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BCL11B/CTIP2 is highly expressed in GABAergic interneurons of the mouse somatosensory cortex

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ABSTRACT

In the nervous system, BCL11B is crucial for the development of deep layer corticospinal projection neurons and striatal medium spiny neurons and is often used as a marker for the aforementioned cell types. However, the expression of BCL11B in subtypes of non-excitatory neurons in the primary somatosensory cortex (S1) has not been reported in the mouse. In this study we show that BCL11B is extensively expressed in S1 GABAergic interneurons, throughout the three main subgroups (somatostatin-, parvalbumin- and 5HT3a-expresssing). Almost all BCL11B positive cells in the upper S1 layers were GABAergic interneurons and surprisingly, almost 40% of the BCL11B positive neurons in layer V were GABAergic interneurons. Single cell mRNA sequencing data revealed higher *Bcl11b* expression in S1 interneurons compared to deep layer pyramidal neurons. The highest levels of *Bcl11b* expression were found within the 5HT3a population, specifically in putative neurogliaform interneuron subclasses (5HT3a-positive but not expressing vasoactive intestinal peptide). In the light of our findings we suggest caution using BCL11B as a single marker to identify neurons.

1. Introduction

To elucidate the function of the brain despite its complexity we need to understand the diversity of the cellular components, the neurons, and how they interact with each other in networks. To investigate the outcome of a manipulation to the system, known molecular markers are often used as a proxy for cell identity and therefore also function. B-cell leukemia/lymphoma 11B (BCL11B) also called Chicken ovalbumin upstream promoter transcription factor interacting protein 2 (CTIP2) or radiation-induced tumor suppressor gene 1 (Rit1), is a zinc finger transcription protein important for development of the central nervous system, skin, hair and lymphocytes (Kominami, 2012). Mutant mice lacking both alleles with *Bcl11b* die shortly after birth with defects in the immune system, central nervous system, skin and hair (Kominami, 2012). The action of BCL11B is known to be necessary for the development of corticospinal projection neurons mainly found in cortical layer V (Arlotta et al., 2005; Chen et al., 2008; Leyva-Diaz and Lopez-Bendito, 2013). In the absence of BCL11B these neurons fail to project axons into the spinal cord (Arlotta et al., 2005). BCL11B is also crucial for the development of medium spiny neurons (MSNs) in the striatum (Arlotta et al., 2008). Thus BCL11B is often used as a marker for deep layer subcerebral projection neurons (Leyva-Diaz and Lopez-Bendito, 2013; Alcamo et al., 2008; Leone et al., 2015; Molyneaux et al., 2005; Britanova et al., 2008) as well as striatal MSNs (Delli Carri et al., 2013). In the striatum, in addition to the striatal MSNs, BCL11B was recently shown to be partly expressed by at least one group of GABAergic interneurons (Muñoz-Manchado et al., 2016), but the expression in subtypes of non-excitatory neurons in the neocortex has not been characterized.

Cortical inhibitory neurons is a very heterogeneous group of neurons. This diversity has been studied using features such as morphology, electrophysiology, membrane properties, molecular markers, cellular location, connectivity and/or recently single cell RNA-sequencing (Markram et al., 2004; Fishell and Rudy, 2011; Rudy et al., 2011; Ascoli et al., 2008; Zeisel et al., 2015; DeFelipe et al., 2013). Although complex, GABAergic interneurons can be divided based on three non-overlapping molecular markers (Rudy et al., 2011). These are: parvalbumin (PV), a calcium-binding protein which is expressed by 40% of the neocortical GABAergic interneuron, and the neuropeptide somatostatin (SST), and 5-hydroxytryptamine (serotonin) receptor 3A (5HT3a), each marking two groups of 30% of the interneurons. The group expressing 5HT3a can be further subdivided with regards to the presence or absence of vasoactive intestinal peptide (VIP) which constitute 40% and 60% of 5HT3a expressing interneurons respectively (Lee et al., 2010). These two 5HT3a-populations still contain more than one

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subtype of interneurons based on morphology and firing patterns (Prönneke et al., 2015; Miyoshi et al., 2010). In this study we show that BCL11B is extensively expressed in neocortical GABAergic interneurons of all three major subgroups and thus its expression is not restricted to subcerebral projections cells.

