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Role of GABA-induced Ca\(^{2+}\) signal in axonal outgrowth in hippocampal newborn granule cells

해마 신생과립세포의 축삭 성장에서의 GABA에 의한 칼슘 신호의 역할

2013년 2월

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이 현 수
ABSTRACT

The hippocampal dentate gyrus undergoes neurogenesis throughout life. Neural network activity regulates the development of hippocampal newborn granule cells (GCs). Excitatory GABAergic input is known to be a key player in this regulation. Although calcium signaling is thought as a downstream mediator of GABA, GABA-induced calcium signaling in newborn GCs is not well understood, and little is known which type of voltage dependent calcium channel (VDCC) mediates the calcium signal in newborn neurons. I investigated Ca$^{2+}$ signaling and its regulatory role in axon and dendrite outgrowth in newborn GCs which are identified in the organotypic slice culture of early postnatal rat hippocampus. Here, I report that hippocampal network activity can induce calcium transients (CaT) in newborn GCs during the first post-mitotic week via GABAergic inputs. The GABA-induced CaTs were mediated mainly by L-type Ca$^{2+}$ channels. Furthermore, I found that inhibiting any step in the signaling pathway, network activity $\rightarrow$ GABA $\rightarrow$ L-type Ca$^{2+}$ channels, selectively suppressed the axonal outgrowth and pruning of newborn GCs, but not the dendritic outgrowth. The GABA$_A$ receptor blocker bicuculline significantly suppressed axonal outgrowth, despite increasing network activity, thus
indicating an essential role of GABAergic inputs. Therefore, I conclude that the network activity-dependent GABAergic inputs open L-type Ca\(^{2+}\) channels and promote the axonal outgrowth in newborn GCs during the first post-mitotic week.

Among subtypes of VDCCs, the L-type VDCCs are known as a key player in the development of neuron. CaV1.2 and CaV1.3 are the most widely expressed L-type subunits in the brain. Because newborn GCs receive only GABAergic inputs in early developmental stage and CaV1.3 channel open at lower membrane potential than CaV1.2 channel, I focused on the role of CaV1.3. To test whether calcium influx through CaV1.3 is necessary for normal development of newborn GCs, I knocked down the CaV1.3 gene in a newborn GCs-specific manner by using retroviral constructs that co-express GFP and short-hairpin RNA against CaV1.3. I confirmed the effectiveness of knock-down of endogenous CaV1.3 gene by western blotting and immunohistochemistry. Nevertheless, the peak current-voltage curves of total calcium current were not distinguishable between control and infected GCs, suggesting compensation of calcium current by CaV1.2. However, the proportion of nimodipine-sensitive calcium current was higher in the infected GCs, indicative of CaV1.3 depletion. The GABA-induced CaTs were abolished in the somata and the growth cones of the
CaV1.3-depleted GCs. The current amplitude induced by direct puff application of GABA or stimulation of perforant pathway was significantly lower in the CaV1.3-depleted GCs, implying that the CaV1.3 expression is essential for GABAergic synapse formation in newborn GCs. Furthermore, the axonal outgrowth was impaired in the CaV1.3-depleted GCs, implying the essential role of GABAergic inputs in normal development of newborn GCs. Therefore, I conclude that the CaV1.3 channel is essential for the GABAergic synapse formation in newborn GCs during the 1st post-mitotic week.

Key Words: GABA, postnatal neurogenesis, hippocampal granule cells, axon outgrowth, L-type calcium channel, CaV1.3, synaptic formation

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BrdU</td>
<td>5-bromo-3’-deoxyuridine</td>
</tr>
<tr>
<td>CaMKI</td>
<td>calcium/calmodulin-dependent protein kinase I</td>
</tr>
<tr>
<td>CaMKKK</td>
<td>calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CaT</td>
<td>calcium transient</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>EBSS</td>
<td>Eagle’s balanced salt solution</td>
</tr>
<tr>
<td>EC</td>
<td>entorhinal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>granule cell</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GPSC</td>
<td>GABAergic postsynaptic current</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NADA</td>
<td>N-arachidonoyl dopamine</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na-K-Cl cotransporter-1</td>
</tr>
<tr>
<td>OGB</td>
<td>Oregon green BAPTA</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PP</td>
<td>perforant pathway</td>
</tr>
<tr>
<td>TrkB</td>
<td>tropomyosin-related kinase B</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential canonical channel</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VDCC</td>
<td>voltage dependent calcium channel</td>
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</table>
GENERAL INTRODUCTION

Generation of functional neurons from progenitor cells, which is called neurogenesis, had been traditionally thought to occur only during embryonic stages in mammals (Ramon y Cajal, 1928). However, Altman and his colleagues provided the autoradiographic evidence for the production of new neurons in the dentate gyrus of the hippocampus and in the subventricular zone of adult rats (Altman & Chorover, 1963; Altman & Das, 1965). After several decades of neglect, adult hippocampal neurogenesis was rediscovered in the adult rat and the adult primate by developing 5-bromo-3’-deoxyuridine (BrdU) which labels mitotically active cells and is detected by immunohistochemistry (Gould et al., 1992; Gould et al., 1997). Furthermore, neurogenesis in the dentate gyrus was established in human postmortem tissue by using BrdU (Eriksson et al., 1998).

After initial discovery of neurogenesis in the postnatal rat hippocampus, Altman suggested that newborn neurons were crucial for learning and memory (Altman, 1967). The first causative link between neurogenesis and memory was that blocking hippocampal neurogenesis by methylazoxymethanol, antimitotic agent, disrupted trace eye-blink conditioning but not delay eye-blink conditioning (Shors et al., 2001).
Inversely, promoting the hippocampal neurogenesis by electrically stimulation of entorhinal cortex (EC), which projects major inputs to the dentate gyrus, facilitated the spatial memory in rodents (Stone et al., 2011). Although the change of neurogenesis was not observed, enhancement of spatial memory by deep-brain stimulation of EC was also reported in human (Suthana et al., 2012).

The process of neurogenesis includes multiple complex steps that begin with the proliferation of progenitor cells, followed by differentiating to a neuronal phenotype, morphological and physiological maturation, and integrating to exiting neural circuit (reviewed by Ming & Song, 2005). In this thesis, I focused on the maturation of newborn neuron in early developmental stage when they receive only GABAergic inputs and give rise axonal neurites to the hilus and that on the verge of the CA3 region.
CHAPTER 1

GABA mediates the network activity-dependent facilitation of axonal outgrowth
Introduction

Accumulating evidence indicates that neural network activity regulates neurogenesis and post-mitotic development of hippocampal newborn granule cells (GCs), which are eventually integrated into pre-existing circuits in the hippocampus (Deisseroth et al., 2004; Tozuka et al., 2005; Ge et al., 2006; Overstreet-Wadiche et al., 2006). Direct stimulation of afferent fibers to the dentate gyrus promotes the survival of 1-week-old GCs (Bruel-Jungerman et al., 2006; Kitamura et al., 2010). Phasic and tonic GABAergic inputs associated with the hippocampal network activity have emerged as a key player in coupling neural activity and the structural plasticity of newborn neurons (Ben-Ari, 2002; Ge et al., 2007; Markwardt et al., 2009; Markwardt et al., 2011). GABA induces depolarization of immature GCs (Wadiche et al., 2005; Ge et al., 2006). It is obscure, however, whether such GABA-induced depolarization is sufficient to induce a Ca\(^{2+}\) signal in newborn GCs (West et al., 2002; Lohmann & Wong, 2005; Greer & Greenberg, 2008).

Immature GCs show initial neurite outgrowth within the first post-mitotic week (Zhao et al., 2006). The development of the mossy fibers of GCs during the early postnatal period has been well documented (Amaral & Dent, 1981; Dailey et al., 1994). Recently, it has been demonstrated that the mossy
fiber terminals of newborn GCs in the adult hippocampus make functional synapses on target cells (Toni et al., 2008). Little is known, however, about whether the axonal outgrowth of immature GCs is regulated by neural network activity.

Ca$^{2+}$ and its downstream signaling pathway involved in axonal outgrowth have been well studied in dissociated cortical neuron culture. It has been reported that axonogenesis, axonal extension and growth cone motility are regulated by the CaMKK-CaMKI\(\alpha\) pathway (Wayman et al., 2004; Ageta-Ishihara et al., 2009). However, the factors upstream of the Ca$^{2+}$-signaling that regulates axon outgrowth in the context of neural network activity are not well understood. Newborn GCs in the hippocampal dentate gyrus are an excellent model for studying axonal extension because their axonal outgrowth is uniformly toward the CA3 field. Nevertheless, it is not easy to demonstrate the axon outgrowth of newborn GCs labeled in vivo because axon fibers are often severed during the preparation of acute brain slices (Zhao et al., 2006; Toni et al., 2008). To circumvent this problem, I explored the axonal outgrowth of newborn, retrovirus-tagged GCs in the organotypic hippocampal slice culture.

Using these techniques, I tried to address the following questions: 1) Do GABA inputs increase the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in
newborn GCs during the first post-mitotic week? 2) If so, which type of calcium channels is responsible for the \( \text{Ca}^{2+} \) influx? 3) Is the axonal outgrowth of newborn GCs regulated by GABA inputs and neuronal circuit activity? Here, I demonstrate that the \([\text{Ca}^{2+}]_i\) response in the somata and axonal growth cones of newborn GCs can be evoked by stimulation of the perforant pathway (PP) or local puff application of GABA to the soma. Furthermore, the axonal outgrowth of newborn GCs is regulated by GABA, which mediates network activity.
Materials and Methods

**Organotypic slice culture.** All experiments were performed with the approval of the animal experiment ethics committee at the Seoul National University College of Medicine (approval Nos. SNU-091001-1 and SNUMC IBC 08-001). Postnatal day 7-10 (P7-P10) Sprague Dawley rats (Orient Charles River Co., Seoul, Korea) were deeply anesthetized by hypothermia, and their brains were removed according to the animal welfare guidelines of Seoul National University. The posterior part of the brain was cut into 300-μm-thick transverse slices using a vibratome (ZERO 1; Dosaka, Kyoto, Japan) in ice-cold Eagle’s balanced salt solution (EBSS) supplemented with 12.5 mM HEPES. The entorhino-hippocampi were dissected out and cultured using membrane interface techniques (Stoppini et al., 1991; De Simoni & My Yu, 2006). Briefly, slices were placed on sterile 30-mm-diameter membranes (Millicell-CM; Millipore, Bedford, USA) and transferred into six-well tissue culture trays. Cultures were fed with 1 ml of 50% minimal essential medium (Gibco-Invitrogen), 25% horse serum (Gibco-Invitrogen), and 25% EBSS enriched with 5.6 mM glucose and kept in a humidified incubator at 37°C in 5% CO₂. The medium was changed to serum-free medium (Neurobasal-A medium with B27 complement, 5 mM
glucose, 2.5 mM L-glutamine) at 4 days after dissection to ensure the development of newborn GCs ex vivo (Raineteau et al., 2004).

**Labeling newborn GCs with retrovirus.** I used VSV-G pseudotyped recombinant retrovirus encoding GFP (EGFP-CL) to label newborn GCs in slice culture (Kim et al., 2007b). I transfected the retroviral vector plasmid into 293gpg packaging cells (Lipofectamine; Invitrogen) and harvested the supernatant, which contained viral particles at 3–10 days after transfection. The supernatant was centrifuged at 50,000 g in a Beckman SW28 rotor (Beckman Instruments, Palo Alto, CA) for 2 h at 4°C. The pellet was re-suspended in 1/100 of the original volume of DPBS (Gibco-Invitrogen). Viral titer was adjusted to 1 x 10^8 particles/ml. The virus solution (0.5 µl) was delivered onto a hippocampal slice culture within 5-10 min after placement on the membrane (Namba et al., 2007).

