 80-90%. The extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25 Na_2HPO_4, 25 NaCO_3, 25 glucose, 1 MgCl_2, and 2 CaCl_2 adjusted to pH7.4 by bubbling with a mix of O_2/CO_2. Drugs (Tocris Bioscience) were added to bath solution 1mM kynurenic acid (KY), 10 μM CNQX, 50 μm APV, 10 μM DHβE, 10 μM Carbachol, and 3 μM TTA-P2 (Alamone Labs). Capillaries for patch electrode (World Precision Instruments, USA) and double-barreled theta glass capillary (Hilgenberg GmbH, Germany) for bipolar stimulator were pulled on a P97 horizontal puller (Sutter Instrument). Currents were recorded at a holding potential of -70 mV and potentials were measured in current clamp mode by LFVC (low frequency voltage clamp, in PatchMaster). Signals were digitized at 10-40 kHz and Bessel filtered at 2.9 kHz.
6-8. Juxtasynaptic stimulation

Giant terminals were visualized using real-time multi-channel confocal scanning-gradient-contrast microscopy (Upright Leica TCS SP5 confocal microscope (Leica Mikrosysteme Vertrieb GmbH, Germany) equipped with an additional external PMT and Dodt-contrast tube to generate an infrared scanning-gradient-contrast image (Wimmer et al., 2004). As established before, 0.05 mM Alexa 594 fluor hydrazide (Invitrogen) filled bipolar stimulation pipette with tip diameters 0.5 to 1 µm was introduced into the target terminal by simultaneously overlying the two fluorescence channels (red for postsynaptic neuron and green for presynaptic bouton) (Groh et al., 2008). Thereafter, biphasic pulse was applied to stimulate labeled presynaptic terminals close to the soma and or the first dendritic branches using a stimulus isolator (custom built by F. Rödel, Max Plank Institute for Medical Research, Heidelberg, Germany).

6-9. Immunohistochemistry

3 weeks after virus incubation, mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. Tissues were postfixed in 4% PFA/PBS overnight at 4°C. 50 µm vibratome coronal sections (Sigmann Elektronik, Hüffenhardt, Germany) were permeabilized for 90 min at RT in a PBS- based solution containing 10% normal goat serum (NGS) (Jackson Immunoresearch Laboratories) and 1% Triton X-100 (TX-100). Then, slices were stained with primary antibody, rabbit anti-Cre (1:2500, Millipore), anti-glutamate receptor 1 (1:500, Millipore), anti-glutamate receptor 2&3 (1:500, Millipore), anti-glutamate receptor 4 (1:500, Millipore), and anti-Ca3.1 (1:500, Alamone Labs) that were diluted in PBS- based solution containing 1% NGS and 0.5% TX-100 over night at 4 °C. On the following day slices were washed 3 x 15 min in 2% NGS and Alexa Fluor 488 or 568 conjugated goat anti-rabbit secondary antibody (1:2000, Invitrogen), which were diluted in the same solution of the primary antibodies, were incubated for 90 min at RT, in the dark. Upon 2 x 10 min washing in 1% NGS, the sections were rinsed with PBS, embedded in SlowFade® Gold antifade reagent (Invitrogen, Darmstadt, Germany) and kept at 4°C. All staining and washing steps were in 24 well plate under shaker.
CHAPTER 6

6-10. Image acquisition

Virus-mediated expression of a synaptophysin-EGFP fusion protein and mOrange were studied from fixed slices incubated with antibodies for immunohistochemistry using confocal microscopy. High-resolution confocal images were acquired using a Leica TCS SP5 laser scanning microscope equipped with Argon 488 nm, 561 nm, and 633 nm laser lines to excite EGFP/Alexa Fluor 488, mOrange, and Alexa Fluor 568, respectively using 63 x glycerol immersion objective (NA 1.3). To avoid cross talk between different fluorophores sequential scans were made same laser settings were used under all conditions. Glycerol-based immersion medium with refractive index of 1.43 was used and the pinhole was adjusted to 1 airy unit. Images, oversampled to meet the Nyquist criterion, had a voxel size of 57.32 x 57.32 nm in axial and 50 nm in vertical dimensions. Overview images were acquired without digital magnification and are presented as a collapsed z-stack of optimized consecutive optical frames.

