ミクログリアの発生と分化

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ミクログリアの発生と分化過程

脳の免疫機能担当細胞として長らく知られてきたミクログリアは脳細胞の5%を占める．Macrophage 1 antigen（Mac1），major histocompatibility complex（MHC）class Ⅱ，CD68，F4/80やfragment，crystallizable（Fc）受容体，low density lipoprotein（LDL）受容体といった細胞表面抗原を発現していること，貪食能，サイトカイン分泌といった機能を有することなど，モノサイトやマクロファージに類似点が多いため，脳内炎症レベルの決定細胞として長らく炎症を伴う病態研究の対象となってきた．ミクログリアをモノサイトやマクロファージと決定的に区別できる細胞表面抗原がまだ見つかっていないため，その起源についてもモノサイトやマクロファージと共通であると長らく考えられてきた．しかし2010～12年にかけて，ミクログリアが系統的に発生過程の早い時期にマクロファージと分かれていることが明らかにされた．ミクログリアは胎生7.5日齢に骨髄前駆細胞から分化し胎生8.5～9.5日齢にはすでに脳内に移行している[1,2]．Ginhouxらは，造血幹細胞（モノサイトやマクロファージのもとになる幹細胞）の完成前である胎生7日齢に，ミクログリア前駆細胞がすでに卵黄嚢で発生しており，胎生9日齢までにこれらの細胞が脳に移行してミクログリアとなることを実験的に証明した[1]．これは神経幹細胞であるラジアルグリアが脳内に発生する（胎生10.5日齢）よりも早いタイミングの出来事である．このミクログリア前駆細胞はCsf1受容体を発現したMyb（－），PU.1依存的であることも[2]，Myb（＋）な造血幹細胞とは異なる点である．一方，病態時には血液脳関門のバリア機能低下により血球系細胞の脳内浸潤が起こるが，これらの細胞がミクログリアにはならないことも示されている．モノサイトやマクロファージのみがgreen fluorescent protein（GFP）標識されたマウスに実験的脳脊髄炎（experimental allergic encephalomyelitis：EAE）を発症させると，EAE症状の重篤度に応じてモノサイトが脊髄に浸潤するが，炎症終息後にGFP陽性細胞は消失した[3]．以上のことから，脳内で生理的な機能を持つミクログリアのほとんどが胎生7.5日齢に脳内に移行したミクログリア前駆細胞の子孫たちであると考えられる．ところが，このように共通の先祖を持ちながらミクログリアは中枢神経系の発達，機能分化とともに，ミエリン，血管系，血液脳関門，周囲細胞の細胞外マトリックス，神経伝達物質，といった周囲環境との相互作用によって，その領域，その時期に特異的な形態，抗原発現パターン，増殖パターン，生理機能を獲得する[4,5]．さらに言えば，一領域中のミクログリア集団の中に感染や外傷に対して非常に劇的に反応する集団とそうでもない集団が含まれていることもあり[6]，ミクログリアと微細な周囲環境との相互作用が起こっていると考えられる．

ミクログリアは，正常脳でよくみられるような，細かく発達した多くの突起を持つ「静止型」と神経炎症等の病変部に集積しているアメボイド状の「活性化型」との間を環境の変化に応じて行き来する細胞である．しかし最近の研究で，静止型ミクログリアはその発達した突起により周囲環境を実に積極的に探索していることが明らかとなっている[5]．したがって「静止型」，「活性化型」はもはや言葉通りに捉えきれなくなっている．現在，ミクログリアの活性状態はM0，M1，M2ステージというカテゴリーで表現されるが，この概念はそもそもマクロファージ研究から導入された[7]．マクロファージは外部刺激に対する反応によってM1，M2a，M2b，M2cというカテゴリーに分けられるが[8]，ミクログリアと異なる点は，分類に有効な35種類の表面抗原が明らかになっている点である．M1ミクログリアは炎症誘発特性，M2ミクログリアが抗炎症特性を持つ，と言われるが，そのどちらにも入らないM0ミクログリアという集団も発見されている[4]．M1，M2の中間とも言えるミクログリアも存在する．生後初期マウスのミクログリアはM1遺伝子（iNOS，TNFα）とM2遺伝子（Arginase-1）の両者を高発現している．M0，M1，M2ステージの他に

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HoxB8というホメオボックスタンパク質の発現によってミクログリアを分類しているグループもいる[9]．HoxB8(＋)細胞はCD11b(＋)ミクログリアの15％を占め，卵黄嚢から分化するミクログリアとは異なる細胞集団であると報告されている．HoxB8を欠損したマウスは強迫性障害スペクトラムに伴う脱毛症と非常によく似た症状が現れるため，病態解明への応用が期待されている．

脳室下帯ミクログリアに関する最近の知見

脳室帯(ventricular zone：VZ)と脳室下帯(subventricular zone：SVZ)は一生を通じて神経新生やグリア新生が起こる領域であることが明らかとなって久しい．主要な投射神経は胎生期に，アストロサイトは胎生期から生後初期まで，オリゴデンドロサイトは胎生後期から生後初期にかけて，介在神経は胎生後期以降新生される[10]．一方，上で述べたように胎生8.5〜9.5日齢にはアメボイド型のミクログリアが脳内にすでに存在している[1,2]．ミクログリアは生後初期に爆発的に増加するが[11]，発達が進むにつれアメボイド型のミクログリアは減少し成体においてほとんど静止型になる[12]．しかし，胎生期の神経新生にミクログリアがどのように関わっているかについては2013年にCunninghamらが報告するまで，ほとんどわかっていなかった[13]．彼らは胎生期の大脳皮質神経細胞新生過程の後半において，ミクログリアが貪食作用によって神経前駆細胞プールの細胞数を調節していることを明らかにした．このとき，ミクログリアが貪食した細胞はアポトーシスを起こしていない点は興味深い．成体海馬歯状回顆粒細胞層(subgranular zone：SGZ)での神経新生の場合，ミクログリアはアポトーシスを起こした細胞を貪食しているからである[14]．発達期SVZと成体SGZでの神経新生とミクログリアの関連について，さらなる解明が待たれる．

生後SVZの神経新生におけるミクログリアの役割については胎生期に増してほとんど情報がなかったが，2006年に，SVZのように神経新生が活発な領域とそれ以外の領域とでミクログリアの活性化や機能に差があることが指摘された[15]．最近われわれは，生後初期に活性化型ミクログリアがSVZに一過的に集積しており，成熟に伴い，より突起の発達したミクログリアが白質まで分散するようになることを見出した．われわれは生後初期SVZでこのように特異な挙動を示すミクログリアが，SVZにおける神経新生およびオリゴデンドロサイト新生を促進することを明らかにした[16](図1)．SVZは一生を通じて神経新生が継続するものの，年を重ねるにつれ新生細胞数は激減する．われわれのデータでも，生後30日齢のSVZに活性化型ミクログリアはすでにほとんど存在せず，SVZ神経新生動態と相関がある．興味深いことに，SVZでの神経新生に対するミクログリアの作用はSVZ中の位置によって異なるようである．われわれも体軸の中間あたり，線条体と海馬が対面するあたりが最もミクログリア密度が高いことを見出している．この細胞分布の偏りは血管系が関与している可能性がある．血管は成体脳SVZにおいて神経新生ニッチとなることが知られているが[17,18]，SVZは血管叢と呼ばれるほど血管が発達しており[19,20]，特にSVZ中心部は腹側から大きな血管が来ている[20,21]．血管から多種の液性因子が放出されていることはよく知られているが[17]，大脳皮質にミクログリアが移行するのに重要なシグナルカスケードであるCXCL12/CXCR4シグナル[22]の関与などが考えられる．成体脳における脳室上衣下層(subependymal zone：SEZ)では吻側尾側方向，背側腹側方向に高度に領域化されており，各領域からは異なる神経細胞系列が新生してくることも知られている[23]．また，背側SEZではより多くのオリゴデンドロサイト新生が起こることも知られている[24]．今後，SVZ領域をさらに細分化してミクログリアの役割について検討する必要があろう．

成体脳SVZからとってきた神経幹細胞や神経前駆細胞の場合，Th2ヘルパーT細胞由来サイトカインによって刺激されたミクログリアが神経新生やオリゴデンドロサイト新生を促進する[25]．このとき，ミクログリアから放出されるIGF-1が作用に関与していることが示唆されている．しかし，生後初期SVZにおいてはIGF-1を発現しているミクログリアはわずかに確認されるが，IGF-1はミクログリアの神経新生促進作用の作用本体ではなかった[16]．われわれは，ミクログリアが神経新生，オリゴデンドロサイト新生を促進するメカニズムとして生後4〜9日齢のSVZにおいて一過的に濃度が上昇するサイトカイン群(IL-1β，IL-6，TNFα，IFNγ)が重要であることを明らかとした．興味深いのは，神経幹細胞塊(neurosphere)とミクログリアの共培養系を用いた実験で，上記4種のサイトカインのう