**Brain slice preparation.** Transverse hippocampal brain slices were prepared from P10-P15 Sprague Dawley rats as described previously (Lee et al., 2007). After brain removal (detailed above), the brain was chilled in ice-cold low-calcium artificial CSF (aCSF) containing (in mM) 124 NaCl, 26 NaHCO_3, 3.2 KCl, 0.5 CaCl_2, 7 MgCl_2, 1.25 NaH_2PO_4, 10 glucose, 2 Na-
pyruvate, and 3 ascorbate with the pH adjusted at 7.4 by saturation with carbogen (95% O₂, 5% CO₂). The posterior part of the brain was cut into 300-µm-thick transverse hippocampal slices using a vibratome. The slices were incubated at 34°C for 30 min in the same solution. Thereafter, they were maintained at room temperature until required.

Electrophysiology. After preparation, slices were placed in a chamber under an upright microscope (BX51WI; Olympus, Tokyo, Japan). The chamber was perfused with normal aCSF (the composition of which was identical to the low-calcium aCSF except 1.3 mM MgCl₂ and 2.5 mM CaCl₂ were included, and the pyruvate and ascorbate were excluded). Whole-cell patch recording was performed under visual control using differential interference illumination. GFP+ cells were identified by a fluorescence image visualized using a CMOS camera (ZTD33CD, ZOOTOS Corporation, Korea). Whole-cell patch data were collected using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany) and Pulse software (version 8.67). Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a two-stage puller (PC-10; Narishige, Japan). To evoke synaptic responses, a bipolar tungsten electrode (WPI, TM53CCINS-05B) was placed in the medial perforant pathway (200 µs duration; 5-20 V
intensity). The lateral distance of the stimulating electrode was at least 500 µm away from the recorded cell. Post-synaptic currents were recorded in newborn GCs using a patch pipette (3-4 MΩ) filled with a solution containing (in mM) 140 CsCl, 1 MgCl₂, 10 HEPES, and 4 Na₂ATP (pH 7.2 with CsOH). For recording mature GCs, patch pipettes were filled with a solution containing (in mM) 140 K-gluconate, 5 di-Tris-phosphocreatin, 5 NaCl, 4 MgATP, 0.4 Na₂GTP, 15 HEPES, and 2.5 Na-pyruvate (pH 7.3 with KOH). To measure the passive electrical properties of GCs, I used a pipette solution containing 130 mM K-gluconate, 20 mM KCl, 2 mM MgCl₂, 4 mM K₂ATP, 0.3 mM NaGTP, 10 mM Na₂-phosphocreatine, and 10 mM HEPES (pH 7.2 with KOH) (Lee et al., 2009). Input resistance was measured from a voltage excursion elicited by current input that hyperpolarized the membrane potential approximately 5 mV in current clamp mode. The membrane time constant was measured by curve-fitting a voltage trace created from the average of 10 raw traces evoked by the hyperpolarizing current pulse. Cell capacitance was calculated by dividing the membrane time constant by the input resistance.

Cytosolic Ca²⁺ measurements. Ca²⁺-indicator dye was introduced into newborn GCs using single cell electroporation as previously described
Borosilicate patch pipettes were filled with Oregon Green Bapta-1 (OGB-1, 1 mM dissolved in distilled water, Molecular Probes, Eugene, OR). The pipette was placed close to the cell under visual control. Voltage pulses for electroporation were generated with voltage stimulator (MASTER-8; AMPI, Jerusalem, Israel) triggered by a TTL pulse from an EPC-10 amplifier. Single square voltage pulses (10 ms duration; 5-10 V intensity) were sufficient to load cells with the OGB-1. The same pipette could be used for electroporating multiple cells. Ca$^{2+}$ imaging was performed using a confocal laser-scanning system (FV300; Olympus) with a 60x water-immersion objective (numerical aperture 0.9, Olympus). To imaging CaTs evoked by local application or PP stimulation, I scanned a region of interest at 5 Hz for 20 s. To minimize photo-bleaching, minimal laser powers (2.5 $\mu$W for 488 nm Ar laser measured under the objective) and maximal pinhole size (300 $\mu$m) were used. Fluorescence values were averaged over an area of interest selected within the fluorescence image. Relative intracellular Ca$^{2+}$ levels were expressed as the change in fluorescence divided by the resting fluorescence ($\Delta F/F_0$). The applied equation was $\Delta F/F_0 = (F - F_0)/(F_0 - F_b)$, where $F$ is the average fluorescence of the region of interest (ROI) in an individual image, $F_0$ is the baseline fluorescence of the ROI averaged over at least 3 sec prior to stimulation or
local puff, and \( F_b \) is the background fluorescence measured in a region devoid of dye-filled structures. All representative traces of CaTs are averages of more than three trials.

**Local puff application of drugs.** For puff application of GABA, GABA was dissolved in extracellular bath solutions that either contained or did not contain antagonists. A puffer pipette was placed at 40-50 \( \mu \text{m} \) away from the cell soma. GABA was applied via the pipette with a pressure of \(< 2 \text{ psi}\) (Pressure System IIe; Toohey Company, Fairfield, USA). To test the efficiency of puff application of drugs in my experimental setup, the fluorescence intensity of fluorescein (100 \( \mu \text{M}, \Sigma \text{igma} \)) was imaged while the drug was applied using the same method as GABA. At the typical distance from the cell (40 – 50 \( \mu \text{m} \)), the fluorescence intensity was 64.5 \( \pm 5.5\% \) (\( n = 12 \)) of that at the tip of the pipette (Fig. 1-1). All chemicals were obtained from Sigma or Tocris, except \( N \)-arachidonoyl dopamine (Alexis Biochemicals). Stock solutions were stored at -20\( ^\circ \text{C} \) and diluted to the desired concentration in aCSF just before use.

**Morphological analysis and immunohistochemistry.** I analyzed the morphology of newborn GCs in the hippocampal slices obtained from P7-P8
rats to minimize age-dependent variance in the speed of maturation. At 6 days in vitro (DIV 6), slices were fixed overnight at 4°C by submersion in 4% paraformaldehyde dissolved in 0.05 M phosphate buffered saline (PBS, pH 7.4). Slices were washed in PBS and placed on the stage of the upright microscope. GFP+ cells in cultured slices were imaged using a confocal laser-scanning system with a 40X water-immersion objective (NA 0.8, LUMPlan FI/IR, Olympus). To analyze the morphology of GFP+ cells, I captured a z-stack of confocal images of the entire dentate gyrus and the hilus. A typical z-stack was composed of 30-50 optical sections taken at 2 μm intervals. To extract the 2D-projection image of individual GFP+ cells, I carefully traced the GFP+ profiles on every adjacent section using the ImageJ plug-in Simple Neurite Tracer (Abràmoff et al., 2004; Longair et al., 2011). We excluded any incompletely reconstructed neurons from the analysis. Moreover, we excluded GFP+ cells that have no or more than five primary processes or that exhibit no dendrite-axon polarization from the analysis, since newborn cells with such morphological features were not immunoreactive for doublecortin (DCX) (Fig 1-3C). Axon length and the number of axonal branches were determined from the reconstructed images using the NeuronJ extension of ImageJ (Meijering et al., 2004).
Data analysis. Data were analyzed using Igor-Pro (version 6.2; WaveMetrics, Lake Oswego, OR). Statistical data are expressed as the mean ± the SEM, and \( n \) indicates the number of cells or growth cones studied. Statistical analyses were performed using Student’s \( t \) tests. The Kolmogorov-Smirnov test was used to compare the length of neurites of the newborn GCs between control slices and drug-treated slices.
Results

It has been well established that GCs are generated from endogenous stem cells and develop normally in hippocampal organotypic slice cultures (Kamada et al., 2004; Raineteau et al., 2004; Namba et al., 2007; for review Lossi et al., 2009). Moreover, the developmental and physiological properties of mossy fibers are retained in organotypic hippocampal slice cultures (Dailey et al., 1994). I investigated the calcium signals of newborn GCs in organotypic hippocampal slice cultures obtained from P8-P10 rats. A retrovirus encoding eGFP was used to label dividing cells. I confirmed that the retrovirus-infected newborn GCs were immunoreactive for doublecortin, a marker of immature GCs, on the 7th post-injection day in vivo and that they underwent normal development in vivo (Fig. 1-2). On DIV5-6, infected newborn GCs which were immunoreactive for doublecortin (83.5% of total GFP+ cells, n = 103 from two independent experiments; Fig. 1-3B) exhibited the following morphological characteristics: 1) the dendritic arbor was composed of one branched apical dendrite with no spine and was confined to the granule cell layer of the dentate gyrus; 2) the axonal arbor was confined to the hilus, and the length of the longest axonal process was between 20 and 150 µm (59.14 ± 10.90 µm, n = 13, see Figs. 1-4B and 1-7A)
for an exemplar cell morphology). However, infected non-neuronal cells that were not immunoreactive for doublecortin exhibited no such polarization of dendritic and axonal processes but have multiple short filopodia-like processes around the soma (16.5% of total GFP+ cells, n = 103 from two independent experiments; Figs. 1-3B, 1-3C), which is the characteristic morphology of oligodendrocyte or astrocyte. Together with their electrophysiological characteristics (Table 1), these morphological features of GCs on DIV5-6 correspond to those of immature GCs that receive only GABAergic inputs (Esposito et al., 2005) or those GCs of class 2-II that receives only slow GABAergic synaptic inputs (Ambrogini et al., 2004). I observed phasic GABA inputs to immature GCs as early as the fifth post-mitotic day (Fig. 1-4F). This is four days earlier than phasic GABA inputs are observed in newborn GCs in the adult hippocampus (Esposito et al., 2005). Similar discrepancies in the development of newborn GCs between the postnatal and adult hippocampus have been reported (Zhao et al., 2006).

GABAergic synaptic inputs mediate the CaT evoked by perforant path stimulation in newborn GCs

To image calcium transients (CaTs), I loaded GFP-expressing GCs on DIV5-6 with OGB-1 using single-cell electroporation techniques. These
techniques enabled us to measure the CaTs of newborn GCs without perturbing intracellular chloride concentrations (Nevian & Helmchen, 2007). When minimal laser intensity was used, the fluorescence of GFP-expressing newborn GCs after loading OGB-1 was $14.6 \pm 3.3$ (n = 22) times higher than that before loading OGB-1. Thus, the error caused by the overlap of the emission spectra between OGB-1 and eGFP was negligible.

I imaged the changes in the fluorescence of OGB-1 dye that were induced by stimulation of the PP at high frequency (50 Hz for 5 s) to test whether hippocampal network activity can induce CaTs in newborn GCs. PP stimulation increased the cytosolic calcium concentration ([Ca$^{2+}$]$_i$) not only in the soma ($\Delta F/F_0 = 0.29 \pm 0.06$, n = 5; Fig. 1-4C) but also in the growth cone ($\Delta F/F_0 = 1.57 \pm 0.32$, n = 6; Fig. 1-4D). CaTs were observed even in growth cones located 100 μm away from the soma. Bath application of bicuculline (50 μM), a GABA$_A$ blocker, significantly reduced the CaTs evoked by PP stimulation both in the somata ($\Delta F/F_0 = 0.08 \pm 0.02$, 24.2 ± 3.2% of control, n = 5, p < 0.01) and in the growth cones ($\Delta F/F_0 = 0.35 \pm 0.12$, 19.93 ± 3.91% of control, n = 6, p < 0.01; Fig. 1-4E), indicating that GABAergic synaptic inputs mediate the CaT evoked by PP stimulation. I confirmed that PP stimulation evoked postsynaptic GABAergic currents in newborn GCs (Fig. 1-4F). The above results indicate that PP stimulation
induces CaTs in the somata and growth cones of newborn GCs via activation of GABA_A receptors.