2. Material and methods

2.1. Mouse lines

In this study we used outbred wild type CD1 mice (6 adult animals of both sexes) and the transgenic mouse line 5HT3a^{EGFP} (GENSAT project at Rockefeller University) on a CD1 background (5 animals, age P21-P22, females). All animal handlings were according to local ethical regulations under the permit N567/11 from *Stockholms norra djurförsöksetiska nämnd*, Sweden.

2.2. Tissue preparation

Mice were transcardially perfused with 4 °C phosphate buffered saline (PBS) solution followed by ice cold 4% paraformaldehyde (PFA) in PBS. The brains were dissected and post-fixated for 1 h in 4% PFA in PBS at 4 °C. These were then rinsed in PBS and cryoprotected in 30% sucrose in PBS overnight at 4 °C. Cryoprotected brains were embedded in optimal cutting temperature (OCT) compound, frozen and kept at −80 °C. Subsequently 40 μm thick coronal sections were obtained in a Leica cryostat and kept free floating in cryoprotect solution (30% Ethylene Glycol, 30% Glycerol, 10% 1× PBS in H_2O) at −20 °C until immunostaining.

2.3. Immunohistochemistry

The brain sections were washed in PBS, 0.1% Tween20 in PBS, 0.3% Tween20 in PBS and PBS again at room temperature and subsequently blocked in blocking buffer (5% normal goat serum (NGS), 0.5 M NaCl, 2.5% bovine serum albumin (BSA) and 0.3% Tween20 in 1x PBS) for one hour at room temperature, followed by incubation with the primary antibodies overnight at 4 °C in blocking buffer without NGS. Wild type mice were double stained with rat anti-BCL11B (1:500; Abcam; monoclonal [25B6]) (Arlotta et al., 2005; Leone et al., 2015; Molyneaux et al., 2005) and either rabbit anti-VIP (1:500; Immunostar; polyclonal), mouse anti-PV (1:1000; Sigma; monoclonal [PARV-19]) or rabbit anti-SST (1:500; Diasorin; polyclonal). 5HT3a^{EGFP} mice were stained with rat anti-BCL11B (as above) and the EGFP signal was detected by using chicken anti-GFP (1:2000; Abcam; polyclonal). After being washed in 0.1% Tween20 in PBS at room temperature the sections were then incubated in appropriate secondary antibodies (raised in goat) conjugated with Alexa Fluor dyes 488, 555 or 647 (Invitrogen) overnight at 4 °C. After washes with 0.1% Tween20 in PBS nuclear counterstaining was performed on all sections with 4,6-diamidino-2-phenylindole (1:1000 in H_2O; Molecular Probes) for 10 min at room temperature. The free floating sections were then mounted with Fluoromount-G (Southernbiotech) on microscope slides.

2.4. Image acquisition and data collection

The images were acquired by Carl Zeiss LSM700 confocal microscope with Plan Apochromat 10X/0.45 DIC II M27 (resolution: 0.74 μm) objective. We used automatic stitching made possible by the motorized stage to acquire a single image spanning all 6 layers of cortex (average area of cortex in each image was $0.77 \pm 0.12\ mm^2$). Two images from S1 per animal were acquired. S1 was determined according to The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2008). The cells were then counted using the ImageJ software (U.S. National Institutes of Health). The cortical area was manually delineated and measured by ImageJ's area measurement function. All data is presented as means ± standard error of the mean (SEM).