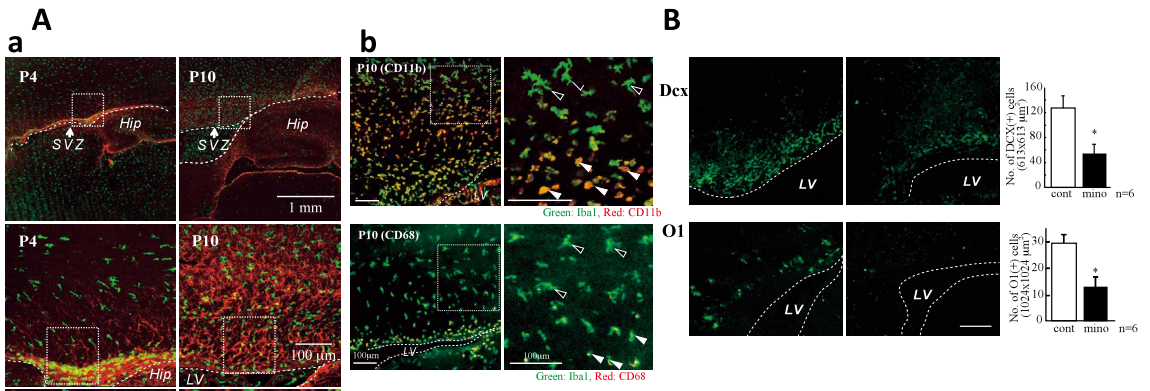

図 1　生後初期 SVZ ミクログリアの神経新生およびオリゴデンドロサイト新生における役割

A-a）生後初期 SVZ に活性化型ミクログリアが集積していることを見出した．

A-b）集積ミクログリアの活性化型マーカー（CD11b，CD68）の発現．白矢頭は Iba1（＋）marker（＋）細胞を表す．

B）ミノサイクリンを P1-4 で腹腔内投与すると，神経前駆細胞マーカー Dcx とオリゴデンドロサイト前駆細胞マーカー O1 を発現する細胞数が有意に減少した．*p＜0.05 vs. control group, student's *t* test（N＝6）．（Shigemoto-Mogami ら[16]より改変）

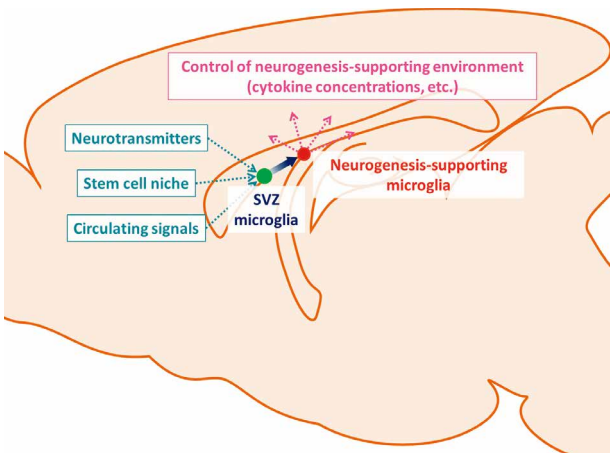

図 2

SVZ ミクログリアは脳内・脳外環境からの信号に応じてサイトカイン環境を調節する‘Hub’として機能しているのではないか．

ち 1 種のサイトカイン機能を機能中和抗体で阻害してもミクログリアの作用に何ら影響はなかったが，全てのサイトカインを同時に阻害するとミクログリアの作用が消失したことである．この結果は神経新生やオリゴデンドロサイト新生を複数のサイトカインが相補的に促進していることを示唆している．これまで，神経新生に関するサイトカインの作用としては，神経前駆細胞が IL-1β，IL-1R1，IL-1R2 を発現し，IL-1β は神経前駆細胞の増殖と分化を調節していること[26]，IL-6 が IL-6R を介して神経新生を促進すること[27]などが報告されていたが，複数サイトカインによる相補的な作用は報告がなかった．Li らは IFNγ の作用はミクログリア存在下では表現系が異なることを示しており，われわれが示した相補的作用を支持するものである[28]．そもそも，IL-1β，IL-6，TNFα，IFNγ は炎症性サイトカインとして，LPS（lipopolysaccharide）刺激[29]，EAE[30]，status epilepticus（SE）[31]といった病理的条件下では神経新生を抑制する本体として研究されてきた．しかし，より穏やかな実験条件では異なる作用が発揮される．炎症モデル作成のために使われる LPS は，適用条件（適用時間など）をわずかに調整することでミクログリアの神経新生への影響が変化する[32]．サイトカイン自身も濃度によって作用が変化する．TNFα は 1 ng/mL では神経幹細胞を増殖する一方，10 ng/mL 以上の濃度でアポトーシスを起こす[33]．われわれのデータでは，ミノサイクリンがミクログリアの活性化を抑制すると，神経前駆細胞の数は半分以下に減少するのに対し，それぞれのサイトカインレベルの減少は皆一様に穏やかであった．サイトカインはミクログリアだけでなく

アストロサイトなどの周囲細胞からも放出される．おそらく，ミクログリアからのサイトカイン放出が‘cytokine storm’[34]には遠く及ばない程度の，‘cytokine drizzling’とも言える連続的なサイトカイン放出トリガーとなっていることが予想される．

むすび

われわれにとって次の課題は，生後初期ミクログリアの活性化機構の解明である．重大なヒントとして胎生期から生後にかけてSVZでのミクログリア機能が大きく変化する点があげられる．体内を循環している種々のメディエーターは誕生の前後で質，量ともに大きく変化する[35]．われわれは，SVZミクログリアはこのような脳内・脳外環境からの信号に応じてサイトカイン環境を調節する‘Hub’として機能しているのではないかと考えている(図2)．このような脳外から脳内への情報デリバリー，それに伴うミクログリアによる神経新生の調節メカニズムの解明は，発達障害を含む神経障害治療にもブレークスルーをもたらすことが期待される．

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REVIEW ARTICLE

Effects of Microglia on Neurogenesis

Kaoru Sato

This review summarizes and organizes the literature concerning the effects of microglia on neurogenesis, particularly focusing on the subgranular zone (SGZ) of the hippocampus and subventricular zone (SVZ) of the lateral ventricles, in which the neurogenic potential is progressively restricted during the life of the organism. A comparison of microglial roles in neurogenesis in these two regions indicates that microglia regulate neurogenesis in a temporally and spatially specific manner. Microglia may also sense signals from the surrounding environment and have regulatory effects on neurogenesis. We speculate microglia function as a hub for the information obtained from the inner and outer brain regions for regulating neurogenesis.

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Key words: microglia, subventricular zone, subgranular zone, neurogenesis, cytokine

Introduction

In humans, approximately 5% of brain cells are microglia (Pelvig et al., 2008). Originally, microglia attracted attention because these cells determine the levels of inflammation in the cellular environment, which subsequently determines whether newly generated neurons survive. However, increasing evidence has demonstrated that microglia play diverse roles in neurogenesis in both the embryonic and postnatal adult stages. In this review, I summarize and organize the data concerning microglial effects on neurogenesis, particularly focusing on the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and subventricular zone (SVZ) of the lateral ventricles, in which the neurogenic potential is progressively restricted during the life of the organism. Recent research regarding the origin of microglia (Ginhoux et al., 2010; Schlegelmilch et al., 2011; Schulz et al., 2012) has demonstrated conclusively that these cells are derived from myeloid progenitors at approximately embryonic day 7.5 (E7.5) in mice and infiltrate the brain through blood vessels between E8.5 and E9.5. This process occurs immediately before the formation of early radial progenitors for neurons and glia, which occurs at E10.5 (Dahlstrand et al., 1995). Microglial progenitors are Myb-negative and PU.1-dependent cells that express the Csf1 receptor; these cells differ from Myb-dependent hematopoietic stem cells, which differentiate into macrophages and monocytes (Ransohoff and Cardona, 2010). A recent study that combined parabiosis and myeloablation revealed that monocytes that infiltrate the brain during inflammation only contribute to a transient population of macrophages that disappear once the inflammation is resolved (Ajami et al., 2011). Therefore, most microglia that have functional roles in neurogenesis in the brain originate from embryonic microglia that differentiated from infiltrating myeloid progenitors at E7.5. Although these cells all have common ancestors, microglia have temporally and spatially specific morphologies, antigen expression profiles, proliferative potentials, and brain functions (Butovsky et al., 2014; Olah et al., 2011). The effects of microglia on neurogenesis also appear to be diverse in a temporally and spatially specific manner.