**L-type Ca2+ current mediates the elevation of \([Ca^{2+}]_i\) evoked by a local GABA puff**

To study direct effects of GABA on \([Ca^{2+}]_i\) in newborn GCs, I imaged CaTs induced by local puff application of GABA. This experiment was not feasible in the cultured slice because the surface of the cultured slice was covered with a thin layer of glia (Kamada et al., 2004). Thus, I examined immature GCs that displayed the aforementioned developmental characteristics of DIV5-6 GFP-expressing newborn GCs in an acute slice (Fig. 1-5A; Table 1). Local puff application of GABA (5 \(\mu\)M) induced CaTs in the somata (\(\Delta F/F_0 = 0.79 \pm 0.04, n = 54\)) and growth cones (\(\Delta F/F_0 = 0.77 \pm 0.13, \text{length of axon, 142.03 } \pm 33.32 \ \mu\text{m, } n = 8\); Figs. 1-5A, B). The GABA-induced CaTs in the growth cones cannot be attributed to direct activation of GABA_A receptors on the growth cones because I always positioned the growth cone opposite or out of the puff stream (Fig. 1-5A). Additionally, the CaTs at the growth cones evoked by the somatic puff of GABA were observed in the presence of TTX (0.5 \(\mu\)M) indicating that the CaTs in the growth cones depended on depolarization mediated by
electrotonic conduction rather than sodium-dependent action potential generation (Fig. 1-5C). Because bath application of TTX (0.5 μM) did not substantially affect the GABA-induced CaTs in the somata or growth cones of immature GCs (Fig. 1-5C), all local puff experiments shown in Fig. 1-5D and Fig. 1-6 were conducted in the presence of TTX to inhibit spontaneous synaptic or extra-synaptic inputs to the immature GCs. The GABA-induced CaTs in the somata and growth cones were completely blocked by application of bicuculline (for somata, ΔF/F₀ = 0.05 ± 0.01, 4.8 ± 0.7% of control, n = 6, p < 0.001; and for growth cones, ΔF/F₀ = 0.06 ± 0.02, 10.5 ± 3.9% of control, n = 8, p < 0.01; Figs. 1-5B, 1-5D).

The above results suggest that GABA is able to induce depolarization that is sufficient to open voltage-dependent calcium channels (VDCCs) and increase [Ca²⁺], in newborn GCs. Next, I examined which type of calcium channel is responsible for the GABA-induced CaTs in the soma of immature GCs. Previous studies have shown that immature GCs have TTX-resistant, but Ni²⁺-sensitive, calcium spikes and suggested that these spikes are mediated by low voltage-activated calcium channels (Ambrogini et al., 2004; Schmidt-Hieber et al., 2004). However, application of Ni²⁺ (50 μM) resulted in only a partial block of GABA-induced CaTs in the presence of TTX (70.5 ± 1.7% of control, n = 4, p < 0.01; Fig. 1-6A). Because Ni²⁺ is not
specific for T-type calcium channels but inhibits L-type calcium channel even at low micromolar concentration (Zamponi et al., 1996), I tested other T-type channel blockers. Mibefradil blocks T-type calcium channels with an IC$_{50}$ in the submicromolar range (Arnoult et al., 1998; Cribbs et al., 1998) and has an affinity for T-type channels that is more than 10 times higher than that for L-type channels (Martin et al., 2000). In the presence of TTX, mibefradil had no significant effect on the GABA-induced CaTs at concentrations up to 10 μM (96.7 ± 2.8% of control at 5 μM, p = 0.29, 92.4 ± 10.9% of control at 10 μM, n = 4, p = 0.43; Fig. 1-6B). I also applied N-arachidonoyl dopamine (NADA), which has recently been reported to be a strong inhibitor of T-type channels (Ross et al., 2009). NADA (5 μM) also had no significant effect on GABA-induced CaTs (99.5 ± 6.7% of control, n = 4, p = 0.57; Fig. 1-6E). The above results suggest that T-type VDCCs contribute little to GABA-induced CaTs.

I tested whether high voltage-activated VDCCs are involved in GABA-induced CaTs. Treatment with ω-conotoxin MVIIC (2 μM) to block N- and P/Q-type calcium channels failed to block GABA-induced CaTs (98.4 ± 5.6%, n = 13, p = 0.55; Fig. 1-6E). On the other hand, the L-type calcium channel blockers nifedipine (30 μM) and nimodipine (20 μM) significantly suppressed GABA-induced CaTs in the newborn GCs (53.7 ± 2.9% of
control, n = 7, p < 0.001; 39.5 ± 4.2% of control, n = 9, p < 0.001; Figs. 1-6C, 1-6E). Diltiazem (100 μM) also inhibited GABA-induced CaTs (51.9 ± 4.0% of control, n = 4, p < 0.01; Fig. 1-6E). Moreover, BayK8644 (10 μM), an L-type VDCC agonist, enhanced GABA-induced CaTs (211.2 ± 15.1% of control, n = 6, p < 0.01; Fig. 1-6D). Altogether, these results suggest that L-type VDCCs are the main calcium entry pathway for GABA-induced CaTs in newborn GCs.

**GABA-induced calcium influx via L-type calcium channels facilitates axonal outgrowth and pruning of newborn GCs**

Although I examined the Ca\(^{2+}\) influx pathway activated by somatic puff application of GABA, considering the high input resistance and small capacitance of newborn GCs, it can be reasonably expected that L-type VDCC may be the major Ca\(^{2+}\) influx pathway activated by network activity-dependent release of GABA in DIV5-6 GCs. Because the axonal outgrowth of newborn GCs is a developmental hallmark of the first mitotic week (Hastings & Gould, 1999), I inquired whether the axonal outgrowth from DIV5-6 newborn GCs is affected by the signaling elements involved in GABA-induced CaTs. To address this question, I examined the axonal outgrowth of newborn GCs on DIV6 in the cultured slice treated for 48 hrs
with TTX (1 µM), bicuculline (50 µM) or nimodipine (20 µM); each of these antagonists inhibit neural network activity, GABA_A receptors, and L-type VDCCs, respectively. I measured the length of the longest axon fiber in the axonal arbors arising from individual GFP-expressing GCs on DIV6 by analyzing their confocal images (Fig. 1-7A; see Materials and Methods). The cumulative distributions of axonal lengths of newborn GCs under different conditions are shown in Fig. 1-7C. The length of the longest axon fiber varied from approximately 50 µm to 800 µm under control conditions (137.8 ± 7.8 µm, n = 155; Fig. 1-7C1). When slices were treated with the above drugs for 48 hrs from DIV4 to 6, the axonal outgrowth was significantly suppressed in all three cases (89.5 ± 4.4 µm, n = 83, p < 0.001 for TTX; 111.5 ± 5.4 µm, n = 104, p < 0.05 for bicuculline; 63.1 ± 4.0 µm, n = 63, p < 0.001 for nimodipine; Figs. 1-7B, 1-7C1). In contrast to the axonal outgrowth, the total dendritic length of newborn GCs were not affected by any of TTX, bicuculline and nimodipine (228.1 ± 15.1 µm, n = 35 for control; 233.7 ± 16.6 µm, n = 40, p = 0.94 for TTX; 219.0 ± 13.0 µm, n = 50, p = 0.63 for bicuculline; 217.7 ± 16.5 µm, n = 37, p = 0.63 for nimodipine; Fig. 1-7C2). These results suggest that network activity-dependent GABA release and L-type VDCC-mediated Ca^{2+}-signaling specifically promotes the axonal outgrowth but not the dendritic outgrowth of the newborn GCs at
I note that bicuculline suppressed axonal outgrowth even though it enhanced overall network activity. It has been reported that GABA$_A$ receptor blockers increase the network activity of hippocampal slices *ex vivo* (Ikegaya, 1999; Nakahara *et al.*, 2009) and increase the expression of brain derived neurotrophic factor in the mossy fiber tract (Koyama *et al.*, 2004); this might affect the axonal outgrowth of newborn GCs. To study the effects of bicuculline on network activity, I simultaneously recorded mature and newborn GCs in current- and voltage-clamp mode, respectively. Bath application of bicuculline on the cultured hippocampal slice on DIV5-6 induced spontaneous epileptic-like activity in the mature GC. In contrast, bicuculline abolished the postsynaptic currents recorded at -60 mV in the newborn GC, except for small inward currents that might be caused by glutamate spillover (n = 3 pairs of GCs; Fig. 1-7D). This finding confirms the previous notion that GABA is the major neurotransmitter that conveys neural network activity to newborn GCs (Markwardt *et al.*, 2009; Markwardt *et al.*, 2011). Furthermore, this finding, together with the inhibitory effect of bicuculline on axonal outgrowth, implies that activation of GABA$_A$ receptors in the newborn GC is more important for the axonal outgrowth than the increase of network activity itself.
Recently, it has been reported that the axonal growth cones of immature hippocampal neurons express functional NMDA receptors (Wang et al., 2011). To test the possibility of NMDA receptor-mediated regulation of axonal outgrowth, I examined the effects of aminophosphonovaleric acid (APV) an NMDA receptor blocker. I found that it had no significant effect on axon outgrowth (145.3 ± 10.0 µm, n = 80, p = 0.57; Fig. 1-8), which implies that the axon outgrowth of newborn GCs in the slice culture is not associated with activation of NMDA receptors.

To investigate the possible downstream pathway of the calcium-dependent regulation of axonal extension in newborn GCs, I treated hippocampal slice cultures with STO-609, a calcium/calmodulin-dependent protein kinase kinase (CaMKK) inhibitor, for 48 hrs (DIV4-6). Consistent with a previous study in cortical neurons (Wayman et al., 2004), STO609 significantly suppressed axonal outgrowth (75.9 ± 4.8 µm, n = 89, p < 0.001; Figs. 1-7B, 1-7C). This result implies that the downstream components of Ca\(^{2+}\)-signal regulated axonal outgrowth in newborn GCs are similar to those of other cortical neurons.

It has been reported that spontaneous CaTs regulate axonal pruning (Singh & Miller, 2005; Hutchins & Kalil, 2008). Competition between axonal neurites at an immature stage culminates in the outgrowth of only one main
axon that reaches the end of the CA3 region (Amaral & Dent, 1981). I investigated the involvement of GABA-induced CaTs in axonal pruning. To quantify the axonal branches, I examined the branch indices (branch number/100 μm of total axonal length) of newborn GCs. Under control conditions, the branch index of newborn GCs was 0.61 ± 0.07 (n = 155; Fig. 1-7E). Compared to the control condition, the branch index was significantly increased by treatment with TTX (0.99 ± 0.11, n = 83, p < 0.01), bicuculline (0.90 ± 0.08, n = 104, p < 0.01), nimodipine (0.99 ± 0.16, n = 63, p < 0.05) or STO609 (1.24 ± 0.11, n = 89, p < 0.001) (Fig. 1-7E). The increased number of branch points suggests that the same signaling involved in axonal outgrowth facilitates competition between axonal neurites and consequent axonal pruning. Altogether, I conclude that calcium influx through L-type VDCCs is essential for GABA-induced Ca\textsuperscript{2+}-signaling, which is the mechanism by which hippocampal network activity promotes maturation of axonal arbors during the 1st post-mitotic week of newborn GCs.
Figure 1-1. Local puff application of fluorescein. A, z-projection image of fluorescein fluorescence during local puff of fluorescein (100 µM) through a patch pipette (< 2 psi). B, z-section image along the broken line in A. Scale bar: 10 µm in A. C, Fluorescence intensity slice (while line in B). Target GCs were positioned in the range indicated by the black line in the present study.
Figure 1-2. Normal development of the newborn GCs infected by retrovirus *in vivo*.