6-11. Quantification of terminals and soma size

Labeled synaptic boutons and relay cells were visualized using a real time multichannel confocal imaging in the same session electrophysiological recordings from acute brain slices were done. Images of EGFP expressing giant terminals were captured with a bipolar stimulation pipette at the z point corresponding the maximal size of the bouton. The cell body of thalamic relay cell was visualized either by Alexa 594-filled patch-pipette or AAV-mediated expression of mOrange. Therefore, all pictures represent 2D planes. Individual images were imported into ImageJ for quantification and the diameters of somata and boutons were measured. The distances between soma and terminal correspond to the length from the center of the terminal to the starting branch point of the dendrite. To analyze the relationship between terminal size and EPSC magnitudes at the same relay cell, the synaptic bouton size was normalized by the smallest value and corresponding EPSC amplitude was followed from the same data set.

6-12. Data Analysis

All the data was measured with custom written routines using Igor Pro (Wavemetrics Inc., Lake Oswego, OR). Igor algorithms were kindly provided by Dr. Francisco Urra.
The data was analyzed using Prism software (GraphPad software Inc., California). For all statistical comparisons involving a number of observations below 8 the normal distribution of the data can not be assessed using the D’Agostino & Pearson omnibus test. Hence, in those cases data were assumed to be not normally distributed and the Mann-Whitney test (MW test) was applied. For comparisons involving 8 or more observations normality was tested and the Student’s t-test or Mann-Whitney tests were used to determine statistical significance when comparing two conditions and ANOVA or Kruskal-Wallis test were used for multiple comparisons (unless otherwise indicated). Numbers are presented as mean ± SEM unless otherwise noted.
Chapter 7.

General discussion

Neurons communicate with one another through synapses that build up the networks such as CTC loops in the brain. Understanding the structural and functional connectivity of synapses and underlying cellular and molecular mechanisms at the level of single synapse is one of the fundamental goals of neuroscience. So far, only few studies have addressed the detailed properties of identified synaptic connections in the thalamus in spite of its basic anatomical description and systemic function.

We characterized structural and functional properties of a mouse corticothalamic synapse connecting L5B neurons of the somatosensory cortex to relay neurons of the thalamic POm nucleus. This L5B-POm giant synapse drives excitation of POm relay neurons in a strongly frequency-dependent manner. Individual presynaptic action potentials triggered multiple postsynaptic action potentials, constituting a synaptic signal amplifier (Chapter 2). Deletion of the GluA4 subunit strongly reduced the synaptic current and resulted in a marked delay from the presynaptic action potential to the first postsynaptic action potential, while maintaining the number of postsynaptic action potentials triggered by a single stimulus (Chapter 3). Knockdown of the Cav3.1 subunit in POm relay neurons abolished the amplifier function at hyperpolarized potentials, essentially silencing the synapse. Only at depolarized postsynaptic membrane potentials a one-to-one spike transfer could be achieved (Chapter 5). Additionally, we found POm neuronal firing by L5B input in the presence of α4β2 nicotinic receptor blocker (Chapter 4).

These perturbations demonstrate that the GluA4 subunit provides a major portion of the glutamatergic current required to activate the synaptic amplifier within a short latency after the presynaptic action potential, while the Cav3.1 subunit is essential to establish the synaptic amplifier function of this giant synapse. The two perturbations characterized here will help in future experiments to uncover the physiological significance of membrane potential-dependent synaptic amplification at this remarkable giant synapse. We found that loss or inactivation of these genes of
interest results in altered synaptic transmission by changes in firing rate and temporal distribution of postsynaptic action potentials.

7-1. Key features of the mouse rosebud synapse
Consistent with our published study in rats, mouse corticothalamic synapse also functions as a driver triggering multiple postsynaptic APs by a single L5B cortical input (Groh et al., 2008). The cortical synaptic input is amplified on the thalamic neuron in a voltage-dependent manner, which is dynamically regulated by voltage-gated T-type calcium channels with the LTS-mediated synaptic delay (Fig. 2-8 and Fig. 5-3, 4). In addition, postsynaptic high conductance glutamate AMPARs combined with presynaptic high release probability allows reliable signal transmission from L5B to the POm at intervals of silence or synchrony in L5B neurons due to strong frequency-dependent STD (Fig. 2-2, 6 and Fig. 3-4 to 7) (Groh et al., 2008).

Morphological and synaptic physiological characteristics of rosebud synapses resemble the synapses formed by periphery lemniscal driver afferents in the VPM (Hoogland et al., 1991; Reichova and Sherman, 2004). However, its systemic function is expected to participate in more complex and higher brain function, top-down control on somatosensory information processing, which underlies cortical involvement such as attention, working memory, motor planning and state-dependent firing (Zompa and Chapman, 1995; Komura et al., 2001; Guo et al., 2014).