Neurogenesis in the Subgranular Zone

In mammals, the SGZ is one of the major sites for the birth of new neurons from radial glial progenitors (Eckenhoff and Rakic, 1988; Kaplan and Hinds, 1977; van Praag et al., 2002). In the hippocampus, granule and pyramidal neurons exhibit different developmental patterns (Taupin, 2008). In contrast to the cornu ammonis (CA), which begins to develop during the prenatal period, 15% of DG granule cells are generated before birth; 70% are generated during the first 2 weeks of life; and 15% are generated after postnatal day 16 (P16) in mice. Beginning at E15.5, Prox1-positive cells migrate from the dentate neuroepithelium, which is adjacent to the fimbria, to the granular layer of the DG (Nakahira

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and Yuasa, 2005). At E18, the characteristic V-shape of the DG is highlighted by Prox1 staining in the granular layer and hilar region. Within the hilar region, neuronal precursors proliferate to generate additional granule neurons. In the postnatal and adult SGZ, neural stem cells (NSCs) have been shown to be long-lived NSCs (Li et al., 2013). They initially originate from the ventral hippocampus during late gestation and then relocate into the dorsal hippocampus. These NSCs can produce not only neurons, but also stem cells, and the ratio of NSCs to neurons depends on experiences of the animal or the location of the NSCs (Dranovsky et al., 2011). The proliferating radial and nonradial precursors give rise to intermediate progenitors, which in turn generate neuroblasts (Gage et al., 1998; Ming and Song, 2011). Immature neurons migrate into the inner granule cell layer and differentiate into dentate granule cells in the hippocampus (van Praag et al., 2002). Through transcriptome analysis, neurogenesis-related transcription factors crucial for SGZ neurogenesis have been identified (Miller et al., 2013). Wnt/β-catenin signaling cascade (Lie et al., 2005; Varela-Nallar and Inestrosa, 2013) and circadian molecular clock (Bouchard-Cannon et al., 2013) are shown to be important in the adult SGZ neurogenesis. New-born functional granule cells contribute to cognitive functions (Kempermann et al., 2004) and temporal memories (Aimone et al., 2006). Aimone et al., have suggested that immature neurons provide a low specificity yet densely sampled representation of cortical inputs, whereas mature granule cells provide a highly specific yet sparse representation of an event (Aimone et al., 2011). This combined representation maximizes the information encoded by hippocampal memories, thus increasing the memory's resolution (behavioral discrimination). Kempermann et al. (1998, 2002) demonstrated that SGZ neurogenesis continues in senescent mice. Based on these back ground, the regulation of SGZ neurogenesis has received abundant attention because of the link between neurogenesis and cognitive function (Lie et al., 2004), which has been indicated by many reports.

Regarding the effects on neurogenesis, microglia originally gained attention because these cells determine the inflammation levels in the cellular environment, which then determines whether newly generated neurons survive. Monje et al. (2003) showed that inflammation caused by LPS inhibited neurogenesis in the adult rat hippocampus via the microglial release of IL-6 and TNFα. The *in vitro* data of these authors indicated that the neurogenic lineage has greater sensitivity to inflammation than does the gliogenic lineage (Monje et al., 2003). Ekdahl et al. (2003) also demonstrated that inflammation-associated microglial activation impairs both basal and insult-induced hippocampal neurogenesis in adult rats. Studies that are more recent have clarified that TNFα signaling via TNFR2 is required for basal neurogenesis, whereas signaling via TNFR1 impairs neurogenesis (Chen and Palmer, 2013). Aging is associated with a substantial decrease in hippocampal neurogenesis (Kempermann et al., 1998; Kohman et al., 2012; Rao et al., 2006; van Praag et al., 2005; Walter et al., 2011), which is associated with cognitive deficits (Bizon et al., 2004; Drapeau et al., 2003; Merrill et al., 2003). Aging also alters microglial activity and drives microglia toward an inflammatory phenotype (Dilger and Johnson, 2008). Gemma et al. (2007) reported that IL-1β released from microglia reduced neurogenesis in aged rats. Age-related priming of microglia contributes to a prolonged neuroinflammatory response following an immune challenge, resulting in the exaggerated expression of sickness behaviors and cognitive deficits (Dilger and Johnson, 2008; Godbout et al., 2005; Kohman et al., 2007).

Some models in which aging-induced neurogenesis is ameliorated have suggested that microglia contribute to SGZ neurogenesis. An enriched environment (EE) is a housing manipulation that increases physical and social stimuli (Diamond et al, 1976) and that is reported to stimulate neurogenesis in the aged brain, leading to better performance in a water maze (Kempermann et al., 1998). Ziv et al. (2006) demonstrated that EE-induced SGZ neurogenesis is associated with T cell recruitment and microglial activation with increased MHCII expression. An EE increased microglial Iba1+ expression only in the DG but not in the CA1 or CA3 and blunted the proinflammatory hippocampal response to LPS (Williamson et al., 2012), suggesting that the effects of an EE on microglia may be specific to the DG. Wheel running, i.e., voluntary physical exercise, also ameliorated several of the behavioral consequences of aging and reversed the decrease in neurogenesis to 50% of that in the young control animals (van Praag et al., 2005). In this case, despite increased levels of neural precursors and newborn neurons, microglia remain in a resting state morphologically and antigenically, and T cells and MHCII-expressing microglia are not present in the DG in the wheel running model (Olah et al., 2009). Voluntary wheel running suppressed the age-associated increase in the number of microglia, increased the proportion of microglia that express IGF-1, and enhanced the survival of new neurons simultaneously (Kohman et al., 2012). Running induces a neuroprotective microglia phenotype and promotes neurogenesis by reducing the expression of proinflammatory cytokines such as TNF-α (Vukovic et al., 2012), and by increasing the expression of anti-inflammatory cytokines such as IL-1ra or the chemokine CX3CL1 (Pervaiz and Hoffman-Goetz, 2011). Wheel running has also been reported to prevent the infection-induced reduction in hippocampal BDNF expression in sedentary rats (Barrientos et al., 2011) and increased neurogenesis in healthy adult mice (van Praag et al., 1999). Gebara et al. (2013) monitored microglia

and the proliferation of adult hippocampal stem/progenitor cells in young adult and aged mice with or without wheel running and demonstrated that the number of microglia in the DG correlated inversely with the stem/progenitor cell number and the cell proliferation rate in the granule cell layer. The interesting data from this study have indicated that both the aging-induced decrease and voluntary running-induced increase in the radial glia number highly correlate with the number of microglia, suggesting that the regulation of radial glia number correlates with the regulation of microglia number. In the case of the DCX+ or Tbr2+ cell number, the correlation coefficients remained high but lower than were those of RGLs, suggesting that other mechanisms that are independent of the regulation of the microglia number are involved in neuronal differentiation. In contrast, some studies have demonstrated that running induces the transcription of genes involved in inflammation, including genes related to MHCI (β2-microglobulin, H2-D1) and elements of the complement system (C4A, C3, and C1q) or the inflammatory response (COX-2 and CX3C; Kohman et al., 2011; Tong et al., 2001). The endpoint of running may depend on the exercise intensity. The largest difference between the embryonic and postnatal adult brains may be the degree of neuronal network completion. Data from status epilepticus (SE) models have suggested that excitatory inputs influence both hippocampal neurogenesis and microglial effects on neurogenesis. Granule cell generation is induced in electrically evoked SE models, and the cells that do not die during the first month after SE induction survive for 6 months despite chronic inflammation (Bonde et al., 2006). Pilocarpine-induced SE can trigger the activation of CRE-mediated gene expression (Lee et al., 2007), including IGF-1 expression predominantly in activated microglia near the SGZ (Choi et al., 2008). Recent studies have indicated that microglia express a variety of neurotransmitter receptors, i.e., alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptors, metabotropic glutamate receptors (mGluRs), gamma-aminobutyric acid (GABA) B receptors, purinergic receptors, and adenosine receptors, as well as adrenergic, dopaminergic, cannabinoid, and opioid receptors (Pocock and Kettenmann, 2007). The neurogenic effects of microglia may be modified by the signalings of these neurotransmitters. *In vitro* data have suggested that VIP, a neuropeptide released by DG interneurons, enhances the proliferative and proneurogenic effects of microglia via the VPAC1 receptor and that these effects are mediated by IL-4 release from microglia (Nunan et al., 2014). Notably, SE-induced neurogenesis differs from physiologically coordinated neurogenesis. Aberrant neurogenesis following seizure activity has been reported to contribute to cognitive impairment (Jessberger et al., 2007). More recently, Parkhust et al. (2013) demonstrated that microglia promote the formation of synapses and the achievement of multiple learning tasks in young adult mice that carry CXCR1CreER to drive the diphtheria toxin receptor allowing cell ablation after diphtheria toxin administration. Therefore, the effects of microglia on learning should be interpreted as the composite results of the effects on neurogenesis and synaptogenesis.