Retrovirus encoding GFP were stereotaxically injected into the dentate gyrus of P11 rats. Rats were sacrificed at 7, 14, 21 and 28 days post viral injection (dpi) as indicated. GFP-labeled GCs (white arrow) expressed immature neuronal marker, doublecortin (DCX, red), at 7, 14 dpi. After 14 dpi, GFP-labeled GCs (white arrow) expressed the mature neuronal marker, neuronal nuclei (NeuN, blue).
Figure 1-3. Morphological features of GFP+ newborn cells that are differentiated to DCX+ neurons and DCX- nonneurons ex vivo.
Figure 1-3. Morphological features of GFP+ newborn cells that are differentiated to DCX+ neurons and DCX- non-neurons ex vivo. 

A, Representative confocal images of GFP+ newborn cells (green) in an organotypic slice culture stained with DCX (red) are shown. DCX+ newborn cells (upper panel, white arrow) exhibited an apical dendrite and more slender axonal process on the opposite side. In contrast, DCX- newborn cell (lower panel, white arrow) showed multiple filopodia-like processes. Scale bar: 30 μm. B, Proportions of DCX+ and DCX- newborn cells from two independent slice cultures. C, Z-projection fluorescence images of all DCX- and GFP+ cells in a hippocampal cultured slice on DIV6. Note that DCX- and GFP+ cells had no or more than five primary processes with no dendrite-axon polarization, which is different from the DCX+ newborn cells. Scale bar: 30 μm. D, Summary of number of primary processes from total DCX- and GFP+ cells.
Figure 1-4. GABA<sub>A</sub> receptor activation is crucial for the CaTs evoked by perforant pathway stimulation in newborn GCs.
**Figure 1-4. GABA$_A$ receptor activation is crucial for the CaTs evoked by perforant pathway stimulation in newborn GCs.**

**A.** Representative confocal image of GFP+ GCs (green) located in the granule cell layer of a organotypic slice culture stained with DAPI (blue). Scale bar: 30 μm. **B.** The newborn GC was loaded with OGB1 by single cell electroporation. Insert (bottom right) shows the higher magnification image of the axonal growth cone from the boxed region. Scale bar: 10 μm, 2μm (insert). **C and D,** Top panels show representative time-lapse confocal images of the soma (C) and the growth cone (D) are shown. Stimulation of perforant pathway was started at 0 s. Bottom traces show corresponding fluorescence intensity profiles of the newborn GC. The CaTs evoked by stimulation of perforant pathway were abolished by bath application of bicuculline (gray trace). Black arrows indicate the time point at 0 s for time-lapse confocal images in top panels. **E,** Summary for the peak fluorescence intensity before and after bicuculline application. Data were obtained from four rats. Mean ± SEM, **p < 0.01,** paired $t$ test. **F,** Representative GABAergic postsynaptic current of the newborn GC evoked by single stimulation of perforant pathway. It was abolished by bath application of bicuculline (gray trace).
Figure 1-5. Direct local puff application of GABA elicited the increase of intracellular calcium ([Ca^{2+}]_{i}) in the immature GCs. A, The immature GC in the subgranular zone of an acute hippocampal slice was loaded with OGB1 by single cell electroporation. Insert (bottom right) show the higher magnification view of the axonal growth cone from the boxed region. Scale bar: 10 μm, 2 μm (insert). B and C, Representative fluorescence intensity profiles from the soma and the growth cone of the immature GC are shown. The horizontal line above each trace indicates the period of puff application. Puff application of GABA (5 μM) induced CaTs (black traces), and it was abolished by bath and puff application of bicuculline (50 μM, gray traces in
B). TTX (0.5 μM, gray) did not block the GABA-induced CaTs in the soma (left) and the growth cone (right). D, Summary for the peak fluorescence intensity before and after bicuculline application. Data were obtained from six rats. Mean ± SEM, ** p<0.01, *** p<0.001, paired t test.
Figure 1-6. The effects of calcium channel blockers on the GABA-induced CaTs of immature GCs. A-D. Representative fluorescence intensity profiles from the soma of the immature GC show the GABA-induced CaTs before and after bath and puff application of nickel (A), mibebradil (B), nimodipine (C), or Bay K8644 (D). GABA (5 μM) or GABA plus a blocker was puffed during the period indicated by the horizontal line above each trace. E, Summary for relative changes of the peak fluorescence intensity after drug application. Number of cells and rats (inside parenthesis) that were examined for each condition is indicated. Mean ± SEM, ** p<0.01, *** p<0.001 versus before drug application, paired t test. N.S. means statistically non-significant.
Figure 1-7. Involvement of GABA-induced Ca^{2+} signaling in the axonal outgrowth and pruning of newborn GCs.
Figure 1-7. Involvement of GABA-induced Ca\textsuperscript{2+} signaling in the axonal outgrowth and pruning of newborn GCs. A, z-projection fluorescence image of GFP+ GCs in a hippocampal cultured slice on DIV6 was made from z-stack confocal images spanning 30 μm (left panel). The image of the GCs was reconstructed by tracing each GC over the z-stack confocal images. Note that the newborn GCs in slice culture are located in the dentate-hilus border (black broken line). B, Representative images of newborn GCs reconstructed from the cultured slices treated for 48 hrs (DIV4-6) with a drug indicated below in each panel. Scale bar: 30 μm. C1, Cumulative distributions of the lengths of the longest axon of individual newborn GCs obtained from the control slice (red) and the slice treated with TTX (blue), bicuculline (gray), nimodipine (green) or STO609 (magenta). Data under each condition were obtained from six slices cultured from three rats. * p<0.05, *** p<0.001, Kolmogorov-Smirnov test. C2, Cumulative distributions of the total dendritic lengths of individual newborn GCs obtained from the control slice (red) and the slice treated with TTX (blue), bicuculline (gray) or nimodipine (green). D, Effect of bicuculline on the spontaneous post-synaptic activities in newborn GC (upper green trace) and in mature GC (lower black trace). Newborn and mature GCs were recorded in voltage clamp mode at -60 mV holding potential and in current clamp.
mode simultaneously. Bicuculline abolished most post-synaptic current in the newborn GC except small slow inward currents, while it induced the epileptiform activity in mature GC. E, Axonal branch index of newborn GCs under different conditions (Con, control; BMI, bicuculline; Nimo, nimodipine; STO, STO609). * p<0.05, ** p<0.01, *** p<0.001, Student t test.
Figure 1-8. NMDA receptor blocker, APV, had no effect on the axonal outgrowth of newborn GCs. A, Representative GC images reconstructed from the cultured slices treated with APV for 48 hrs (DIV4-6). Scale bar: 30 µm. B, Cumulative distributions of lengths of the longest axonal neurite of individual newborn GCs from control (red) and APV (blue) conditions.
**Table 1**

Comparison of passive electrical properties between newborn GCs of culture slices and immature GCs of acute slices

<table>
<thead>
<tr>
<th></th>
<th>Newborn GCs in slice culture (n = 8)</th>
<th>Immature GCs in acute slice (n = 10)</th>
<th>Statistical significance</th>
</tr>
</thead>
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<tr>
<td>RMP (mV)</td>
<td>-54.2 ± 3.1</td>
<td>-58.2 ± 2.4</td>
<td>0.33</td>
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<tr>
<td>Input resistance</td>
<td>2.6 ± 0.3</td>
<td>3.1 ± 0.3</td>
<td>0.34</td>
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<tr>
<td>(GΩ)</td>
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<tr>
<td>Capacitance (pF)</td>
<td>19.6 ± 3.9</td>
<td>24.6 ± 4.8</td>
<td>0.44</td>
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</table>

Mean values ± SEM are presented. Statistical significance was determined by Student t test.
Discussion

In this study, I present two novel findings on the development of newborn GCs during the 1st post-mitotic week. First, L-type VDCCs are essential for Ca\textsuperscript{2+}-signaling induced by GABA, and this signaling conveys hippocampal network activity to newborn GCs. Second, GABA-induced Ca\textsuperscript{2+}-signaling promotes axon outgrowth and pruning in newborn GCs. The first idea is supported by following lines of evidence: 1) stimulation of PP elicited CaTs in the somata and growth cones of newborn GCs, and these CaTs were abolished by bicuculline (Fig. 1-4E); 2) GABA-induced CaTs were most strongly inhibited by L-type VDCC blockers (Fig. 1-6). In support of the second idea, I demonstrated that inhibiting any of the signaling pathway, network activity → GABA → L-type VDCCs, suppressed axonal outgrowth and pruning of newborn GCs. The involvement of L-type VDCCs downstream of GABA signaling is in line with previous reports that GABA-induced CaTs mediated by L-type VDCCs promote neuritogenesis in the early developmental stage of different types of neurons (Tapia et al., 2001; Borodinsky et al., 2003; Gascon et al., 2006; Sernagor et al., 2010); this implies that the Ca\textsuperscript{2+}-signaling involved in the regulation of axonal maturation is not substantially different between newborn GCs and other
type of neurons (Esposito et al., 2005; Sernagor et al., 2010).

**Role of L-type VDCCs in GABA-induced Ca\(^{2+}\) signaling**

I showed that PP stimulation increases [Ca\(^{2+}\)]\(_i\) of newborn GCs via activation of GABA\(_A\) receptors. Although it is well known that an enriched environment (van Praag et al., 2005; Tashiro et al., 2007) or direct electrical stimulation (Bruel-Jungerman et al., 2006; Kitamura et al., 2010; Stone et al., 2011) promotes survival of newborn neurons *in vivo*, the underlying mechanisms are controversial (Olson et al., 2006; Kempermann et al., 2010). Neurotrophic factors or extra-synaptic glutamate released from mature GCs have been suggested to mediate the enhanced survival of newborn GCs caused by direct electrical stimulation during the first post-mitotic week (Bruel-Jungerman et al., 2006; Kitamura et al., 2010). In contrast, depolarizing GABA signals, which lead to phosphorylation of cAMP response element-binding protein (CREB) have been suggested to be an important factor for the survival of 2-weeks-old GCs (Jagasia et al., 2009). In the present study, I showed that CaTs can be induced directly by GABA in newborn GCs. Given that Ca\(^{2+}\)-signaling is essential for the survival of newborn neurons (West et al., 2002), my result implies that this Ca\(^{2+}\)-dependent signaling pathway can be activated by network activity-dependent
GABA release in the early developmental stage before newborn GCs receive glutamatergic inputs.