7-1-1. Structure-function relationship at the L5B-POm synapse and comparison to other giant synapses
The relatively large average diameter of 3.3 µm of L5B-POm terminals provides sufficient space to accommodate multiple active zones functioning in parallel, similar to other giant terminals such as the calyx of Held or hippocampal mossy fiber terminals (Rollenhagen and Lubke, 2006). In contrast to the calyx of Held, the parallel active zone design does not result in a reliable relay function for the L5B-POm synapse. This is mostly due to the more pronounced presynaptic short-term depression of the L5B-POm synapse that establishes low-pass frequency filtering of presynaptic spiking patterns. We suggest that the most prominent function of parallel active zones in the L5B-POm synapse is to generate a large postsynaptic current required to drive the relay neuron to firing threshold. In continuation of this thought
one might expect that the size of the terminals would correlate with the magnitude of the postsynaptic EPSC. However, we could not find such a relationship (Fig. 2-5), suggesting a large variability of form and function.

The proximal location (18.59 ± 1.1 μm from soma, n=34) on the thick primary dendrites of POm relay neurons electrotonically couples L5B-POm synapses with the soma and axon initial segment, strongly supporting their role as amplifying driver synapses (Fig. 2-1, 8).

7-1-2. Comparison of rosebuds between mice and rats

The most evident difference between mouse and rat rosebud synapses concerns the ten times smaller EPSC amplitudes (mice: 0.27 ± 0.03 nA, n=79 versus rat: 3.3 ± 1.45 nA, n=26), from which one would expect that mouse rosebuds may not function as driver synapses. However, the difference in amplitudes is at least partially due to the different recording temperature (20-22°C mouse versus 34°C rat). Another factor supporting the driver function in mouse rosebuds despite the smaller EPSC amplitude concerns the electrotonic distance to the axon initial segment. Mouse terminals were typically situated at an average distance from the soma of 18.59 ± 1.1 μm (n=34) versus 29.5 ± 11.6 μm (n=3) in rats. Higher expression levels of T-type calcium channels could also compensate for the smaller EPSC amplitude.

We also found a significant difference in the size of presynaptic terminals (mice: 3.34 ± 0.11 μm, n=55 versus rat: 5.1 ± 0.2 μm, n=8). Given the large variability and lack of structure-function relationship in mouse L5B-POm terminals, the difference in size between rat and mouse terminals may not be relevant functionally.

Finally, there are further differences, some of which may also be due to the different recording temperatures, such as quantal size (mice: 22.19 ± 7.92 pA, n=5 versus rat: 60 ± 16 pA, n=5), size of releasable vesicle pool (mice: 0.45 ± 0.05 nA, n=14 versus rat: 4.1 ± 1.7 nA, n=22) and release probability (mice: 50.48 ± 6.79% versus rat: 80 ± 9%). Some of these features may translate into differences in ultrastructure.

These observations suggest that L5B-POm synapses may have species-specific functions, consistent with functional and structural differences in barrel cortex circuitry and POm reported previously (Bureau et al., 2006; Kichula and
Huntley, 2008). However, in both species L5B-POm synapses act as drivers and synaptic amplifiers in a manner dependent on the resting membrane potential of the relay neuron. Previous work done in the rat stimulated corticothalamic terminals with patterns of activity known from in vivo recordings (de Kock et al., 2007; Groh et al., 2008). Therefore, it is necessary to characterize spontaneous and whisker-evoked activity patterns of L5B neurons in the mouse by in vivo recordings. The rationale behind this is that the faster speed of whisker movements in mice would likely be differently decomposed on frequency content in the L5B-POm synapse compared to rats (Jin et al., 2004). Otherwise, the conduction velocities from L5B neuron to the relay cell of POm may be different because of the difference in distance between the two species.