2.5. Single cell mRNA-sequencing

The single cell mRNA data from somatosensory cortex was obtained from Zeisel et al. (2015). The *Sst* neurons were defined as interneurons with more than 100 *Sst* mRNA molecules. The *Vip* neurons were defined as interneurons with more than 50 *Vip* mRNA molecules. All the FACS sorted EGFP$^+$ interneurons from the 5HT3a^{EGFP} mice excluding any cell without any *Htr3a* (5HT3a gene) mRNA molecules were defined as *Htr3a* interneurons. The different pyramidal neurons were defined as the classification in Zeisel et al. (2015). Since the expression data is not normally distributed we

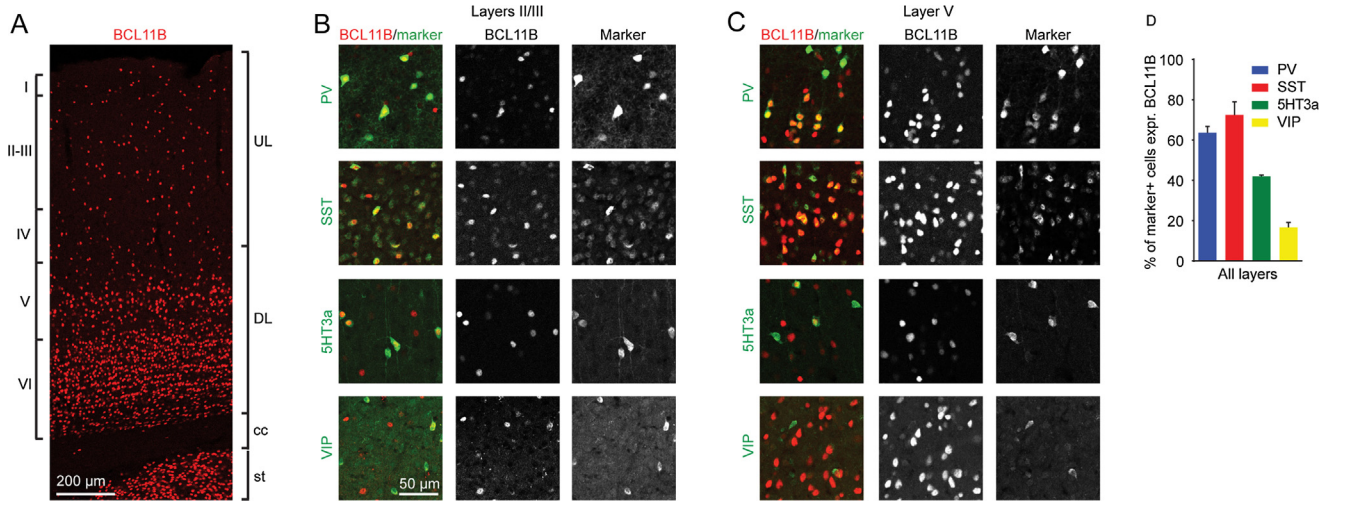

Fig. 1. Immunohistochemistry analysis reveals BCL11B was expressed by GABAergic interneurons. (A) Representative image of a coronal section immunostained for BCL11B showing the entire depth of S1 and parts of corpus callosum and striatum. (B) Representative images of co-expression of BCL11B and the different GABAergic interneuron subgroup markers in the layers II/III of S1. (C) Representative images of co-expression of BCL11B and the different GABAergic interneuron subgroup markers in the layer V of S1. Same scale as B. (D) Proportion of the different GABAergic interneuron subgroups which express BCL11B in all layers. (BCL11B + marker)/marker. Error bars represent SEM. Abbreviations: 5HT3a = 5-hydroxytryptamine receptor 3A, BCL11B = B-cell leukemia/lymphoma 11B, cc = corpus callosum, DL = deep layers, PV = parvalbumin, S1 = primary somatosensory cortex, SST = somatostatin, st = striatum, UL = upper layers, VIP = vasoactive intestinal peptide.