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POSTNATAL DEVELOPMENT OF GABAergic INTERNEURONS IN THE NEOCORTICAL SUBPLATE OF MICE

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Abstract—The subplate (SP) plays important roles in developmental and functional events in the neocortex, such as thalamocortical and corticofugal projection, cortical oscillation generation and corticocortical connectivity. Although accumulated evidence indicates that SP interneurons are crucial for SP function, the molecular composition of SP interneurons as well as their developmental profile and distribution remain largely unclear. In this study, we systematically investigated dynamic development of SP thickness and chemical marker expression in SP interneurons in distinct cortical regions during the first postnatal month. We found that, although the relative area of the SP in the cerebral cortex significantly declined with postnatal development, the absolute thickness did not change markedly. We also found that somatostatin (SOM), the ionotropic serotonin receptor 3A ($5HT_{3A}R$), and parvalbumin (PV) reliably identify three distinct non-overlapping subpopulations of SP interneurons. The SOM group, which represents ~30% of total SP interneurons, expresses neuronal nitric oxide synthase (nNOS) and calbindin (CB) and colocalizes entirely with neuropeptide Y (NPY). The $5HT_{3A}R$ group, which accounts for ~60% of the total interneuronal population, expresses calretinin (CR) and GABA-A receptor subunit delta ($GABA_AR\delta$). The PV group accounts for ~10% of total SP interneurons and coexpressed $GABA_AR\delta$. Moreover, distinct interneuron subtypes show characteristic temporal and spatial distribution in the SP. $nNOS^+$ interneurons in the SP increase from the anterior motor cortex to posterior visual cortex, while CR^+ and CB^+ interneurons the opposite. Interestedly, the majority of $GABA_AR\delta^+$ neurons in SP are non-GABAergic neurons in contrast to other cortical layers. These findings clarify and extend our understanding of SP interneurons in the developing cerebral cortex and will underpin further study of SP function.

Key words: subplate, interneuron, subtype, postnatal development.

INTRODUCTION

The subplate (SP) is a distinctive and highly dynamic structure in the developing neocortex derived from the embryonic cortical preplate (Chun and Shatz, 1989a; Woo et al., 1991; Wood et al., 1992; Molnár and Blakemore, 1995a; Valverde et al., 1995a; Marín-Padilla, 1998). When the layered neocortex starts to form, new migratory neurons split the cortical preplate into two parts, the upper marginal zone and the lower SP zone (Chun and Shatz, 1989a; Woo et al., 1991; Wood et al., 1992; Molnár and Blakemore, 1995a; Valverde et al., 1995a; Marín-Padilla, 1998). The SP zone undergoes programed cell death in early development, and evolves differently across species (Kostovic and Rakic, 1980, 1990; Al-Ghoul and Miller, 1989; Chun and Shatz, 1989a; Naegele et al., 1991; Ghosh and Shatz, 1993; Allendoerfer and Shatz, 1994; Valverde et al., 1995b; DeFreitas et al., 2001). In rodents, some SP cells persist into adulthood and form a compact layer after birth, named the SP layer (Aboitiz and Montiel, 2007; Hoerder-Suabedissen and Molnár, 2015), also known as cortical layer 6b or layer 7 (Aboitiz and Montiel, 2007; Perrenoud et al., 2013).

The SP is located at the interface between cortical layer 6 and the white matter (WM) in the neocortex at the postnatal stage (Reep and Goodwin, 1988; Vandevelde et al., 1996; Clancy and Cauller, 1999; Reep, 2000), and contains glutamatergic excitatory neurons and GABAergic interneurons as well as various corticofugal, corticopetal and corticocortical projections (Lavdas et al., 1999; Hevner and Zecevic, 2006; Kanold and Luhmann, 2010; Wang et al., 2010; Perrenoud et al., 2013; Hoerder-Suabedissen and Molnár, 2015). Given that they are the earliest generated cortical neurons (Bayer and Altman, 1990; Hevner and Zecevic, 2006; Wang et al., 2010), SP neurons have been shown to play a critical role in processes such as the guidance of thalamocortical and corticofugal projection (McConnell et al., 1989; Ghosh et al., 1990; Carlos and O'Leary, 1992;

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Abbreviations: $5HT_{3A}R$, serotonin receptor 3A; CB, calbindin; CGE, caudal ganglionic eminence; CR, calretinin; DAPI, 4′,6-diamidino-2-phenylindole; GABA, γ-aminobutyric acid; $GABA_AR\delta$, GABA-A receptor subunit delta; L1, layer 1; L2/3, layer 2 and Layer 3; L4, layer 4; L5, layer 5; L6, layer 6; MC, motor cortex; MGE, medial ganglionic eminence; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; Nurr1, nuclear receptor-related 1; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PV, parvalbumin; SC, somatosensory cortex; SOM, somatostatin; SP, subplate; VC, visual cortex; VIP, vasoactive intestinal polypeptide; WM, white matter.

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Ghosh and Shatz, 1993; Molnár and Blakemore, 1995b), the maturation and plasticity of thalamocortical circuits (Kanold et al., 2003; Kanold and Shatz, 2006; Kanold, 2009), establishment of ocular dominance and orientation columns (Grossberg and Seitz, 2003; Kanold et al., 2003; Kanold and Luhmann, 2010), supporting corticocortical connectivity (Kostovic and Rakic, 1990; Friedlander et al., 2009; Kostović et al., 2011), and generating oscillatory activity (Balkowiec and Katz, 2000; Dupont et al., 2006; Heck et al., 2008; Hanganu et al., 2009). Moreover, SP abnormalities have been implicated in various neural disorders, including schizophrenia (Bunney and Bunney, 2000; Eastwood and Harrison, 2006; Suárez-Solá et al., 2009), Alzheimer's disease (Suárez-Solá et al., 2009) and autism spectrum disorders (Bunney and Bunney, 2000; Hutsler and Casanova, 2015).