7-1-3. Physiological impact of strong short-term depression at the L5B-POm synapse

The mouse L5B-POm synapse undergoes strong short-term depression at frequencies exceeding a few Hertz (Fig. 2-6A), similar to the situation found in the rat L5B-POm synapse (Groh et al., 2008). In the line with this finding, consecutive EPSCs of similar magnitude were induced only for intervals that were longer than about 400 ms (Fig. 2-6B). In addition to the replenishment of releasable vesicles on the presynaptic side, it is conceivable that also the recovery of T-type calcium channels from inactivation on the postsynaptic side may occur during such a time window, with both mechanisms optimizing the strength of synaptic transmission after a period of quiescence. Indeed, this time window correlates well with the period of hyperpolarization that is needed for de-inactivation of T-type calcium channels (Perez-Reyes, 2003; Groh et al., 2008). Hence, burst response in the POm can be preferentially triggered by low-frequency stimuli (Swadlow and Gusev, 2001; Groh et al., 2008; Rothman et al., 2009). In addition, hyperpolarization of the POm relay neuron by GABAergic and M2 cholinergic inputs will boost the response to subsequent depolarizing L5B inputs through de-inactivation of T-type calcium channels (Barthó et al., 2002; Bokor et al., 2005a; Urbain and Deschênes, 2007).
7-1-4. Voltage-dependent switch of synaptic transfer mode

At hyperpolarized membrane potentials, the L5B-POm synapse functions as a driver and amplifier synapse. Essential for this function are T-type calcium channels expressed in POm relay neurons. The EPSP generated by a single L5B-POm synapse suffices to activate T-type calcium channels, resulting in a delayed and long-lasting depolarization that ultimately yields a burst of action potentials (Fig. 2-8A). Hence, every presynaptic spike will get translated into multiple postsynaptic spikes. Only at more depolarized potentials, the L5B-POm synapse will switch to a one-to-one transmission mode because of the inactivation of T-type channels. Cortical activity depolarizes thalamic neurons in the range between -70 and -60 mV, resulting in markedly different firing patterns of POm neurons in response to L5B excitation (Fig. 2-8A,C) (Dossi et al., 1992). Also subcortical inputs may adjust the resting membrane potential of POm relay neurons (Lee and Dan, 2012). Thus, although the role of thalamus and cortex in initiation of up states is still a matter of debate, a relatively small change of the resting membrane potential may bring about distinctive differences in POm spiking, thereby reflecting the momentary brain state (Crunelli and Hughes, 2010).

7-2. GluA4 is the dominant AMPAR subunit in POm relay neurons

To explore the molecular mechanisms underlying rosebud synapse function, we examined the phenotype of GluA4 KO and GluA1, 3, and 4 TKO by selectively recording from Cre-infected AMPAR subunit-deficient POm neurons while stimulating labeled presynaptic L5B-POm terminals (Fig. 3-2). Our results show that GluA4 plays a predominant role in L5B-POm synapses (Fig. 3-5 to 12), consistent with previous studies demonstrating that GluA4 receptors are linked to the strength of corticothalamic synapses involving synchrony between cortex and thalamus (Golshani et al., 2001; Beyer et al., 2008; Paz et al., 2011) and are abundantly expressed in the thalamus (Mineff and Weinberg, 2000).

Loss of GluA4 at L5B-POm synapses results in a strong reduction of the EPSC amplitude (65% reduction) and slower kinetics, causing a prolonged spike onset (Fig. 3-5, 10). The latter may have a strong influence on spike-timing-dependent plasticity and may disrupt synchrony in the cortico-thalamo-cortical system (Abbott
and Nelson, 2000; Paz et al., 2011). Both may disrupt the timing of sensorimotor activity.

Given that T-type conductances mediate between the glutamatergic EPSP and the action potential at hyperpolarized membrane potentials (Fig. 2-8A), it remains unclear why particularly GluA4, the ionotropic glutamate receptor subunit conferring the fastest kinetics (Mosbacher et al., 1994), mediates the EPSC in POm relay neurons. We suggest that its rapid kinetics may only be important for precise timing of single action potential transfer at depolarized membrane potentials (Fig. 2-8C).