Results of the present study indicate that L-type VDCCs are the major the calcium influx pathway activated by GABA in the rat newborn GCs (Fig. 1-6). L-type VDCCs have been suggested to regulate neurogenesis in hippocampal neural progenitor cells (Deisseroth et al., 2004) and mediate Ca^{2+}-signaling in migrating newborn neurons of the subependymal layer (Darcy & Isaacson, 2009). However, no study has suggested that L-type VDCCs play a primary role in Ca^{2+}-signaling in newborn GCs. The present study demonstrates that activation of L-type VDCCs triggered by GABAergic inputs regulates axonal neurite outgrowth. This result is in line with previous studies that observed similar phenomena in newborn interneurons of the olfactory bulb and cultured cerebellar GCs (Borodinsky et al., 2003; Gascon et al., 2006).

**Involvement of T-type VDCCs**

Although Ni^{2+} has been proposed as a preferential blocker of T-type VDCCs, only α1H among three T-type VDCCs have exceptionally low IC_{50} (13 μM), whereas IC_{50} values for other T-type subunits are comparable to that of α1C, one of L-type VDCC subunits (127 μM for α1C, Zamponi et al.,...
1996; 216 and 250 μM for α1I and α1G; Lee et al., 1999b). Therefore, it needs great care to interpret the effect of Ni²⁺ on Ca²⁺ signals. Although 50 μM Ni²⁺ reduced the peak amplitude of CaTs by 30%, I have shown that other T-type VDCC blockers such as mibefradil and NADA had little effect on GABA-induced CaTs, implying that the Ni²⁺ effect might be due to block of L-type VDCCs. In contrast to T-type blockers, all the L-type VDCC blockers that I tested (nifedipine, nimodipine, and diltiazem) effectively attenuated GABA-induced CaTs. Finally, Bay K8644, an L-type VDCC activator, greatly potentiated GABA-induced CaTs. These findings are in contrast to previous studies which suggested that the T-type VDCC has an important role in mediating calcium influx in immature GCs (Schmidt-Hieber et al., 2004; Tozuka et al., 2005). This idea, however, is not based on the study of GABA-induced CaTs but on the observation that 50 μM Ni²⁺ abolished the Ca²⁺-spike induced by direct current injection into immature GCs (Schmidt-Hieber et al., 2004). Although there is one report of Ni²⁺-sensitivity in GABA-induced Ca²⁺-signaling (Tozuka et al., 2005), that study was performed in type-2 hippocampal progenitor cells, which are in a different developmental stage from the GCs in the present study, and effect of other T-type blockers are not proven.

I measured the resting membrane potential of newborn GCs as -54.2 ± 3.1
mV in the present study. This value is similar to the previous report on the resting membrane potential (RMP) of immature GCs at the developmental stage of Class 2-II (Ambrogini et al., 2004), which display morphological and passive electrical properties similar to those in the present study (Fig. 1-4B and Table 1). Compared with the RMP of rat immature GCs, more hyperpolarized RMP values have been reported for mouse immature GCs by Schmidt-Hieber et al (2004; -75 mV) and by us (-77 mV after liquid junction potential correction; Lee et al., 2009). Although Esposito et al (2005) reported a more depolarized RMP value for the mouse immature GCs (-45 mV), this value was measured by microelectrode techniques, and thus cannot be directly compared with results from other whole-cell patch clamp studies. Considering that most of T-type VDCCs are inactivated at the RMP of rat immature GCs (-58.2 ± 2.4 mV, Lee et al., 1999a), the depolarized RMP may one of reason for the low contribution of T-type VDCCs to the CaTs induced by puff application of GABA in the rat immature GCs. Contribution of T-type VDCCs, however, might be different in the mouse immature GCs which exhibit more hyperpolarized RMP. A caveat of this idea, however, is that the measured RMP value is prone to be more depolarized than the real value, because it is highly dependent on the seal resistance especially when the input resistance of the cell is on the same order as the seal resistance of the
recording as in immature GCs.

Finally, it should be noted that still appreciable amount of Ca\(^{2+}\) influx remained even in the presence of L-type blockers, suggesting that calcium influx pathways other than L-type VDCCs may be activated by GABA. Given that the affinity of blockers for VDCCs can be affected by co-expressed accessory subunits (Zamponi et al., 1996), the concentration of blockers used in the present study may not be sufficient to block the target VDCCs in the newborn GCs.

**The role of calcium influx in axon outgrowth**

I showed that axonal outgrowth and pruning of newborn GCs are regulated by the GABA-induced calcium signaling. Although all of GFP+ newborn cells are not differentiated to neuron, the majority of them (83.5%) were differentiated to GCs judging from DCX immunoreactivity (Fig. 1-3). Moreover, non-neuronal newborn cells displayed distinct morphological features from DCX+ young GCs (see Results). Since I excluded the newborn cells that did not meet the morphological criteria for newborn neuron described in Results from the analysis of axonal outgrowth and pruning, it is plausible to assume that the measurement of axonal length was generally taken from newborn GCs.
The causal link between calcium signaling and axon extension has been well studied in cultured cortical neurons. The CaMKK-CaMKI cascade has been suggested to be a downstream component of the Ca\(^{2+}\) signaling that regulates the axonogenesis (Wayman et al., 2004; Ageta-Ishihara et al., 2009). Consistent with this, I observed that treatment of the slice with STO609, a CaMKK inhibitor, for 48 hrs severely suppressed axonal outgrowth from newborn GCs, suggesting that the downstream mechanisms of GABA-induced Ca\(^{2+}\) signaling in newborn GCs are similar to those of cortical neurons.

Previous \textit{in vitro} studies support the idea that axon elongation requires a redistribution of limited resources to an elongated axon fiber (Singh & Miller, 2005; Singh et al., 2008b). Consistently, I found that branching index was higher in the GCs where axonal outgrowth was inhibited (Fig. 1-7E). Given that the mossy fiber of a mature GC displays extensive ramification within the hilar region, one can raise a question whether competition between axonal branches, which is important for axon outgrowth, occurs within the hilar region. It should be noted, however, that a dense fasciculus of mossy fibers is first found in the stratum lucidum of CA3 field before mossy fibers make an extensive ramification within the hilus in the postnatal hippocampus (Amaral & Dent, 1981). This finding implies that
axonal outgrowth precedes the hilar branching during the developmental course of mossy fibers. Consistent with the report, Raineteau et al. (2006) reported that branches of axon in DIV20 were higher than that in DIV10 when the axonal tip of newborn GCs was already reached the end of CA3 region. In light of these findings, axonal competition between mossy fiber branches may occur in the GCs in the present study. The inverse alterations of branching index and axonal outgrowth by inhibition of Ca\(^{2+}\)-signaling suggest that GABA-mediated Ca\(^{2+}\)-signaling promotes the axonal competition and outgrowth in the DIV4-6 GCs.

To my surprise, I found that GABA-induced Ca\(^{2+}\) signaling selectively promotes outgrowth of axon, but not of dendrite in the newborn GCs at DIV4-6. This result was unexpected because GABAergic depolarization was necessary for dendritic arborization of immature GCs in the second postmitotic week (Ge et al., 2006). The discrepancy may be caused by differences in the developmental stage of the GCs (1st vs. 2nd post-mitotic week), in the experimental conditions (ex vivo vs. in vivo) and/or in the age of the rat (early postnatal vs. adult). Similar to the present study, however, the axon-specific effect of GABA-induced Ca\(^{2+}\) signaling has been previously demonstrated in immature cortical neurons. It has been shown that GABA specifically stimulate axonal outgrowth via CaMKI\(\alpha\), while
BDNF selectively promotes the dendritic outgrowth via CaMKIγ (Takemoto-Kimura et al., 2007; Ageta-Ishihara et al., 2009). These studies together with the present study were performed for neurons in the earlier developmental stage than Ge et al. (2006), and thus imply that GABA-induced Ca\(^2+\) signaling promotes specifically axonal outgrowth rather than the dendritic outgrowth of the newborn GCs in the first post-mitotic week.

I showed that PP stimulation or local puff administration of GABA to the somata elicits CaTs simultaneously in the somata and growth cones of newborn GCs. Previously, it has been shown that sub-threshold dendritic depolarization propagates to mossy fiber boutons even in mature GCs, which have low input resistance (Alle & Geiger, 2006). Considering the high input resistance of immature GCs (Ambrogini et al., 2004; Schmidt-Hieber et al., 2004; Lee et al., 2009), it is highly plausible that concurrent depolarization in the growth cone is caused by electrotonic conduction of GABA-induced somatic depolarization. CaTs in the growth cones of dissociated cortical neuron are an important regulator of axonal outgrowth, and they are mediated by L-type calcium channels (Kater & Mills, 1991; Henley & Poo, 2004; Hutchins & Kalil, 2008). Thus, activity-dependent CaTs in the growth cone may participate in competing axon outgrowth of immature GCs. In support of this view, blocking CaTs or a downstream calcium-dependent
kinase not only suppressed axonal growth in newborn GCs but also impaired pruning of axonal branches. Therefore, I conclude that network activity-dependent tonic and phasic GABAergic inputs to newborn GCs may induce Ca^{2+} influx, not only in somata but also in axonal growth cones, and thereby regulate axonal outgrowth and pruning.

Axonal outgrowth and dendritic arborization are essential for newborn GCs to reach their postsynaptic target and be integrated into pre-existing neural circuits; this integration may be a pre-requisite for the cell survival. The present study provides compelling evidence for the idea that GABA-induced Ca^{2+} signals mediate network-dependent regulation of axonal outgrowth.

**Comparison of the present study with adult neurogenesis**

Recently, the development of newborn GCs has been studied in the context of adult neurogenesis. Because the present study dealt with newborn GCs labeled in organotypic slice cultures taken from the early postnatal rat hippocampus, it remains to be addressed whether my findings are correct for newborn GCs in the adult hippocampus. I have already mentioned two discrepancies between my findings and previous ones from adult hippocampus. First, GABAergic synapse formation of newborn GCs that I
studied was earlier than that of newborn GCs in the adult hippocampus (Esposito et al., 2005). The second one was the effect of blocking GABAergic signaling in the dendrite arborization of the newborn GCs. These discrepancies may arise from the age of rat (Zhao et al., 2006) and/or the experimental conditions (ex vivo vs. in vivo). However, it has been reported that the development of newborn GCs in the adult hippocampus recapitulate the embryonic development (Esposito et al., 2005). Moreover, maturation of newborn GCs in the organotypic slice culture is comparable or slightly delayed in comparison to that in age-matched in vivo conditions (Namba et al., 2007). The trisynaptic circuits of hippocampus are conserved in the organotypic culture (Raineteau et al., 2006), and I showed that newborn GCs labeled ex vivo were normally integrated in the pre-existing circuit (Figs. 1-4F, 1-7D). Furthermore, I showed that the electrophysiological property of newborn GCs labeled in vitro was similar to that of immature GCs in acute slices from postnatal rats (Table 1). Therefore, newborn GCs labeled in the slice culture could be a useful ex vivo model for studying the maturation mechanism of newborn GCs.
CHAPTER 2

CaV1.3 is essential for GABAergic synapse formation in newborn granule cells
Introduction

GABA is a key player in the development of newborn granule cells (GCs). It is well established that GABA depolarizes immature neurons which display high cytosolic [Cl\(^-\)] (Ben-Ari et al., 1989). GABA-induced depolarization and consequent calcium signaling are essential for the normal development of newborn GCs (Ge et al., 2006). GABAergic synapse rather than ambient GABA are the primary source of GABAergic inputs to newborn GCs at least during the second post-mitotic week (Esposito et al., 2005; Markwardt et al., 2011). Therefore, GABAergic synapse formation is a crucial step for the developmental of newborn GCs.