In addition to regulating the proliferation and differentiation of NSCs/neural progenitor cells (NPCs), microglia also control the resulting number of newborn neurons via phagocytosis. Sierra et al. (2010) reported that unchallenged microglia (CD11b-low and CD68-low) maintain the homeostasis of the baseline neurogenic cascade in the young adult SGZ in a microglial activation-independent manner. The primary critical period occurs in the first 4 days of a cell's life, specifically during the transition from late amplifying neuroprogenitors to neuroblasts. The cells that are committed to apoptosis in the first 4 days of cell life interact with unchallenged microglia to induce phagocytosis (Sierra et al., 2010). Interestingly, although the numbers of newly generated neurons and apoptotic cells decrease with age and acute inflammation, the phagocytosis index (the percentage of apoptotic cells that undergo microglial phagocytosis) remains constant, suggesting that once microglia become ramified, their phagocytic activity is unaffected by the surrounding environment (Sierra et al., 2010). Two distinct functional types of phagocytic receptors have been characterized in microglia (Neumann et al., 2009). The first group, including TLRs, recognizes microbes; these receptors support the removal of pathogens and simultaneously stimulate a proinflammatory response in phagocytes. The second group of receptors recognizes apoptotic cellular materials, such as phosphatidylserine (PS); these receptors are important for ingesting apoptotic cell corpses and for stimulating an anti-inflammatory response in phagocytes (Ravichandran, 2003). The latter pathway may be involved in the microglial phagocytosis of newborn cells in the SGZ. Furthermore, *in vivo* studies have shown that DAP12-CD11b-ROS signaling actively contributes to the developmental death of postnatal hippocampal neurons (Wakselman et al., 2008).

Mechanisms That Underlie the Neurogenic Effects of Microglia in the Postnatal and Adult SGZ

The interaction between T cells and microglia is important for the EE-induced neurogenic effects of microglia (Ziv et al., 2006). An *in vitro* study demonstrated that microglia activated by T helper cell type 2 (Th2)-derived cytokines, interleukin-4 (IL-4) and a low level of IFN-γ differentially induce neurogenesis and oligodendrogenesis in adult NSCs/NPCs. NPCs co-cultured with IL-4-activated microglia are

biased toward oligodendrogenesis, whereas NPCs co-cultured with IFN-γ-activated microglia are biased toward neurogenesis. The effect of IL-4-activated microglia is mediated by IGF-1 release (Butovsky et al., 2006). Increased IGF-1 expression has also been observed in a voluntary wheel running model (Kohman et al., 2012). In addition to the antiapoptotic effects of IGF-1 during development (Chrysis et al., 2001), IGF-1 reduces the G1 phase length and total cell cycle length but increases NPC cell cycle reentry (Hodge et al., 2004). The interaction of CNS-specific autoimmune T cells (T cells directed to myelin basic protein, MBP) with resident microglia is important for the effects of an EE on spatial learning and memory, as well as for BDNF expression (Ziv et al., 2006). However, whether this effect is mediated by direct cell-cell contact between T cells and microglia remains unclear. Because T cells can barely be detected in healthy CNS parenchyma, T cells likely interact with microglia via secreted factors (Ziv and Schwartz, 2008b).

CX3CR1 is expressed primarily in microglia (Cardona et al., 2006; Harrison et al., 1998) and is located in hippocampal neurons (Sheridan and Murphy, 2013). Its ligand, CX3CL1/fractalkine, can act as a signaling molecule when cleaved (Chapman et al., 2000). Wheel running increases the expression of CX3CL1 (Pervaiz and Hoffman-Goetz, 2011) and the phenotypic transition of microglia from non-neurogenic to neurogenic fates by facilitating the CX3CL1/CX3CR1 signaling axis, which underlies the exercise-induced reversal of age-related decreases in neurogenesis (Vukovic et al., 2012). Furthermore, CX3CL1 administration resulted in the recovery of the age-related decrease in hippocampal neurogenesis and an increase in the number of microglia with ramified morphology (Bachstetter et al., 2009). Generally, CX3CL1 limits the activation of microglia and the expression of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α 2009 (Bachstetter et al., 2009; Rogers et al., 2011), which act directly on neural progenitors (Koo and Duman, 2008; Monje et al., 2003). Therefore, CX3CL1 likely contributes to the preservation of a neuronal niche that is optimal for SGZ neurogenesis by maintaining a milieu skewed toward quiescence. Hippocampal neurogenesis is decreased in mice that lack CX3CR1; these mice have significant deficits in cognitive functions and LTP induction (Rogers et al., 2011). In contrast, another report demonstrated that CX3CR1$-/-$ mice exhibited enhanced neurogenesis and better hippocampus-dependent memory, with increased numbers and soma sizes of hippocampal microglia (Reshef et al., 2014). CX3CL1/CX3CR1 signaling is multifunctional in various processes of neuronal development, including the synaptic pruning process during postnatal synaptogenic development (Paolicelli et al., 2011). The effects of CX3CL1/CX3CR1 signaling manipulation may vary in a manner dependent on the developmental stage.

Neurogenesis in the SVZ

The ventricular zone (VZ) and SVZ are germinal zones during the embryonic period. Although several major classes of neocortical neural precursor cells have been identified in these areas, the lineal relationships and molecular profiles of these cells remain largely unknown. Using a novel fate-mapping approach, neural precursors have been divided into distinct subtypes based on their lineage profile, morphology, and transcription factor expression *in vivo*. These results have suggested that neocortical neurons are produced via multiple indirect routes during embryonic development (Tyler and Haydar, 2013). Until the report by Cunningham et al. (2013), few reports had been published regarding the role of microglia in embryonic SVZ neurogenesis. Cunningham et al. (2013) demonstrated that microglia regulate the size of the neuronal precursor pool via phagocytosis of Tbr2+ and Pax6+ cells during the late stages of cortical neurogenesis, as analyzed during embryonic stages in these experiments. Notably, most precursor cells that were targeted by microglia did not exhibit signs of cell death or apoptosis. These authors also demonstrated that maternal immune activation reduces the size of the neural precursor cell pool via microglial phagocytosis. The phagocytosis that occurs in the embryonic SVZ differs from that in the adult SGZ, in which unchallenged microglia phagocytose apoptotic cells as discussed previously (Sierra et al., 2010). The precise mechanisms for embryonic SVZ phagocytosis remain to be clarified. Sultan et al. (2013) used doxycycline (Dox) and liposomal clodronate to deactivate microglia and demonstrated that Dox directly increased neurogenesis in the adult mouse SGZ (6 w), emphasizing that multiple tools should be used to suppress microglial activation and the reproducibility of the results should be confirmed to study about the contribution of microglial activation to the actions. More recently, using multiple mouse models, including cell-depletion approaches and cx3cr1$-/-$, CR3$-/-$, and DAP12$-/-$ mutants, embryonic microglia in the forebrain were shown to act as modulators of dopaminergic axon outgrowth and neocortical interneuron positioning (Squarzoni et al., 2014). The microglial functions during brain development have increasingly attracted more attention to provide a framework for understanding the etiology of neuropsychiatric diseases.

The SVZ is a niche of life-long neurogenesis and oligodendrogenesis. Neurogenesis is initiated in quiescent type B stem cells that, upon activation to their proliferative state (activated type-B cells), give rise to type C transit amplifying progenitors, which subsequently generate type A neuroblasts (Doetsch et al., 1999; Garcia-Verdugo et al., 1998; Ihrie and Alvarez-Buylla, 2011). Adult-born SVZ type A cells migrate along the rostral migratory stream (RMS) to the OB, where they differentiate into GABA- and dopamine (DA)-releasing interneurons (Lledo et al., 2006; Luskin, 1993). In contrast,

newly generated oligodendrocytes migrate toward the corpus callosum, the striatal white matter tracts and the fimbria fornix (Gonzalez-Perez and Alvarez-Buylla, 2011; Hack et al., 2005; Menn et al., 2006). The SVZ NSCs are therefore important in terms of cell lineage determination and their potential application in clinical therapy. Although it has been shown that microglia play bidirectional roles for the maintenance of proper circuitry, i.e., microglia eliminate unnecessary cells, axons, and synapses, while support the neighboring ones (Ueno and Yamashita, 2014), limited information is available especially regarding the interaction between microglia and postnatal-adult SVZ neurogenesis. During the first few days after birth, a marked increase in the number of microglia is observed in the rodent brain (Sminia et al., 1987). As brain development progresses, amoeboid microglia become less abundant, with a concomitant increase in the number of ramified microglia, which acquire a surveillance role (Ginhoux et al., 2010; Prinz and Mildner, 2011; Schlegelmilch et al., 2011). However, the microglia in the neurogenic zone, such as the SVZ, have different functions compared with those in the non-neurogenic regions in terms of constitutive and postlesion levels of microglial activation (Goings et al., 2006).