On the other hand, we recorded spontaneous EPSCs of POm neurons presumably arising from a variety of presynaptic sources including cortical and subcortical inputs (Sherman and Guillery, 2013) and found a small but significant change in the spontaneous activity in the absence of GluA4 (10% reduction) (Fig. 3-3). This difference between spontaneous and evoked EPSCs from GluA4 KO suggests that there is an activity-dependent AMPAR recruitment at the synapse between L5B and POm. The level of attenuated spontaneous EPSC peak for GluA4 KO is comparable in thalamic reticular nuclei (11% reduction, p > 0.1) where GluA4 is most strongly expressed, from the global GluA4 KO mice (Paz et al., 2011). Results from us and other group propose that AMPARs are not uniformly distributed on thalamic cells and GluA4 subunits are densely expressed at the postsynaptic sites receiving cortical inputs, such as rosebud synapses. But it still remains to determine the relative contribution of each AMPAR subunit. Hence, the future study is needed to quantitatively and qualitatively analyze the consequence of AMPAR perturbations by 3D-immunohistochemistry and serial sectioning scanning electron microscopy. This analysis study will also provide a clue concerning the endogenous distribution of AMPARs and presumed altered subcellular distribution of AMPARs after deletion of either GluA4 or three synaptic AMPARs. L.Y.Wang’s group argued that slower responses for GluA4 KO at the calyx of Held-MNTB synapse is likely resulted from not properly apposed synaptic AMPA receptors to the release sites (Yang et al., 2011).
7-3. Cav3.1 activates synaptic amplifier at hyperpolarized membrane potentials

We found the signal amplification in the synapse connecting L5B neurons with thalamic relay cells, which is regulated by voltage-gated T-type calcium channels with distinct synaptic delay (Fig. 2-8 and supplemental Fig. 2). In attempting to investigate how thalamic response is changed in the absence of T-type calcium channel subunit Ca,3.1, we introduced AAV encoding Ca,3.1-specific shRNA into the POm (Fig 5-1, 2). Knockdown of Ca,3.1 brings about not only failure of burst firing but also shortening the onset latency of tonic spike by the presynaptic L5B input at hyperpolarized and relatively depolarized membrane potential, respectively (Fig 5-3, 4). In contrast, the post-inhibitory rebound burst of APs is preserved in our mismatch and scramble control groups, which is repressed by the T-type calcium channel blocker TTA-P2 (Fig. 5-2, 5). Hence, T-type calcium channels play an essential role in determining the spike timing and pattern with voltage-sensitive LTS generation at the rosebud synapse.

Slow kinetics in de-inactivation of T-type calcium channels may introduce the feature selectivity on a long time scale to the intrinsic excitability of POm neurons. When POm cells are hyperpolarized, T-type calcium channels are unable to follow high frequency stimulation because of low-pass filter property at the giant corticothalamic synapse (Fig. 2-6 and 3-11). Thus kinetics of the stimulus can regulate the outcome of POm, slow inputs may preferentially bring about a burst of action potentials. There is improvement in signal-to-noise ratio in burst mode providing better detectability on the activating postsynaptic cell, leading to increased information content within AP patterns and all-or-none character of thalamic cell response (Guido and Weyand, 1995; Swadlow and Gusev, 2001). Latest work from Svoboda group probed that, prior to the action itself, sensory information from S1 is evaluated for decision with a delay at the level of the frontal regions of the neocortex, especially the anterior lateral motor cortex (ALM) (Guo et al., 2014). LTS-mediated burst firing at the L5B-POm synapse is well suitable to all these features (Fig. 2-8, and supplemental Fig. 2). Nevertheless, S1 indirectly communicates to the ALM either via the posterior nucleus of thalamus (PO) including the POm or S2 from which S1 requires transthalamic connection by the POm, suggesting potential engagement of L5B-POm synapses in tactile decision during a delay period (Fig. 1-2) (Theyel et al., 2010; Mao et al., 2011; Guo et al., 2014).
7-4. Methodological considerations

The selectivity, specificity and fidelity of our stimulation paradigm has been supported here and our previous rat study, the EPSCs, of which rise time is fast and invariant, are evoked in an all-or-none manner and are blocked by glutamate receptor antagonists (Chapter 2) (Groh et al., 2008). To ensure these features, we limited the stimulation intensities and duration (<100 µs) for the minimal condition and guaranteed the delay between stimulus artifact and postsynaptic response. Also, the stimulation method used is exquisitely distance dependent. Moving the tip of the pipette only 1 µm away results in a loss of the postsynaptic response. Therefore, stimulation of the other bouton is extremely unlikely. Moreover, the single Gaussian fit in the distribution of spontaneous activities supports the single entity (Fig. 3-3). However, some difficulties in introducing a stimulation pipette to the synaptic bouton depending on its position in plane of brain slice, where it is localized too close to the patch pipette or too deeper than cell body, resulted in low yield of successful recordings. Focal photostimulation by optogenetic approach may overcome this drawback and be capable to excite repeatedly multiple terminals at the same cell, instead of serial stimulation as in the present study (Fig. 2-5C).