Single cell knock-out of BDNF in cortical neurons revealed that BDNF released from postsynaptic target neurons promotes the formation or maturation of GABAergic synapses through paracrine actions (Kohara et al., 2007). BDNF released from postsynaptic neurons promotes maturation of GABAergic synaptic terminals (Fiorentino et al., 2009) and stabilize the surface expression of GABA\(_\Lambda\)-R (Porcher et al., 2011). BDNF is secreted from neurons in response to high levels of electrical activity that elevates cytosolic calcium (Wetmore et al., 1991; reviewed in Gottmann et al., 2009; Leßmann & Brigadski, 2009), Such calcium signaling is triggered by
calcium influx through L-type VDCC or NMDA-receptor (NMDA-R) (Kolarow et al., 2007). NMDA-R may not be essential for maturation of newborn GCs in the first four post-mitotic weeks because the newborn GC–specific NR1 knock-out did not affect the maturation of newborn GCs (Tashiro et al., 2006). L-type VDCCs have been proposed to mediate the positive feedback loop between GABA$_{A}$-R activation and regulated secretion of BDNF (Porcher et al., 2011). In Chapter 1, I described that L-type VDCCs are the major calcium influx pathway in newborn GCs probed by GABA puff experiments.

Two major L-type VDCCs expressed in central neurons are CaV1.2 and CaV1.3 (Hell et al., 1993). Newborn GCs in early post-mitotic stage prior to GABAergic synapse formation express non-synaptic GABA$_{A}$-Rs. Such nonsynaptic GABA-Rs are expected to sense lower concentration of GABA than synaptic GABA$_{A}$-Rs, because they will sense ambient GABA or transient release of GABA from potential presynaptic partners (Lohmann & Bonhoeffer, 2008). These GABAergic inputs may cause only a weak depolarization in newborn GCs. CaV1.3 is activated by lower depolarization than CaV1.2, and exhibits different sensitivity to dihydropyridines (Xu & Lipscombe, 2001). Recently, it has been reported that CaV1.3 interacts with RyR2 in hippocampal neurons (Kim et al., 2007a) and GABA$_{B}$-R (Park et al.,...
Furthermore, activation of GABA$_B$-R in immature hippocampal neurons induces calcium signaling and secretion of BDNF (Kuczewski et al., 2011). These circumstantial pieces of evidence raise a possibility that CaV1.3 may be a major player for calcium influx pathway that triggers BDNF secretion and GABA synapse formation in a newborn GC that receives non-synaptic GABAergic inputs. Here, I tested the role of CaV1.3 in the formation of GABAergic synapses onto newborn GCs in the postnatal hippocampus.
Materials and Methods

Organotypic slice culture. All experiments were performed with the approval of the animal experiment ethics committee at the Seoul National University College of Medicine (approval Nos. SNU-091001-1 and SNUMC IBC 08-001). The enorhino-hippocampi of postnatal day 7-10 (P7-P10) Sprague Dawley rats (Orient Charles River Co., Seoul, Korea) were cultured using membrane interface techniques as described in Chapter 1.

Labeling newborn GCs with retrovirus. I used VSV-G pseudotyped recombinant retrovirus encoding GFP and shRNA under the control of U6 promoter in same vector (Fig. 2-1A). The following short hairpin sequences were cloned into the retroviral vector using restriction enzyme HpaI/XhoI: TAAATATGTCAAATGGTTTCC (CaV1.3-targeting shRNA, shCaV1.3); TAAGGCTA TGAAGAGATAC (non-targeting shRNA, shNT). The highly concentrated virus solution were prepared and delivered onto a hippocampal slice culture as previously described in Chapter 1.

Electrophysiology. Slices were placed in a chamber under an upright microscope (BX51WI; Olympus, Tokyo, Japan). The chamber was perfused
with normal artificial CSF (aCSF) containing (in mM) 124 NaCl, 26 NaHCO₃, 3.2 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1.25 NaH₂PO₄, and 10 glucose with the pH adjusted at 7.4 by saturation with carbogen (95% O₂, 5% CO₂). GFP+ cells were identified by a fluorescence image visualized using a CMOS camera (ZTD33CD, ZOOTS Corporation, Korea). Whole-cell patch recording was performed using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany) and Pulse software (version 8.67) as previously described. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a two-stage puller (PC-10; Narishige, Japan). To evoke synaptic responses, a bipolar tungsten electrode (WPI, TM53CCINS-05B) was placed in the medial perforant pathway (200 μs duration; 5-20 V intensity). The lateral distance of the stimulating electrode was at least 500 μm away from the recorded cell. Post-synaptic currents and GABA-induced currents were recorded in newborn GCs using a patch pipette (3-4 MΩ) filled with a solution containing (in mM) 140 CsCl, 1 MgCl₂, 10 HEPES, and 4 Na₂ATP (pH 7.2 with CsOH). To measure calcium currents, the chamber was perfused with aCSF containing (in mM) 10 TEACl, 5 CsCl, and 0.5 μM TTX. Patch pipettes were filled with a solution containing (in mM) 110 Cs-gluconate, 20 tetraethylammonium-Cl, 10 NaCl, 20 HEPES, 10 BAPTA, 4 Mg-ATP, 5 Na₂-phosphocreatine.
Cytosolic Ca\(^{2+}\) measurements. Ca\(^{2+}\)-indicator dye, Oregon Green Bapta-1 (OGB-1, 1 mM dissolved in distilled water, Molecular Probes, Eugene, OR), was introduced into newborn GCs using single cell electroporation as previously described in Chapter 1. Ca\(^{2+}\) imaging was performed using a confocal laser-scanning system (FV300; Olympus) with a 60x water-immersion objective (numerical aperture 0.9, Olympus). To imaging CaTs evoked by local application, I scanned a region of interest at 5 Hz for 20 s. Fluorescence values were averaged over an area of interest selected within the fluorescence image. Relative intracellular Ca\(^{2+}\) levels were expressed as the change in fluorescence divided by the resting fluorescence (ΔF/F\(_0\)) which was calculated as previously described in Chapter 1. All representative traces of CaTs are averages of more than three trials.

Local puff application of drugs. For puff application of GABA or KCl, GABA was dissolved in aCSF, and KCl was replaced with NaCl in aCSF. A puffer pipette was placed at 40-50 μm away from the cell soma. GABA was applied via the pipette with a pressure of < 2 psi (Pressure System Ile; Toohey Company, Fairfield, USA). All chemicals were obtained from Sigma or Tocris, Stock solutions were stored at -20°C and diluted to the desired
concentration in aCSF just before use.

*Morphological analysis and immunohistochemistry.* I analyzed the morphology of newborn GCs as previously described in Chapter 1. In brief, at 6 days in vitro (DIV 6), slices were fixed in 4% paraformaldehyde, and imaged using a confocal laser-scanning system with a 40X water-immersion objective (NA 0.8, LUMPlan FI/IR, Olympus). For immunostaining, I processed the slices as previously described (Lee et al., 2012) and used the following antibody: goat anti-CaV1.3 (Santa Cruz, 1:400), Cy5-conjugated donkey anti-goat secondary antibody (Jackson, 1:500). I analyzed the morphology of GFP+ cells by using ImageJ as previously described in Chapter 1.

*Westen blot analysis.* To validate the specificity and efficiency of the shRNAs, retroviral shRNA vectors and an expression construct for CaV1.3 (generously provided by D. Lipscombe) were co-transfected into HEK293 cells. Cell lysates were prepared for Western blot analysis of CaV1.3 expression using anti-CaV1.3 (Alomone labs, 1:200) as previously described (Lim et al., 2009).
Data analysis. Data were analyzed using Igor-Pro (version 6.2; WaveMetrics, Lake Oswego, OR). Statistical data are expressed as the mean ± SEM, and \( n \) indicates the number of cells or growth cones studied. Statistical analyses were performed using Student’s \( t \) tests. The Kolmogorov-Smirnov test was used to compare the length of neurites of the newborn GCs. The two-factor ANOVA test was used to compare the input-output curves of GPSCs.
Results

I used a retroviral approach to label newborn granule cells (GCs) of hippocampal slice culture. To knock down the expression of endogenous CaV1.3 in newborn GCs, I engineered retroviral constructs that co-express GFP and short-hairpin RNA (shRNA; Fig. 2-1A). CaV1.3 heterologously expressed in HEK293 cells was effectively knocked down by co-transfection of shRNA targeted to CaV1.3 (shCaV1.3) but not by that of non-targeting shRNA (shNT) (Fig. 2-1B). Because the granule cell layer and the inner molecular layer are immunoreactive for anti-CaV1.3 antibody (Hell et al., 1993; Leitch et al., 2009), we compared the expression of CaV1.3 in the proximal dendrite within 10 µm from soma. The CaV1.3 immunofluorescence in the proximal dendrite (F_{GFP+}) of the newborn GCs expressing shCaV1.3 (shCaV1.3/GFP+ cells) was significantly lower than that of the adjacent GFP negative GCs (F_{GFP-}) (F_{GFP+}/F_{GFP-} = 44.1 ± 2.8%, n = 15, p < 0.001; Fig. 2-1C), whereas that in newborn GCs expressing shNT (shNT/GFP+ cells) was not lower than the adjacent GFP negative GCs (F_{GFP+}/F_{GFP-} = 101.2 ± 2.5%, n = 22, p = 0.63; Fig. 2-1C), indicating that the expression of CaV1.3 was reduced by shCaV1.3 in newborn GCs. To examine the knockdown of CaV1.3 functionally, I characterized the calcium
current of newborn GCs. Calcium currents were recorded in shNT/GFP+ cells and shCaV1.3/GFP+ cells (Figs. 2-1D, 2-1E). To my surprise, the peak current amplitude-potential relationship (IV curve) was indistinguishable between two groups (n = 14 of shNT/GFP+ cells, n = 13 of shCaV1.3/GFP+ cells; Fig. 2-1E). To test possibility for compensatory up-regulation of CaV1.2 in shCaV1.3/GFP+ cells, I examined the calcium current sensitive to low concentration nimodipine (2 μM), which blocks CaV1.2 channels but barely blocks CaV1.3 channels (Xu and Lipscombe, 2001). The amplitudes of the nimodipine-sensitive current were significantly larger in shCaV1.3/GFP+ cells than in shNT/GFP+ cells, indicating that the proportion of CaV1.2 channels in shCaV1.3/GFP+ cells is higher than in shNT/GFP+ cells (n = 10 of each group; Fig. 2-1F). Above results suggest that my retroviral vector effectively knocked down the CaV1.3 gene in newborn GCs. Nevertheless, I found no significant difference in the I-V relationship of calcium current between shCaV1.3/GFP+ cells and their non-targeting control cells.