Recently, we demonstrated that activated microglia first accumulate in the SVZ during the early postnatal period and then disperse to white matter where they became more ramified. In addition, the number of activated microglia was highest in the medial SVZ throughout the study period (P1–P30). Using a combination of *in vivo* and *in vitro* approaches, we demonstrated that these activated microglia in the early postnatal SVZ enhanced neurogenesis and oligodendrogenesis via cytokine release (Shigemoto-Mogami et al., 2014). The addition of microglia-conditioned medium to cultured P8 mouse SVZ cells increased neuroblast production (Walton et al., 2006), which is consistent with our data. Despite the lifelong presence of NSCs, a progressive reduction in neurogenesis is also observed in the aging SVZ (Tropepe et al., 1997). Our data also demonstrated that microglia adopt a more ramified shape by P30 (Shigemoto-Mogami et al., 2014). Walton et al. (2006) demonstrated that conditioned medium from adult-derived microglia is less effective in reconstituting inducible neurogenesis; however, this medium is markedly more effective in promoting the rapid morphological development of axonal processes. Taken together, these data suggest that the SVZ microglia undergo phenotypic changes during aging.

The effects of microglia on SVZ neurogenesis appear to differ in the rostrocaudal and dorsoventral locations. In our study, the number of activated microglia in the early postnatal SVZ was highest in the center plane along the rostrocaudal axis (Shigemoto-Mogami et al., 2014). Blood vessels may be related to this rostrocaudal distribution. The vasculature may be a critical niche compartment for stem cells in the adult SVZ (Goldberg and Hirschi, 2009; Quaegebeur et al., 2011). The SVZ is extensively vascularized by a rich plexus of blood vessels (Ihrie and Alvarez-Buylla, 2011; Shen et al., 2008; Tavazoie et al., 2008), and the central SVZ has large blood vessels that originate from the ventral aspect (Dorr et al., 2007; Shen et al., 2008). Among the soluble factors released from blood vessels (Goldberg and Hirschi, 2009; Shen et al., 2004), CXCL12/CXCR4 signaling may be involved in the accumulation of microglia in the center plane because microglia express CXCR4 and are recruited in the developing cerebral cortex by CXCL12/CXCR4 signaling (Arno et al., 2014). The adult SEZ (subependymal zone to discriminate from the embryonic SVZ when no ependymal cells are present) is highly regionalized, with neuronal progeny of distinct identities being generated in different areas along its dorsoventral and rostrocaudal axes (Brill et al., 2009; Merkle et al., 2007). The dorsal SEZ has an increased rate of oligodendrogenesis compared with the lateral SEZ (Ortega et al., 2013). The contribution of SVZ microglia to each progeny can be clarified if we specifically focus on the subregions in the SVZ along the dorsoventral and rostrocaudal axes.

Mechanisms That Underlie the Neurogenic Effects of Microglia in the Postnatal and Adult SVZ

As mentioned previously, the interaction between microglia and T cells is important for SGZ neurogenesis (Ziv et al., 2006). In an *in vitro* study, NSCs/NSPs were collected from the adult SVZ to demonstrate that neurogenesis and oligodendrogenesis are enhanced by microglia stimulated by Th2-derived cytokines (Butovsky et al., 2006). In our study (in the early postnatal SVZ), although the small population of activated microglia produced IGF-1, IGF-1 did not play a primary role in the neurogenic effects of activated microglia (Shigemoto-Mogami et al., 2014). We demonstrated that the neurogenic effects of early postnatal SVZ microglia were mediated by a combination of cytokines. We determined that the levels of IL-1β, IL-6, TNF-α, and IFN-γ in the SVZ cytosol during the early postnatal period were increased transiently compared with other periods (P1–P30) and that minocycline suppressed both neurogenesis and the cytokine levels significantly. Given that we could reproduce the neurogenic effects of activated microglia and the suppressive effects of minocycline in an *in vitro* neurosphere assay, we further examined the contribution of each cytokine to the effects of activated microglia. Interestingly, in our *in vitro* co-culture experiments, the enhancement of neurogenesis was suppressed by a mixture of function-blocking antibodies (anti-IL-1β, anti-IL-6, anti-TNF-α, and anti-IFN-γ) but not by any single

antibody. These results suggest that these cytokines enhance neurogenesis and oligodendrogenesis cooperatively. In support of this model, among these four cytokines, we confirmed that only IL-1β and IFN-γ enhanced neurogenesis, whereas only IL-1β and IL-6 exhibited the potential to enhance oligodendrogenesis. Previous reports have demonstrated that neural progenitor cells express IL-1β, IL-1RI, and IL-1RII and that IL-1β regulates the proliferation and differentiation of neural progenitor cells (Wang et al., 2007). Furthermore, IL-6 and IL-6R have been reported to promote neurogenesis (Islam et al., 2009). Li et al. (2010) showed that the effects of IFN-γ are modified in the presence of microglia, supporting a model of complementary interactions between cytokines. IL-1β, IL-6, TNF-α, and IFN-γ are proinflammatory cytokines that have been reported to suppress neurogenesis in pathological conditions, such as chronic LPS stimulation (as described in the SGZ chapter; Monje et al., 2003), allergic encephalomyelitis (EAE; Ben-Hur et al., 2003), and SE (Iosif et al., 2006; Koo and Duman, 2008). However, recent reports have indicated that a slight modification of the LPS application protocol induces a phenotypic change in microglia (Cacci et al., 2008). The microenvironment and ambient conditions may regulate the combination and concentrations of the cytokines that are released by microglia. In fact, some reports have suggested that the effects of cytokines change in a concentration- and context-dependent manner (Bernardino et al., 2008; Cacci et al., 2008; Das and Basu, 2008; Russo et al., 2011). Bernardino et al. (2008) demonstrated that TNFα results in NSC proliferation at 1 ng/mL but causes apoptosis at 10–100 ng/mL. A more recent study has confirmed that TNFα signaling via TNFR2 is required for basal neurogenesis, whereas signaling via TNFR1 impairs neurogenesis (Chen and Palmer, 2013). Our *in vivo* data indicate that when microglial activation is suppressed by minocycline, the number of neural progenitors decreases to fewer than half of the number observed under control conditions; however, the decrease in each cytokine level was uniform and mild. Cytokines are released by not only microglia but also other glia. Cytokine release from microglia may stimulate cytokine release from other cell types in a manner akin to "cytokine drizzling" (a milder effect than a "cytokine storm"; Clark, 2007).

The next question to be resolved is to determine what activates SVZ microglia in the early postnatal SVZ. A valuable clue is that the role of microglia in postnatal SVZ neurogenesis differs from that in embryonic neurogenesis (Cunningham et al., 2013). Circulating mediators and hormones are altered after birth (Spencer et al., 2008). During pregnancy, maternal proinflammatory responses are suppressed (Aguilar-Valles et al., 2007) and anti-inflammatory responses are increased (Ashdown et al., 2007). The levels of circulating estrogen and progesterone increase and progesterone rapidly decreases immediately before birth (Mesiano and Welsh, 2007). We speculate that SVZ microglia function as a "hub" that may trigger an optimal "cytokine nest" for neurogenesis in response to peripheral signals. Interestingly, neural progenitors contact blood vessels at sites devoid of the blood brain barrier (BBB) in the SVZ (Tavazoie et al., 2008), and neural progenitors regulate microglia, proliferation, migration, and phagocytosis via the secretion of immunomodulatory proteins (Mosher et al., 2012). The interaction between neural progenitors and microglia may also be important in terms of peripheral signal delivery to the brain.

Molecular Signaling Regulates the Activation State of Microglia

The development of microglia, including their renewal, during neuronal development has been largely unstudied. However, this information is important for understanding the temporally and spatially specific effects of microglia on neurogenesis during brain development. Runt-related transcription factor 1 (Runx1), a key regulator of the proliferation and differentiation of hematopoietic stem cells (HSCs; Burns et al., 2005), is expressed in forebrain microglia during late embryogenesis and the first 2 weeks of postnatal development (Zusso et al., 2012). Runx1 regulates microglial proliferation throughout the neurogenic regions (Logan et al., 2013) during development (Ginhoux et al., 2010). Runx1 has also been demonstrated to be necessary for the transition from the proliferative activated amoeboid state to the deactivated ramified phenotype in the postnatal mouse SVZ (Zusso et al., 2012). Runx1 is a major genetic target of Notch signaling (Burns et al., 2005), and amoeboid microglia express Notch1 together with its ligands Jagged-1 and Delta-1 from P1 to P10 (Cao et al., 2008). During this period, microglial populations supporting neurogenesis or oligodendrogliogenesis acquire gene expression of adult microglia (after P4; Butovsky et al., 2014). TGFβ1 signaling is also essential for microglia to adapt to the adult brain environment. TGFβ signaling induces the quiescent microglial phenotypic characteristics of adult microglia *in vitro*, and most microglia are lost in Tgfb1−/− mice. Similar to tissue macrophages, microglia are dependent on Csf-1R for their development (Erblich et al., 2011; Hamilton, 2008). Recently, a new cytokine, IL-34, has been identified in mice and humans and shown to bind Csf-1R with high affinity (Lin et al., 2008). The expression of IL-34 rescued the phenotype of Csf-1-deficient mice in a Csf-1R-dependent manner (Wei et al., 2010), suggesting that Csf-1 and IL-34 both regulate Csf-1R signaling. In specific areas of the adult brain, including the cortex, olfactory bulb, ventral striatum, and hippocampus, microglia rely on IL-34/Csf-1R signaling for their own maintenance (Greter et al.,

2012). Whether IL-34 and Csf-1 have complimentary or redundant roles remains to be established.