The time course of virus expression in this study was 3 weeks after injection, which is based on the matured state of rosebud synapses in WT and healthy of Cre-infected cells because we found that some of neurons are not healthy even dead after more than 4 weeks of virus incubation (Fig. 2-3, 4). It has been reported that high level of Cre expression can cause cytopathic effects, DNA damage and abnormal brain developments (Loonstra et al., 2001; Forni et al., 2006). Additionally, fluorescent proteins can be toxic, even though we did not find any influence of AVV-mOrange expression in the neuronal property after 3 to 4 weeks of incubation (Chapter 3) (Shaner et al., 2008). However, this would be probably problematic for long-term behavioral tests to define the contribution of these giant synaptic connections to sensory-motor integration. To relate systemic consequence of target gene loss by focal viral injection into the specific thalamic nucleus, it is necessary to estimate the percentage of infected thalamic volume and the infection rate for neurons. The number of infected neurons can be varied by the tilter and volume of injected virus solution.
7-5. Functional implications in sensorimotor integration

7-5-1. Temporal aspect of whisker-related behaviors

POm relay cells are thought to be involved in temporal processing related to sensorimotion control of whisker movement, comparing sensory events with the ongoing cortical activity from motor descending pathways (Ahissar et al., 2000; Urbain and Deschênes, 2007). Perception of pressure, vibration and texture for discriminative touch are haptic information processing, stressing the precise spike timing. The temporal characteristics of signal transfer differed depending on the original synaptic conductance size. The high conductance AMPAR-component in the rosebud synapse is well positioned to detect timing information by evoking precisely timed action potentials with short latencies (Blitz and Regehr, 2003). Therefore, altered synaptic transmission in AMPAR knockout mice with slower postsynaptic response at the L5B-POm synapse may lead to change in temporal control of whisker movement (Fig. 3-4 to 12). Similarly, the lesion in the pulvinar as a visual higher order thalamic nucleus, an analogous to the POm, results in increased reaction times to neglect-like symptoms (Wilke et al., 2010). Nevertheless, less prominent and slower STD development without synaptic AMPARs may provide a change in neuronal gain and adaptation (Fig. 3-7, 8) (Chung et al., 2002; Abbott and Regehr, 2004) that influences the discriminability of vibrissa deflection (Wang et al., 2010b; Ramirez et al., 2014).

Interestingly, synapses between POm relay cells and L4 neurons of S2 are formed by large terminals and function as drivers (Viaene et al., 2011). Recently, it has been reported that two driver inputs for the POm from cortical L5 and brainstem converge at the single thalamic neuron and interact synergistically in time-dependent manner (Groh et al., 2013). Besides sensory inputs, additional driver-like inputs from M1 onto POm has been studied (Yamawaki and Shepherd, 2015). These studies prompt the question whether the loss of synaptic AMPAR-mediated currents in the POm affects differently these driver synapses, if so or not, impaired precise spike timing in the POm neuron may disrupt the temporal interaction in the sensorimotor networks involved in whisker behavior.
7-5-2. State-dependent two firing modes

The switch between different brain states and spontaneous cortical activity strongly influence the transmission of sensory information (Castro-Alamancos, 2002; Crochet and Petersen, 2006; Ferezou et al., 2007; Saalmann et al., 2012). Also, the role of neuromodulation such as cholinergic system in the control of cortical state has been well described (Chapter 4) (Lee and Dan, 2012). However, only a few experiments have recorded neural activity from awake and behaving subjects, majority of studies were performed in anesthetized animals from which POm neurons are difficult to excite by whisker stimulation (Diamond et al., 1992a; Ferezou et al., 2007; Poulet et al., 2012). Strong and rapid STD with fast synaptic recovery of rosebud synapses are suitable to operate sensory processing according to behavioral states (Fig. 2-5). Moreover, the voltage (highly controlled by ongoing cortical state) - dependent thalamic firing in the POm (Fig. 2-8 and supplemental Fig. 2) by L5B input may engage the synchrony across cortical areas (Zompa and Chapman, 1995; Slezia et al., 2011) and can ensure reliable transmission of sensory information during whisking and active touch with more precise spike timing onset (Temereanca et al., 2008). On the other hand, thalamic spike firing has been demonstrated to be necessary for the active cortical states during behaving and it is driven by an internally generated signal within the central nervous system rather than peripheral sensory input (Poulet et al., 2012). Thus, altered firing pattern in Ca,3.1 lacking POm neurons at the strong and reliable corticothalamic giant synapses is expected to give rise to deficits in sensorimotor coordination (Fig. 5-3). In order to prove that, it needs an understanding of how sensory information from S1 is differently coded between two firing modes of POm neurons and transformed into cortical motor-related output. There is experimental evidence of casual link dual thalamic firing pattern to opposing behavioral controls (Lee et al., 2012). Whisker twitching, 7-12 Hz rhythmic whisker movements preceding increased VPM bursting activity is not observed after S1 inactivation, asking to test whether certain whisker movement is relevant to bursting at the L5B-POm connections (Fanselow et al., 2001).
7-6. Future directions