Next, I examined the effect of CaV1.3 depletion on the GABA-induced Ca\(^{2+}\) signaling. To test whether CaV1.3 is essential for GABA-induced Ca\(^{2+}\) transients (CaTs), I compared it with KCl-induced CaTs in the same newborn GCs. The peak [Ca\(^{2+}\)]\(_i\) increment (estimated as ΔF/ΔF\(_0\)) induced by a local puff
of GABA (5 μM) was comparable to that by high concentration KCl (60 mM) in shNT/GFP+ cells (soma: ΔF/F₀ = 0.48 ± 0.09 by high KCl, ΔF/F₀ = 0.40 ± 0.12 by GABA, ΔF_{GABA}/ΔF_{KCl} = 0.759 ± 0.092, n = 9, p = 0.11; growth cone: ΔF/F₀ = 0.77 ± 0.14 by high KCl, ΔF/F₀ = 0.80 ± 0.25 by GABA, ΔF_{GABA}/ΔF_{KCl} = 0.924 ± 0.109, n = 8, p = 0.84; Figs. 2-2A, 2-2C). In contrast, the peak amplitude of GABA-induced CaTs was significantly smaller than high KCl-induced CaTs measured in the same shCaV1.3/GFP+ cells (soma: ΔF/F₀ = 0.48 ± 0.04 by high KCl, ΔF/F₀ = 0.11 ± 0.03 by GABA, ΔF_{GABA}/ΔF_{KCl} = 0.274 ± 0.087, n = 10, p < 0.001; growth cone: ΔF/F₀ = 0.73 ± 0.13 by high KCl, ΔF/F₀ = 0.13 ± 0.05 by GABA, ΔF_{GABA}/ΔF_{KCl} = 0.149 ± 0.034, n = 7, p < 0.001; Figs. 2-2B, 2-2C).

The peak [Ca^{2+}]_i increment induced by high KCl in shCaV1.3/GFP+ cells was not different from that in shNT/GFP+ cells (p = 0.97 at somata, p = 0.97 at growth cone). This result is consistent with my finding that shCaV1.3 did not significantly affect the total Ca^{2+}-current. Even if depolarization induced by GABA is lower than that by KCl, the reduced Ca^{2+}-response to GABA in shCav1.3/GFP+ cells cannot be explained by difference in the activation threshold between CaV1.2 and CaV1.3, because the knock-down of CaV1.3 had little effect on the I-V relationship of total calcium current (Fig. 2-1E). Thus, my finding that the compensatory up-regulation of CaV1.2 channels
does not rescue the GABA-induced CaTs in shCaV1.3/GFP+ cells suggests a unique role of CaV1.3 in the expression of GABA$_A$-Rs or early GABAergic synapse formation in newborn GCs.

I hypothesized that the expression of functional GABA$_A$ receptors may be decreased in shCaV1.3/GFP+ cells. To test this hypothesis, I compared the current amplitude evoked by local application of GABA (200 μM) in shCaV1.3/GFP+ cells to that in shNT/GFP+ cells under the whole-cell patch clamp conditions with high chloride internal solution at -60 mV. Fig. 2-3A shows the representative GABA-induced currents recorded in the two groups. The peak amplitude of GABA-induced current in shCaV1.3/GFP+ cells (-0.53 ± 0.06 nA, -82.8 ± 10.2 pA/pF, n = 17) was significantly lower than that in shNT/GFP+ cells (-1.06 ± 0.09 nA, -255 ± 43.1 pA/pF, n = 13, p < 0.001; Fig. 2-3B), indicating the lower expression of GABA$_A$ receptors is responsible for the reduced GABA-induced CaTs in shCaV1.3/GFP+ cells.

I further studied whether such low expression of GABA$_A$ receptors is associated with defects in the GABAergic synapse formation in shCaV1.3/GFP+ cells. To this end, I examined the GABAergic postsynaptic currents (GPSCs) of the newborn GCs evoked by stimulation of perforant pathway (PP). Input-output curves of GPSCs revealed that GPSCs of shCaV1.3/GFP+ cells were almost abolished, and significantly lower than
that of shNT/GFP+ cells (Fig. 2-3D, $F_{(1,249)} = 39.71; p < 0.001$). These results suggest that the expression of CaV1.3 is necessary not only for the surface expression of GABA$\alpha$ receptors but also for the formation of GABAergic synapses on the newborn GCs.

It has been reported that BDNF release by Ca$^{2+}$ influx through L-type VDCC is necessary for GABAergic synapse formation in developing neurons (Kuczewski et al., 2011). Given that BDNF has a similar role in the newborn GCs, I hypothesized that lack of BDNF release may be responsible for the low GPSCs in shCaV1.3/GFP+ cells. To test this hypothesis, I treated the organotypic culture with BDNF (25 ng/mL) for DIV 4-6. The treatment of BDNF effectively enhanced the GPSC not only in shCaV1.3/GFP+ cells (Fig. 2-3D, $F_{(1,239)} = 39.71; p < 0.001$) but also in shNT/GFP+ cells (Fig. 2-3D, $F_{(1,199)} = 20.23; p < 0.001$). Consistent with my hypothesis, the input-output curves of GPSC were not significantly different between BDNF-treated shNT/GFP+ cells and BDNF-treated shCaV1.3/GFP+ cells ($F_{(1,189)} = 0.17; p = 0.68$), indicating that the defect in GABAergic synapse formation caused by the lack of CaV1.3 is fully rescued by supplement of exogenous BDNF. These results imply that CaV1.3 plays a major role in triggering the secretion of BDNF.

Newborn GCs of the first postmitotic week is characterized by a rapid
axonal outgrowth towards CA3 (Hastings & Gould, 1999). I have shown that GABA-induced Ca\textsuperscript{2+}-signaling promotes axonal outgrowth in DIV5-6 newborn GCs in Chapter 1. Given that depletion of CaV1.3 severely suppresses the GABA-induced Ca\textsuperscript{2+}-signaling in the newborn GCs, it is predicted that the axonal growth might be impaired in shCaV1.3/GFP+ cells. Examining the longest axonal length of shNT/GFP+ cells and shCaV1.3/GFP+ cells, I found that depletion of CaV1.3 significantly suppressed the axonal outgrowth [74.7 ± 4.3 μm (n = 98) vs. 145.1 ± 7.2 μm (n = 119), p < 0.001; Figs. 2-4A, 2-4B]. The branch index of shCaV1.3/GFP+ cells was significantly larger than that of shNT/GFP+ cells (1.18 ± 0.09 vs 0.64 ± 0.07, p < 0.001; Fig. 2-4C). I noted that GABAergic inputs to newborn GCs mediate the network activity-dependent facilitation of axonal outgrowth in Chapter 1. Consistent with this idea, silencing the network activity by treatment with TTX for 48 hrs did not further suppress the axonal outgrowth of the shCaV1.3/GFP+ cells (78.1 ± 9.2 μm, n = 63, p = 0.22 compared to shCaV1.3/GFP+ cells in absence of TTX, Fig. 2-4B), and did not significantly alter the branch index either (1.37 ± 0.15, p = 0.31 compared to shCaV1.3/GFP+ cells in absence of TTX, Fig. 2-4C). Since the effect of shCaV1.3 on GPSC was rescued by BDNF, I tested whether BDNF is also able to rescue its effect on the axonal outgrowth. The axonal
outgrowth of the shCaV1.3/GFP+ cells was significantly increased by
treatment of BDNF in dose-dependent manner (97.1 ± 6.4 μm in 25 ng/ml, p < 0.01, 119.5 ± 7.7 μm in 100 ng/ml, p < 0.001 compared to
shCaV1.3/GFP+ cells in absence of BDNF, Fig. 2-4B). The branch index
was also decreased by treatment of BDNF at 25 ng/ml, although it did not
reach statistical significance at higher dose (0.80 ± 0.11 at 25 ng/ml, p < 0.05,
0.98 ± 0.10 at 100 ng/ml, p = 0.16 compared to shCaV1.3/GFP+ cells in
absence of BDNF, Fig. 2-4C). Therefore, I concluded that the expression of
CaV1.3 is necessary for activity-dependent axonal maturation in newborn
GCs, and that BDNF may be critically associated with the role of CaV1.3 in
the surface expression of GABA\textsubscript{A}-R and axonal maturation.
Figure 2-1. Effects of CaV1.3 on the calcium current of newborn GCs.
Figure 2-1. Effects of CaV1.3 on the calcium current of newborn GCs. A, An expression cassette in the retroviral vector for expressing GFP and shRNA. B, Efficiency of CaV1.3-targeting shRNA (shCaV1.3). Lysate samples from HEK293 cells co-transfected with an expression vector for CaV1.3 and a retroviral vector encoding CaV1.3-targeting or non-targeting shRNA (shNT) were subjected to Western blot analysis for CaV1.3. Ca, Representative confocal images of CaV1.3 immunostaining in newborn GCs infected with retrovirus encoding GFP and shNT (upper row) or shCaV1.3 (lower row) at DIV 6. The proximal dendrite of GFP+ newborn GCs (white arrow) and GFP- cells (white arrow head) in the same section are shown. Scale bar: 10 μm. Cb, Summary for FGFP+/FGFP- (%) in the same visual fields of cultured slices treated with retrovirus encoding GFP and shNT (NT) or shCaV1.3 (CaV) are shown (3 slices from 3 rat). D and E, Representative calcium current traces recorded from shNT/GFP+ (D, black) or shCaV1.3/GFP+ (E, blue) cells. Currents were evoked from a holding potential -55 mV to depolarizing steps between -50 mV and +40 mV. Calcium currents evoked by the steps between -20 mV and +10 mV are shown. F, I-V curves of calcium currents in shNT/GFP+ (black open circle) and shCaV1.3GFP+ (blue open square) cells. G, I-V curves of nimodipine-sensitive calcium currents in shNT/GFP+ (black closed circle) and
shCaV1.3GFP+ (blue closed square) cells.
Figure 2-2. The GABA-induced calcium transients (CaTs) were impaired by depletion of CaV1.3. A and B, Representative CaTs (expressed as ΔF/F₀) from the soma (left) and the growth cone (right) of the shNT/GFP+ cell (A) and the shCaV1.3/GFP cell (B) induced by local puff of 60 mM KCl (gray traces) or 5 µM GABA (black traces). C, Summary for the ratio of peak CaTs evoked by GABA to that by KCl in shNT/GFP+ cells (NT) or shCaV1.3/GFP+ cells (CaV). n, number of cells or growth cones.
Figure 2-3. The essential roles of CaV1.3 in formation of GABAergic synapses. A, Representative current traces evoked by local application of GABA (1 s, black line) in shNT/GFP+ cell (left) or shCaV1.3/GFP+ cell (right). Averaged traces (black) were obtained from at least five trials (gray). B, Summary for GABA-induced currents and current density. Number of cells are indicated in parentheses. C, Representative GPSCs evoked by PP stimulation in shNT/GFP+ cell (left) or shCaV1.3/GFP+ cell (right) cultured in control condition (black) or BDNF-treated condition (gray). D, Input-output curves for GPSCs recorded from shNT/GFP+ cells (open symbols; n = 12 in control condition; n = 8 in BDNF-treated condition) and shCaV1.3/GFP+ cells (filled symbols, n = 13 in control condition; n = 11 in BDNF-treated condition). The stimulus intensity varied from 1 to 10 V.
Figure 2-4. Activity-dependent axonal outgrowth and pruning are impaired by depletion of CaV1.3.
Figure 2-4. Activity-dependent axonal outgrowth and pruning are impaired by depletion of CaV1.3. A, Z-stack confocal images of GFP+ GCs in hippocampal cultured slices on DIV6. The representative images of shNT/GFP+ cells (left) and shCaV1.3/GFP+ cells in the absence (upper middle) or presence of TTX (lower middle) or BDNF (right) are shown. B, Cumulative distributions of the lengths of the longest axon of individual newborn GCs (NT, shNT/GFP+ cells; CaV, shCaV1.3/GFP+ cells). ** p<0.01, *** p<0.001, N.S. for non-significant, Kolmogorov-Smirnov test. C, Axonal branch index of newborn GCs is shown. Mean ± SEM. * p<0.05, *** p<0.001, N.S. for non-significant, Student t test.
Discussion

Despite the importance of calcium signaling for neuronal development, the molecular identity of calcium ion channels triggering the calcium signaling is little understood (Ge et al., 2007; Zhao et al., 2008). In this study, I elucidated the essential role of CaV1.3 in GABAergic synapse formation in the newborn GCs using single cell gene knock-down techniques. Furthermore, the lack of GABAergic synapses in CaV1.3-depleted newborn GCs resulted in the lack of activity-dependent axonal maturation.