Heterogeneity of Microglia and Neurogenesis

As described above, microglia have diverse effects on neurogenesis in a spatially and temporally specific manner. In general, we use the term "activated" to describe the microglial phenotype in contrast to the ramified phenotype. However, recent studies have demonstrated that ramified microglia are not only quiescent, i.e., ramified microglia survey their territory actively with fine processes and receive environmental stimuli as sensory cells (Kettenmann et al., 2011). Currently, the definitions of microglial "activation" are ambiguous. Microglial phenotypes are determined by the microenvironment, including the myelin content, vascular and BBB features, cellular neighborhood, extracellular matrix constituents, prevalent neurotransmitters, and neurochemical milieu (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Lawson et al., 1990). Hippocampal microglia express increased levels of mRNA for TNFα, CD4, and FcγRII compared with microglia in the diencephalon, tegmentum, cerebellum, or cerebral cortex (Ren et al., 1999). NT3 expression is identified selectively in microglia from the cerebral cortex, globus pallidus, and medulla but not from other brain regions (Elkabes et al., 1996). Even within a given region, the reactive phenotypes in response to infection or injury appear to be represented by subsets rather than by a uniform population of microglia (Scheffel et al., 2012). A single population of microglia can potentially adopt various phenotypes; however, whether these phenotypic differences can be accurately classified as "subtypes" remains unclear. Therefore, categorizing the neurogenic microglia present in the SGZ and SVZ may be difficult at present.

Furthermore, the M0, M1, and M2 stages are considered the functional phenotypes of microglia at present. These delineations are based on data regarding monocyte and macrophage biology (Sica and Mantovani, 2012). Macrophages that have been exposed to different stimuli are designated M1, M2a, M2b, and M2c depending on the stimulus and context (Geissmann et al., 2010; Mantovani et al., 2002; Mills, 2012). Approximately 50 surface markers that are characteristic of the mononuclear phagocyte lineage have been identified for macrophages, and 35 of these markers are considered useful for discriminating subsets of macrophages and dendritic cells (Chan et al., 2007). M1 microglia are proinflammatory and cause neurogenesis failure. However, whether anti-inflammatory M2 microglia represent the active neurogenic phenotype remains unknown. The characteristics of M2 microglia are not necessarily associated with positive outcomes; they can induce an inappropriate downregulation of the inflammatory response (Mantovani et al., 2002). Moreover, stimulus-induced transcriptional plasticity of microglia has been described. Because this type of plasticity does not correspond to M1 or M2 plasticity and is distinct from the resting state transcriptome, this stage has been referred to as M0 (Butovsky et al., 2014). Furthermore, the existence of intermediate phenotypes is possible. P3 mouse brain microglia express increased levels of both M1 (iNOS and TNFα) and M2 (Arginase-1) genes compared with adult microglia (Crain et al., 2013). The systemic intraperitoneal administration of LPS (1 mg/kg) to P5 mice increased the proliferation of microglia/microglial precursor cells and caused a transient inhibition of neuronal differentiation in the SVZ (Smith et al., 2014). The Cd86 (M1) and Ym1 (M2) genes were both upregulated at 48 h, whereas iNOS (M1) was upregulated, and IL-6 (M1) was downregulated. These data suggest that multiple microglial phenotypes, which cannot be simply categorized as M1 or M2, may exist. Recent comparative analyses have demonstrated that microglia exhibit distinct phenotypic and functional properties compared with peripheral macrophages regarding their responsiveness to M1-M2 polarization conditions (Durafourt et al., 2012). In addition to the M1-M2 classification, HoxB8 expression also divides microglia into positive and negative groups. HoxB8-expressing cells represent approximately 15% of the CD11b+ cells in the adult brain and may be ontogenically distinct from the yolk sac-derived dominant population of microglia (De et al., 2013). The characterization of this population is important because mice with a complete loss of HoxB8 exhibit excessive grooming, leading to hair loss and skin lesions; these phenotypes are similar to the human phenotype of the OCD spectrum disorder trichotillomania (Chen et al., 2010). The relationship between HoxB8 expression and neurogenic potential remains unknown.

A Note regarding the Experimental Design

An instructive role of microglia in the regulation of neuronal differentiation was first demonstrated by *in vitro* studies. *In vitro* microglial systems can never faithfully reproduce the complex characteristics of the *in vivo* environment; however, these systems allow us to investigate specific aspects of microglia that are masked by surrounding factors. Aarum et al. (2003) demonstrated that microglia can guide the differentiation of precursor cells isolated from the embryonic brain, as well as adult mouse neural precursor cells toward a neuronal phenotype. Reports that are more recent have demonstrated that microglia possess intrinsic, spatially restricted characteristics that are independent of their *in vitro* environment and that they represent unique and functionally distinct populations. SGZ microglia *in vitro* are uniquely capable of providing sustained levels of inducible neurogenesis (Marshall et al., 2014). Furthermore, the neurogenic

phenotype acquired from wheel running is sustained *in vitro* (Ziv and Schwartz, 2008a). However, isolated microglia do not exhibit the highly ramified structure that is typically observed in the healthy brain (Neiva et al., 2014). One of the major reasons for differences between *in vivo* and *in vitro* microglia is serum. In living organisms, microglia reside behind the BBB and are shielded from plasma proteins (Bechmann et al., 2007). *In vitro* cultures include high concentrations of serum (derived from plasma, to which microglia are never exposed in the healthy brain) (Ransohoff and Perry, 2009). Gene expression analysis has indicated that microglial cultures stimulated with cytokines and bacterial cell-wall components expressed genes in a pattern that was more similar to macrophages (challenged with the same stimuli) from the abdominal cavity compared with microglia isolated from animals that received the same cocktail of cytokines and bacterial components injected into the brain (Schmid et al., 2009). Plasma fibrinogen may be a key component of serum (Adams et al., 2007). Fibrinogen-stimulated microglia exhibit increased phagocytic capacity, an effect that is mediated via AKT- and Rho-dependent pathways (Ryu et al., 2009). *In vitro* microglial cultures are useful as long as the differences between *in vivo* and *in vitro* systems are acknowledged. To achieve convincing significance from an *in vitro* system, confirming that the observed phenomena are reproduced *in vivo* and demonstrating that the effect in question is mediated via the same signaling pathways are essential.

At P4, male rats have a significantly greater number of microglia compared with females in many brain regions critical for cognition, learning, and memory, including the hippocampus, parietal cortex, and amygdala (Schwarz and Bilbo, 2012). The same authors also demonstrated that females have a greater number of microglia later in development (P30-60). Most microglia in P4 males have been demonstrated to have an activated/amoeboid morphology, whereas microglia in P30-60 females exhibited a more ramified morphology. Furthermore, the hippocampal and cortical expression profiles of numerous cytokines, chemokines and their receptors shift dramatically during development and are highly sex dependent (Schwarz et al., 2011). In human females, the number of brain microglia has been demonstrated to increase with age (Pelvig et al., 2008). A strong sex bias is known to exist with respect to the prevalence of many neuropsychiatric developmental disorders that exhibit differences in the latency to onset, as well as to strong dysregulation of the immune system. For example, males are more likely to be diagnosed with early-onset developmental neurological disorders, such as autism, dyslexia, and schizophrenia (Bao and Swaab, 2010). Therefore, sex differences should be considered in animal experiments, particularly for studies that investigate animal models of developmental disorders.

Closing Remarks

In this review, I have summarized and organized the data regarding the effects of microglia on neurogenesis, particularly focusing on the SGZ and the SVZ, in which the neurogenic potential is progressively restricted during the life of the organism. As described above, microglia regulate neurogenesis in a temporally and spatially specific manner. To further understand the physiological significance of the role of microglia in neurogenesis, the development of microglia in the brain should also be clarified. The data introduced here raise the possibility that microglia sense signals from the surrounding environment and have regulatory effects on neurogenesis. We speculate that microglia function as a "hub" for information from the inner and outer brain regions during neurogenesis regulation. When the precise mechanisms for the enhancement of neurogenesis, interaction between microglia and NSCs, and delivery of peripheral signals into the brain are clarified, these new findings will be helpful in the clinical therapy of neuronal disorders, including developmental disorders.

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［パネル討論］神経伝達物質を視るデバイスが拓く神経科学の展開 －求められる技術を開発する技術－

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あらまし　生物学的な酵素反応、化学的な表面処理、工学的な導波技術を利用した新しい神経伝達物質可視化デバイスを開発し、多くの医学・生理学研究に利用している。ユーザーであり同時に開発者となった立場から，神経科学者が求めているデバイス開発のあり方を考える。

キーワード　酵素光学法，表面光，酵素担持，神経伝達物質，時空間分布

Development of new device of neuronal development by neuroscientist and engineer, for neuroscientist

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Abstract　We have developed the new enzyme-linked photo assay devices in order to visualize spatiotemporal release of transmitters, and reported their release in developing cerebellar cortex. A model case of requirement-dependent development would be discussed.