Several studies have clearly demonstrated that interneurons in the SP are crucial for functions such as generating neuronal network oscillation in the cerebral cortex (Voigt et al., 2001; Hanganu et al., 2009), and regulating neuronal migration pattern (Manent et al., 2005; Reiprich et al., 2005; Heck et al., 2007; Luhmann et al., 2009). Therefore, further study of SP interneurons is necessary to promote our understanding of SP functions. Although some interneuron markers (somatostatin (SOM), neuropeptide Y (NPY), NOS et al.) were found in SP interneurons in different species (Antonini and Shatz, 1990; Yan et al., 1996; Finney and Shatz, 1998; Río et al., 2000; Robertson et al., 2000; Friedlander et al., 2009; Perrenoud et al., 2013), so far the molecular composition of SP interneurons as well as their developmental profile and distribution remain largely unknown.

In this study, we characterized the developmental changes in SP thickness in the mouse neocortex. We also characterized the subtypes of SP interneurons and quantitatively analyzed their density in distinct cortical regions during postnatal development. Overall, our research conducts an extensive analysis of the neurochemical properties of SP interneurons in the developing neocortex, and will serve as a quantitative resource for future studies of the development and function of SP interneurons.

EXPERIMENTAL PROCEDURES

Animals

CD-1 mice, GAD67-GFP (Δneo) knock-in mice (Tamamaki et al., 2003; Jiao et al., 2006; Ma et al., 2014) and serotonin receptor 3A ($5HT_{3A}R$)-GFP transgenic mice (Vucurovic et al., 2010) were used in this study. GAD67-GFP knock-in mice exhibit specific and efficient GFP-labeling of interneurons in the neocortex. The expression of $5HT_{3A}R$ in neurons was tightly linked with GFP in $5HT_{3A}R$-GFP transgenic mice. The day of birth was noted as postnatal day 1 (P1). All animal procedures were conducted according to the guidelines for the Animal Care and Use Committee of Fudan University.

Data acquisition

We obtained quantitative data from the motor cortex (MC), somatosensory cortex (SC), and visual cortex (VC) from at least three discrete sections from at least three individual animals at each time point. The cortical regions were determined with a stereotaxic map. The SP was a thin layer between layer 6 and WM in postnatal mice (Hoerder-Suabedissen et al., 2009; Luhmann et al., 2009; Hoerder-Suabedissen and Molnár, 2012, 2013, 2015), equivalent to layer 6b or layer 7 (Reep and Goodwin, 1988; Reep, 2000; Aboitiz and Montiel, 2007; Chung et al., 2009; Hoerder-Suabedissen and Molnár, 2013; Perrenoud et al., 2013). The laminar borders of the SP were identified on the basis of cell distribution visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining as previously reported (Reep, 2000; Torres-Reveron and Friedlander, 2007; Viswanathan et al., 2012). By use of nuclear receptor-related 1 (Nurr1), a special molecular marker labels SP (Hoerder-Suabedissen et al., 2009; Hoerder-Suabedissen and Molnár, 2013), we confirmed the precise distinguishing of SP in our experiments (Fig. 1A).

The analysis of SP thickness in development

Using Photoshop CS5 (Adobe Systems) the SP in coronal brain slices was divided into a series of trapezoids (Fig. 1B) and the total area ($\sum_{i=1}^{n}(Si)$) of the trapezoids and total length ($\sum_{i=1}^{n}(Li+li)$) of upper and lower lines of the trapezoids were calculated. L and l indicate the upper and lower lengths of the trapezoids. The average length (*Length*) of the SP in a coronal brain slice was calculated as $\sum_{i=1}^{n}(Li+li)/2$ (Fig. 1B). The average thickness of the SP was calculated as $\sum_{i=1}^{n}Si/Length$ (Fig. 1B). The SP area was divided by the neocortical area to calculate the proportion of the SP in the neocortex in each section.

Immunohistochemistry

Mice of either sex (P2–P32) were perfused intracardially with cold phosphate-buffered saline (PBS, pH 7.4) and cold 4% paraformaldehyde (PFA) in PBS (pH 7.4). The dissected brains were fixed in 4% PFA overnight, washed in PBS and sectioned coronally (60 μm) using a vibratome (VT1000S, Leica, Nusslosh, Germany). After blocking in PBS containing 5% bovine serum albumin, 0.5% Triton X-100 and 0.05% sodium azide for 1.5–2 h, sections were incubated with the primary antibodies (diluted in PBS containing 1% bovine serum albumin, 0.5% Triton X-100 and 0.05% sodium azide solution) for 36–48 h at 4 °C. After five washes in PBST (0.1% Triton X-100 in PBS) for 10 min each, sections were incubated with the appropriate secondary antibodies overnight at 4 °C and subsequently washed five times in PBS for 10 min each.

The following primary antibodies were used: goat anti-Nurr1 (1:100, R&D Systems AB5380, Minneapolis, Minnesota, U.S.A.), chicken anti-GFP (1:1000, Aves #1020, Tigard, Oregon, U.S.A.), rabbit anti-GABA (1:1000, Sigma #A2052), goat anti-SOM (1:500, Santa Cruz #sc-7819, Dallas, Texas, U.S.A.), mouse anti-parvalbumin (PV, 1:400, Millipore #MAB1572, Darmstadt, Germany), rabbit anti-NPY (1:1000, Immunostar #22940, Hudson, Wisconsin, U.S.A.), rabbit