Here, we described the synaptic properties of identified giant corticothalamic synapses connecting L5B neurons from S1 with POm relay cells of the mouse using visually guided recordings. Besides somatosensory, other sensory thalamic systems form giant synapses across species (Rouiller and Welker, 2000). Hence, our recording approach can be applied to study other thalamic giant synapses, for instance the middle line thalamus involving in cognitive functions, and compare the results to the rosebuds (Lee et al., 2012; Xu and Südhof, 2013).

The next step is to investigate the collective actions of thalamic relay cells and interconnected cortical neurons in the context of sensorimotor behaviors beyond the single synapse basis (Jones, 2007). Currently, nothing is known which sensory information is conveyed and how or when this sensory signal is converted into goal-directed action during whisker behavior via the L5B-POm synapse. Thus, spatiotemporally controlled activation or inactivation at specific L5B-POm circuit by optogenetic probes in conjunction with the molecular perturbation strategies developed here will help to answer these questions. Using area/cell type-specific inducible Cre mice, we plan to address the role of presynaptic proteins such as Bassoon/Piccolo or Munc18 to modulate neurotransmitter release at corticothalamic giant terminals. Together with these genetic techniques, newly designed calcium sensors/indicators like GCaMP6 will aid to reveal the neuronal activity change via CTC loops and directly link T-type calcium transients to relevant behavioral change using two photon imaging (Chen et al., 2013a; Chen et al., 2013b). Furthermore, we can manipulate the synaptic delay between L5B and POm and assess the extent of delay to quantifiable behavioral consequences.
Supplemental Figures

Supplemental Figure 1. Synaptic depression in EPSC and EPSP.
Upon repetitive stimulation, postsynaptic responses are depressed compared to the first response in both EPSC (black bottom trace) and EPSP (red upper trace).

Supplemental Figure 2. Voltage and time-dependent burst firing in thalamic neurons.
(A) Relay cell switches to burst spike in respond to depolarizing currents preceding hyperpolarization. Preceding hyperpolarization lasting at least 50-100 ms is required for bursting.
(B) Subtle change in depolarized potentials does not affect tonic firing.
(C) and (D) Spike counts for bursting are regulated by the low-threshold calcium conductance according to membrane potential.
Supplemental Figure 3. EPSC waveforms for AMPAR KO mice, GluA4 KO (blue, n=2) and TKO (red, n=4), are changed by the nicotinic blocker DHβE (checked bar, 5 μM).

Supplemental Figure 4. Spike onset is changed in WT (A and B, black line) and TKO (C and D, red line) by the nicotinic blocker DHβE (5 μM, gray dashed line).
Abbreviations

AAV: adeno-associated viruses  
ACh: acetylcholine  
ALM: anterior lateral motor cortex  
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor  
AP: action potential  
APV: 2-amino-5-phosphonopentanoic acid  
APT: anterior pretectal nucleus  
CNQX: 6-cyano-7-nitroquinoxaline-2, 3-dione  
CTC loop: cortico-thalamo-cotrical loop  
Ctrl: control  
DHβE: dihydro-β-erythroidine hydrobromide  
EGFP: enhanced green fluorescent protein  
EPSC: excitatory postsynaptic current  
EPSP: excitatory postsynaptic potential  
GABA: gamma-aminobutyric acid  
I_t: low-threshold Ca^{2+} current  
IZ: zona incerta  
KD: Knokdown  
KO: Knockout  
KY: kynurenic acid  
L5B: layer 5B  
LTS: low-threshold spike  
LVA: low voltage-activated  
M1: motor cortex  
MM: mismatch  
nAChR: nicotinic receptor  
NMDA: N-methyl-D-aspartic acid  
nRT: reticular nucleus  
POM: posteriomedial nucleus  
Rosebud: synapse between L5B somatosensory neurons to POM thalamic neurons  
PPR: paired-pulse ratio
RRP: readily releasable pool  
S1: primary somatosensory (barrel) cortex  
S2: secondary somatosensory cortex  
SC: scramble version of shRNA  
shRNA: short hairpin RNA  
STD: short-term depression  
STP: short-term plasticity  
TKO: triple knock out  
TTA-P2: 4-Aminomethyl-4-fluoropiperidine  
VPM: ventral posteromedial nucleus
References


de Kock CPJ, Sakmann B (2008) High frequency action potential bursts (&gt;= 100 Hz) in L2/3 and L5B thick tufted neurons in anaesthetized and awake