Keywords　enzyme-linked assay, evanescent light, immobilization, neurotransmitter, spatiotemporal information

1. 神経情報伝達のメカニズム

1.1. 電気的情報伝達と化学的情報伝達

神経細胞の情報伝達メカニズムには、イオン起電力を用いて細胞内を伝わる電気的な情報伝達と、化学物質＝神経伝達物質を用いて細胞間を伝わる化学的な情報伝達が存在する。電気的情報伝達は，微小電極を用いた電気生理学的な手法で研究され，近年は電位感受性色素を用いた空間的情報伝播の研究も進んでいる。

これら電気生理学的な手法は、情報の受け手側のスパイク的な速い反応を見ている。一方，発達過程の神経回路では受け手の神経がまだ未発達で、速い電気的変化を示さない。このような場合，化学的な情報伝達である神経伝達物質の放出量変化を観察し、情報の出し手側の反応を観察することが有用になる。従来化学的な情報伝達は，マイクロダイアリシスと高速液体クロマトグラフィにより観察されてきたが，この方法には空間的な情報が大きく欠落している。また、伝達物資を受け取る受容体タンパク質の発現、またはmRNAの転写の観察では，時間的情報が大きく制限される。

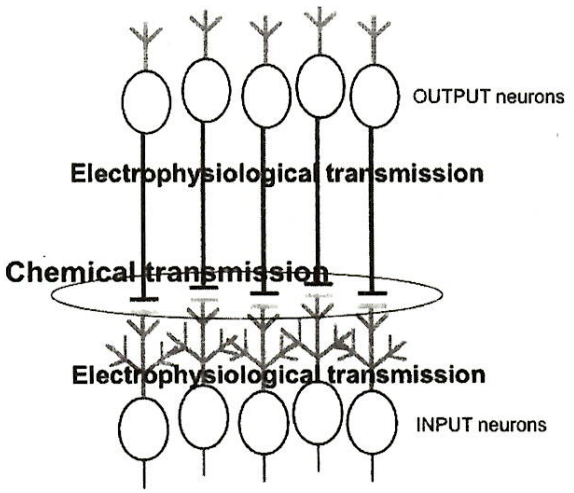

図１　情報出力細胞群から入力細胞群への情報伝達

1.2. 小脳発生過程に必要な情報はなにか

著者の研究対象である発達過程の小脳では，幼弱な神経細胞の増殖・移動・神経回路形成に、時間的に変化する伝達物質放出が関与することが示唆されていたが，上記の従来手法ではこれを観察することが困難だった。伝達物質グルタミン酸、およびγアミノ酪酸（GABA）の小脳皮質内空間分布と時間変化が、研究に必要な情報だった。

2. 酵素光学法による伝達物質観察デバイス

我々が構成しているデバイスは、①酸化還元酵素を用いた蛍光観察、②UV-LED 光源を用い、ガラス導波路表面からの励起による組織障害の軽減、③シラン化剤による酵素のガラス表面固定化、を特徴とする[1]。

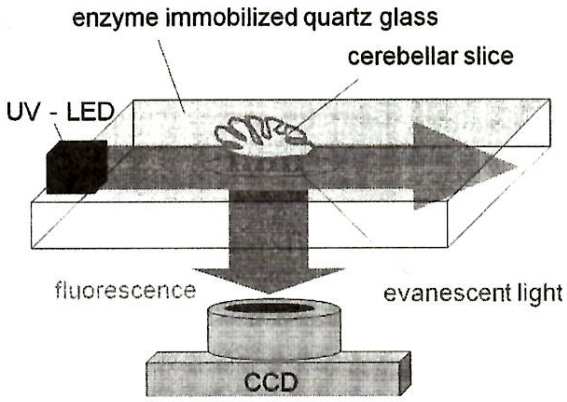

図２　固定化酵素光学法によるデバイスの構成

これは現場の神経科学者がユーザーとしての必要から、従来からある酸化還元酵素を用いた光学測定と、エンジニアの協力を得ながら開発したデバイスである。

3. このデバイスでみえたもの

小脳発達の過程で，神経細胞が未分化の部位・時期に GABA の放出が見られることが確認された。以前より、遺伝子改変動物の研究[2]から、グリア細胞による伝達物質の放出と制御が示唆されていたが、伝達物質の空間分布と時間変化が観察できることで，脳発生におけるグリア細胞の寄与が脳の各所で観察されるようになった[3]。

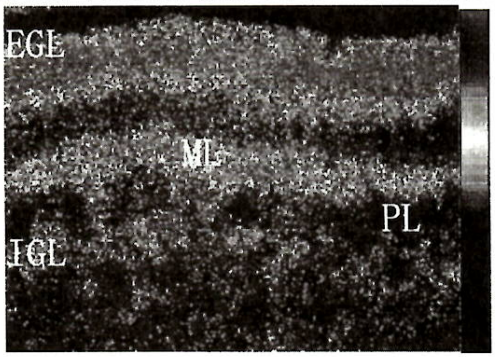

図３　生後３日の小脳皮質での GABA 放出分布

4. デバイスの応用と発展

神経研究者が欲しているのは、自分の欲しい情報を与えてくれる技術である。我々のデバイスは，パーキンソン病の臨床研究に、網膜変性の基礎研究に、あるいは糖尿病の予防効果の研究へと、応用の幅を広げてきた[4]。ATP 放出の空間分布を測定したいというオーダーを受けて新規測定法の開発も行っている。これらはアルツハイマー症、脳虚血などの研究に利用が広がっている[5]。

5. 求められる技術を開発する技術

我々のデバイスは、決して特殊で卓越した技術によって展開されたものではない。これが神経研究者に受け入れられ利用されているのは、彼らの必要とする情報を提供するデバイスだからであり、情報を得るために工学技術を提案し開発するエンジニアと共同研究できるからである。

個々のエンジニアが持つ卓抜した技術が「求められる技術」になるには、ユーザーが求める情報を知る能力、あるいは、ユーザーとエンジニアを仲介する“インターフェース”研究者が必要だろう。ユーザー側の学会で情報収集し求められる技術を汲み取る能力、そしてそれを実機に具体化する高い技術力が、これからのエンジニアに求められている。

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解　　説

発達期小脳アストロサイトの機能と秩序形成

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The Astrocytes as the Conductor of the Cerebellar Development —Their Function and Order—

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概要

小脳神経回路の形成に大きく影響する伝達物質を可視化するため，生物学的な酵素反応，化学的な表面処理，工学的な光導波技術を利用した新しい神経伝達物質可視化デバイスを開発した．これを用い，アストロサイトが発達期小脳において「雰囲気」と時間秩序を作っていることを観察した．

1.　小脳神経回路の発達過程

大きく秩序だった層構造を持つ大脳・小脳は，高等脊椎動物に特徴的な神経系である．ほ乳類の小脳は深い脳溝によって大脳に匹敵する広大な表面積をもち，5種の神経細胞が一定の様式で層状に配列し小脳皮質を形成する．小脳の発生過程では，γ-アミノ酪酸（GABA）作動性神経細胞であるプルキンエ細胞，ゴルジ細胞，バスケット細胞，星状細胞がすべて脳室帯（ventricular zone）で発生し[1]，小脳板表面に順次整列する．唯一のグルタミン酸作動性神経細胞である顆粒細胞は胎生期の菱脳唇で発生し，未分化な状態で小脳板表面に遊走し，小脳皮質の最表面に未分化細胞の層である外顆粒層（external granular layer；EGL）を形成する．これらの神経細胞の発生には，いくつかの特異的な遺伝子の発現が必要であることが示唆される[2]．出生時のラット・マウスの小脳は，外顆粒層と未発達のプルキンエ細胞層（Purkinje cell layer；PL）から構成されるが，生後約2週間かけて顆粒細胞が分裂・分化しプルキンエ細胞下に遊走して内顆粒層（internal granular layer；IGL）を形成，同時にプルキンエ細胞は樹状突起を伸長させ，顆粒細胞および脳室帯から遊走してくるゴルジ細胞，バスケット細胞，星状細胞と神経回路を構成して皮質表面に分子層（molecular layer；ML）を形成し，成熟した小脳の3層構造をかたち作る[3]（Fig. 1）．

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神経回路の構造的秩序は，未分化の神経細胞の集団からどのように作られるのだろうか．小脳の神経回路を制御する要素として細胞内リン酸化因子や細胞表面タンパク質による誘導などがあげられてきたが，本論では神経回路本来の情報伝達分子，特にGABAの機能に注目してみたい．