I have showed that L-type VDCCs are the major calcium entry pathway for GABA-induced CaTs in newborn GCs during the first post-mitotic week in Chapter 1. I hypothesized a dominant role of CaV1.3 in GABA-induced CaTs, because CaV1.3 channels open at more negative membrane potential than CaV1.2 (Xu & Lipscombe, 2001), and they are dominant over CaV1.2 in nuclear pCREB signaling at low level stimulation (Zhang et al., 2006). This rationale prompted me to study the effect of CaV1.3 gene depletion in the newborn GCs. Different from my hypothesis, the voltage dependence of calcium current in shCaV1.3/GFP+ cells was not altered, suggesting that the dominant isoform of CaV1.3 in newborn GCs is not a short splicing variant which are activated at lower voltage (Singh et al., 2008a). Despite the lack
of apparent alteration of calcium current, I found a lack of GABAergic synaptic currents in shCaV1.3/GFP+ cells, indicating that CaV1.3 plays a key role in the GABAergic synaptic formation in newborn GCs, a formation that is not rescued by compensatory up-regulation of CaV1.2.

The mechanism underlying the unique role of CaV1.3 in GABAergic synapse formation remains to be elucidated. Recently, it has been reported that BDNF is essential for GABAergic synapse formation (Kohara et al., 2007) and maintenance of surface GABA_A-Rs in developing cortical neurons (Kuczewski et al., 2011). Both CaV1.2 and the long splice variant of CaV1.3 mediate pCREB signaling and, thereby, lead to activity-regulated transcription of genes essential for neuronal survival including BDNF, which in turn promotes GABAergic synapse formation (Zhang et al., 2005). Furthermore, L-type VDCCs mediate the activity-dependent secretion of BDNF (Kolarow et al., 2007). Our results indicate that expression of CaV1.2 alone is not sufficient for GABA synapse formation, implying indispensable roles of CaV1.3 in gene transcription and/or in the local Ca^{2+}-induced release of BDNF. Consistent with this view, the lack of GABAergic synapse in shCaV1.3/GFP+ cells can be overcome by application of BDNF, suggesting that BDNF signaling occurs downstream of CaV1.3. It may be possible that CaV1.3 is essential for pCREB signaling in early post-mitotic GCs that
receive only nonsynaptic GABAergic inputs that can be regarded as a form of low-intensity stimulation. However, the similarity between the I-V curves of control and CaV1.3-depleted GCs does not support this idea (Fig. 2-1). Alternatively, CaV1.3 may be closely coupled to the machinery for release of BDNF. Recently, it has been reported that CaV1.3 interacts with GABA_B receptors, whose activation can trigger BDNF release (Park et al., 2010; Kuczewski et al., 2011). It remains to be elucidated whether the unique role of CaV1.3 stems from its interaction with GABA_B receptors.
GENERAL DISCUSSION

In Chapter 1, I suggested CaMKK as a downstream signal mediator of L-type VDCCs which were opened by GABAergic input-induced depolarization. In Chapter 2, I proposed that the release of BDNF was mediated by activation of CaV1.3. Thus, it is needed to discuss about the intracellular signal cascade activated by CaMKK and BDNF in the newborn GCs.

Two separate roles of CaMKK in axonogenesis and dendritogenesis in cultured cortical neuron were previously reported (Takemoto-Kimura et al., 2007; Ageta-Ishihara et al., 2009). They suggested that specific subcellular localization of two sister kinases, CaMKIα in axon and CaMKIγ in dendrite, provides the basis of two distinct roles of CaMKK in developmental stage of cortical neuron. In addition, they have suggested that different upstream signals of CaMKK, GABA and BDNF, regulate the axonal development by CaMKIα and the dendritic development by CaMKIγ, respectively. The morphogenic role of BDNF on dendrite was supported by dendritic enrichment of tropomyosin-related kinase B (TrkB) receptor (Suzuki et al., 2004). However, they did not show how BDNF increases the intracellular calcium which leads to activation of CaMKK (Takemoto-Kimura et al.,
Recently, transient receptor potential canonical channels (TRPC) have been suggested as the calcium entry pathway activated by TrkB receptor. The activation of CaMKK by TRPC5/6 mediates the translation and synaptic incorporation of calcium-permeable AMPA receptors through AKT/mammalian target of rapamycin (mTOR) pathway (Fortin et al., 2012).

In this study, exogenous BDNF effectively increased the GPSC of shCaV1.3/GFP+ cells, implying that the activation of TrkB receptor mediates the GABA_A receptor expression and/or GABAergic synaptic formation of newborn GCs. Whereas CaMKK mediates the BDNF-induced AMPA-R trafficking, the phosphoinositide 3-kinase and protein kinase C, another downstream signaling pathway of TrkB receptor might contribute to the GABA_A receptor surface expression of newborn GC by inhibiting endocytosis of GABA_A receptor (Porcher et al., 2011). Recently it has been reported that the dendritic localization of BDNF is important for GABAergic synapse formation and normal differentiation of newborn GCs (Waterhouse et al., 2012). Although Waterhouse et al suggested that little activation of the presynaptic TrkB receptor of parvalbumin positive interneurons is partly responsible for the impaired GABAergic synapse formation in the transgenic mice, an autocrine activation of TrkB receptors in newborn GCs is still plausible. Conversely, the increased GPSC by application of BDNF in
shNT/GFP+ and shCaV1.3/GFP+ cells (Fig. 2-3) might be caused by the activation of presynaptic TrkB receptor in local interneuron.

In contrast to the dramatic increase of GPSC by application of BDNF, the axonal outgrowth of shCaV1.3/GFP+ cells in presence of BDNF did not exceed that of shNT/GFP+ cells (Fig. 2-4). The axonal outgrowth was effectively increased by high dose of BDNF. In contrast, branch index of shCaV1.3/GFP+ cells was not significantly decreased in high dose of BDNF (100 ng/ml), indicating that axon pruning does not effectively occur when BDNF was bath applied. Given that the activation of p75 neurotrophin receptor by BDNF mediates the axon degeneration by suppressing TrkA receptor signaling pathway (Singh et al., 2008b), localized effect of BDNF on axon and growth cone might be essential for the axonal outgrowth and the axon pruning of shCaV1.3/GFP+ cells. Thus, it is needed to elucidate the downstream signal pathway of BDNF in newborn GCs and the localization of TrkB receptor.
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Distinct Morphological Stages of Dentate Granule Neuron
해마 착상회절에서는 일생동안 새로운 신경세포가 생성된다. 이러한 해마신경과립세포는 신경회로의 활동성에 따라 그 발달이 조절되는데, 홍분성 GABA 신호가 중요하다고 알려져 있다. GABA 신호에 의해 막판이 탈분극되어 칼슘이 들어온다고 예상되지만, 그 중요성에 비해 유전 한 신호전달 기전이나 어느 막판 음존성 칼슘통로에 의해 칼슘이 들어오는지에 대해서는 알려져 있지 않다. 본 논문에서는 어린 쥐의 해마를 조직 배양으로 해마 신경과립세포에서의 칼슘 신호전달과 그것이 축삭과 수상돌기의 성장에 어떤 역할을 하는지에 대하여 연구하였다. 먼저 해마 신경회로의 활동에 따라 홍분성 GABA 신호가 신경과립세포로 들어오는 것을 발견하였고, 이에 의해 세포 분열된 이 일주일 이내의 신경과립세포 내 칼슘이 증가하였다. 홍분성 GABA에 의해서 증가하는 미성숙과립세포 내 칼슘은 대부분 L-type 칼슘통로를 통해 들어오는 것이었다. 신경과립세포의 칼슘을 증가시킬 수 있는 신호전달 경로인 신경회로의 활동 → GABA 수용체 → L-type 칼슘 통로 중 하나라도 약물로 막으면, 신경과립세포의 축삭 성장이 감소하였고, 그에 반해 수상돌기 성장에는 영향이 없었다. 그 중 GABA\textsubscript{A} 수용체 저해제인 bicuculline은 해마의 간질성 신경회로 활동을 일으키
다소도 불구하고 축삭 성장을 저해하는 것으로 보아, 신생파립세포에서의 GABA 신호가 축삭 성장에 필수적임을 알 수 있다. 그러므로, 신경회로 활동에 따른 GABA 신호가 L-type 칼슘통로를 통해 칼슘을 증가시킴으로써, 신생파립세포의 축삭 성장을 촉진한다고 결론지을 수 있다.

마취암 의존성 칼슘통로 중에서 L-type 칼슘통로는 신경세포의 성숙과 발달에 중요하다고 알려져 왔다. L-type 칼슘통로 중 CaV1.2과 CaV1.3가 뇌에서 많이 발현되고 있다. 신생파립세포는 발달 초기 단계에는 오직 GABA 신호만 받고, CaV1.2보다 CaV1.3이 좀 더 낮은 마취암에서 열리기 때문에 본 논문에서는 CaV1.3의 역할에 대해 연구하였다. 신생파립세포의 정상적 성숙을 위해 CaV1.3을 통한 칼슘 유입이 중요한지 알아보기 위하여, 레트로바이러스 벡터에 형광표지자인 GFP와 CaV1.3의 발현을 저해할 수 있는 short-hairpin RNA을 동시에 발현하도록 하여 CaV1.3의 발현이 저해된 신생파립세포를 구별할 수 있도록 하였다. 면역조직화학염색법과 Western blot을 이용하여 레트로바이러스가 CaV1.3의 발현이 잘 억제함을 확인하였다. 하지만 CaV1.3 발현을 억제한 세포군과 그렇지 않은 세포군 간의 칼슘 전류의 마취암의존성은 변하지 않았다. 그럼에도 불구하고 CaV1.3 발현을 억제한 세포군에서 낮은 농도의 nimodipine에 의해 칼슘 전류가 막히는 정도가 높
은 결로 보아, CaV1.2가 보상적으로 발현이 증가되었고, CaV1.3의 발현은 감소했음을 확인하였다. CaV1.3의 발현을 억제한 세포군에서 KCl에 의한칼슘 유입은 감소하지 않는 데에 비해, GABA에 의한 칼슘 유입이 감소함을 관찰하였다. 게다가 GABA$_A$ 수용체를 통한 전류 크기가 감소하고, perforant pathway 자극에 의한 시냅스 후 전류의 크기도 감소함을 관찰하였다. 이 결과는 CaV1.3의 발현이 신생파림세포에서의 GABA 시냅스 형성에 중요하다는 것을 의미한다. GABA 신호에 의해 촉진되는 축삭 성장 역시 CaV1.3의 발현을 억제한 세포군에서 저해되었음을 관찰하였다. 그러므로, 세포분열 후 일주일 이내 신생파림세포의 GABA 시냅스 형성에 CaV1.3의 발현이 필수적이라고 결론 내릴 수 있다.

중심 단어: GABA, postnatal neurogenesis, 해마 신생파림세포, 축삭 성장, L-type 칼슘통로, CaV1.3, 시냅스 형성

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