Diamond ME, Armstrong-James M, Ebner FF (1992a) Somatic sensory responses in the rostral sector of the posterior group (POm) and in the ventral posterior medial nucleus (VPM) of the rat thalamus. The Journal of Comparative Neurology 318:462-476.

Diamond ME, Armstrong-James M, Budway MJ, Ebner FF (1992b) Somatic sensory responses in the rostral sector of the posterior group (POm) and in the ventral posterior medial nucleus (VPM) of the rat thalamus: dependence on the barrel field cortex. The Journal of Comparative Neurology 319:66-84.


development leading to microencephaly and hydrocephaly. J Neurosci 26:9593-9602.


Hoogland PV, Welker E, Van der Loos H (1987) Organization of the projections from barrel cortex to thalamus in mice studied with Phaseolus vulgaris-
leucoagglutinin and HRP. Experimental brain research Experimentelle Hirnforschung Expérimentation cérébrale 68:73-87.


Sherman S, Guillery R (2002) The role of the thalamus in the flow of information to the cortex. ... of the Royal ....


Summary

The properties and molecular determinants of synaptic transmission at giant synapses connecting layer 5B (L5B) neurons of the somatosensory cortex (S1) with relay neurons of the posteriomedial nucleus (POm) of the thalamus have not been investigated in mice. We addressed this by using direct electrical stimulation of fluorescently labeled single corticothalamic terminals combined with postsynaptic whole-cell recordings and molecular perturbations. Consistent with their function as drivers, we found large amplitude excitatory postsynaptic currents (EPSCs) and multiple postsynaptic action potentials triggered by a single presynaptic action potential. To study the molecular basis of these two features, ionotropic glutamate receptors and low voltage-gated T-type calcium channels were probed by virus-mediated selective perturbation. Loss of GluA4 almost abolished the EPSC amplitude, strongly delaying the onset of action potential generation, but maintaining the number of action potentials generated per presynaptic action potential. In contrast, knockdown of the T-type calcium channel Ca$_{v}$3.1 abrogated the driver function of the synapse when transmitting single action potentials. Only summation upon repetitive stimulation shifted the membrane potential towards firing threshold, generating postsynaptic action potentials with almost no delay relative to the presynaptic action potential. Hence, GluA4 subunits are required to produce an EPSC sufficiently large to trigger postsynaptic action potentials within a defined time window after the presynaptic action potential. Moreover, Ca$_{v}$3.1 expression is essential to introduce a defined synaptic delay and support L5B-POm synapses to function as drivers. Additionally, we found that nicotinic receptors may participate in controlling thalamic neuronal excitability and output timing at individual L5B-POm glutamatergic synapses.

In conclusion, deleting these genes selectively in relay neurons impair distinct aspects of synaptic transmission at the L5B-POm giant synapse, offering an attractive possibility to study the systemic function of this synapse and the CTC loop in sensory-motor integration.
Acknowledgement

In his heart a man plans his course, but the LORD determines his steps (Proverbs 16:9).

I would like to acknowledge my supervisor Prof. Thomas Kuner for giving the opportunity to work in Anatomy Institute at University Heidelberg and invigorating me throughout my Ph.D. Also, thanks to him and Prof. Verhage (VU, Netherlands) for receiving me for Marie Curie fellowship, BrainTrain program. I am also grateful to Prof. Hee-sup Shin (IBS, Korea) for introducing me to Thalamus and T-type calcium channel.

I would like to express appreciation to Dr. Francisco Urra for teaching me electrophysiology. And many thanks to wonderful technicians Michaela Kaiser and Claudia Kocksch, my work wouldn’t be without your help. I would like to mention all Prof. Thomas Kuner’s lab members, owing to you I had great times inside and outside lab.

To dear my family, your support, encouragement and love was and still is invaluable.