2.　神経発生におけるGABA

GABAは脳の主要な抑制性神経伝達物質であり，また幼弱な神経や損傷した神経では興奮性の伝達物質として働く（GABA興奮）．GABA受容体の阻害剤を用いた研究から，神経発生過程でGABAが増殖刺激や神経細胞の遊走制御，神経細胞の成熟促進などの多様な働きを持つことが示唆されている[4]．小脳発生においてもGABAが重要な分子であることが示唆されていたが，出生直後の小脳ではGABA作動性神経細胞は未発達で，顆粒細胞が増殖・分化する外顆粒層にはほとんど分布していない．神経伝達物質の放出量変化を調べる方法であるマイクロダイアリシスと高速液体クロマトグラフィには空間的な情報が大きく欠落しており，伝達物資を受け取る受容体タンパク質の発現，またはmRNA転写の観察では時間的情報が大きく制限される．発達期の神経回路を創出する物質的ダイナミズムを観察するには，情報分子の空間分布と時間変化を可視化する必要がある．

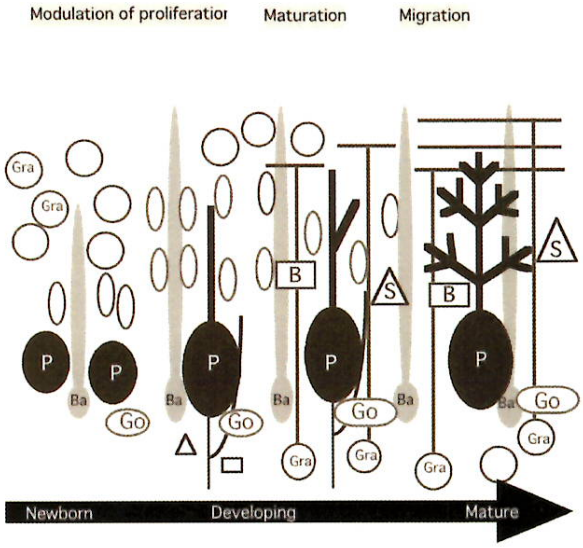

Fig. 1　Diagram of cerebellar development. In newborn cerebellum (1 to 3 days after birth), granule cell progenitors (Gra) proliferate in the EGL while immature Purkinje cells (P) form the PL with Golgi cells (Go) and some Bergmann glia (Ba). On developing, 5 to 7 days after birth, Gra-cells elongate their axon and migrate inside, and P-cells spread their dendrites and connect to other neurons during 2 weeks. B, Basket cell, S, satellite cell

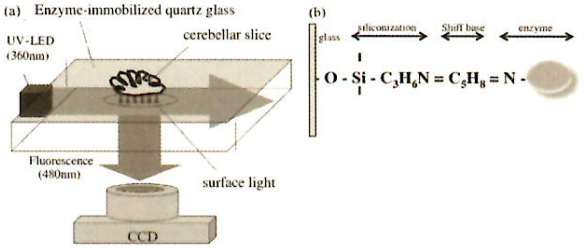

Fig. 2　Enzyme-linked photo assay device with the surface UV excitation lightning and covalent immobilization of enzymes (a). Transmitter molecules released from the sample are degenerated by the enzyme and generated NAD(P)H is excited by UV-LED generating narrow band 360 nm UV light. The cooled CCD catches the fluorescence. (b) N-terminal of enzymes is bound on the siliconized quartz glass surface.

3.　酵素光学法による伝達物質可視化デバイスの開発

そこで我々は，伝達物質 GABA やグルタミン酸の放出分布と時間変化を可視化するために新規光学デバイスを開発した（Fig. 2）．

本デバイスは，①酸化還元酵素を用いた伝達物質放出の蛍光観察，②UV-LED 光源を用い，ガラス導波路表面からの励起によって自家蛍光と組織障害を軽減，③シラン化剤による酵素のガラス表面固定化，を特徴とする[5]．薄切した小脳組織を十分な酸素供給の後ガラス基板上に載せ，組織と基板の界面に放出された GABA およびグルタミン酸は，GABA 分解酵素およびグルタミン酸脱水素酵素の酵素反応により化学量論的に還元型ニコチンアミドアデニンジヌクレオチド（リン酸）（NAD(P)H）を生成する．これを 360 nm で励起し 480 nm の蛍光を発生させ，放出分子の分布を観察する．基質選択性の高い酵素反応を用いるため，放出される伝達物質に特異的に反応して蛍光を発する（特徴①）一方，NAD(P)H は細胞内に普遍的に存在する分子であるため，励起光により細胞内から自家蛍光が発生する．そこで励起光源にガラス導波路を用い，表面からごく浅い範囲に励起光を照射することとした（特徴②）．さらにシラン化剤により酵素をガラス表面に固定化する（特徴③）ことで，簡易なバイオデバイスを構成した．

4.　発達期小脳で放出される GABA とその由来

発生過程の小脳皮質からの GABA 放出を観察したところ，出生直後の外顆粒層からの GABA 放出が観察された．この層には GABA 作動性神経細胞が殆ど分布しておらず，非神経性の GABA 放出であることが示唆された．外顆粒層からの GABA 放出は生後 1 週間程度続き，バスケット細胞・星状細胞が分布して神経性の GABA 放出が始まると消失することが観察された[6]（Fig. 3 a–d）．

外顆粒層では GABA の持続性電流も観察された．これらの GABA は顆粒細胞分裂を促進すると考えられた[7]．

小胞性 GABA トランスポーター（VGAT）遺伝子改変動物の観察[8]から，発達期の小脳皮質でグリア細胞の一種であるアストロサイトが GABA の放出と制御を行っていることが示唆された．さらに小脳以外の脳発生過程でも，伝達物質観察デバイスを用いることで，グリア細胞からの GABA 放出が脳発生に寄与することが観察された[9]．そこで小脳皮質から単離した

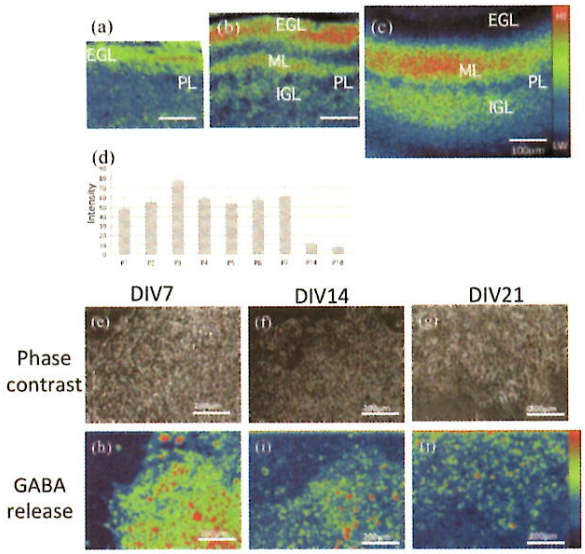

Fig. 3 GABA release from developing cerebellar cortex; (a) postnatal day 1 (P1), (b) P3, (c) P8. (d) shows temporal change of GABA release. (e) to (f) show GABA release from cultured cerebellar astrocytes. DIV: day *in vitro*.

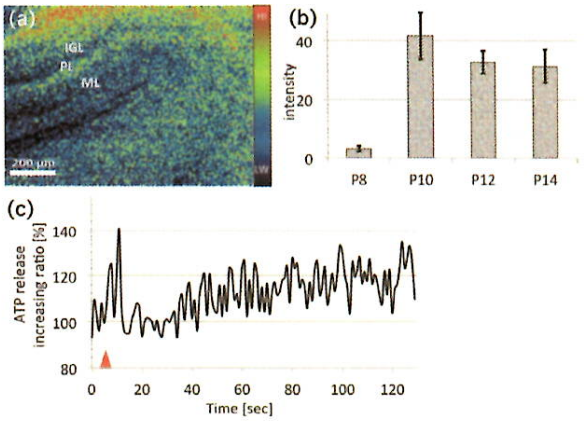

Fig. 4 (a) shows released ATP distribution in P10 cerebellar cortex. ATP release was suddenly increased from P10. (c) shows the time course of ATP release to 100 μM glutamate application.

培養アストロサイトを観察したところ，培養初期の細胞から GABA 放出が観察された (Fig. 3 e–f)．これらは GABA 合成酵素 (GAD) を発現しており，発達期の小脳アストロサイトは神経細胞非存在で GABA を合成し放出していることが示唆された[10]．GABA の放出は培養日齢とともに減少し，同時に GAD, VGAT の発現も急激に減少した．

5. アストロサイトが発生の場を作る

アストロサイトは従来神経細胞をサポートする非神経細胞ととらえられてきたが，近年神経伝達物質の取り込みおよび再放出によって，神経回路の「雰囲気」を作り，情報処理に直接作用することが知られてきた．特に発生期の脳組織では，アストロサイトが未分化の神経細胞の機能を代替することが知られている．我々は発生初期の小脳外顆粒層で，GABA が未分化の顆粒細胞からグルタミン酸放出を誘発することを観察している．未分化の培養顆粒細胞が細胞内リン酸化によってグルタミン酸を放出することは知られていたが，生体内ではアストロサイト由来の GABA によって顆粒細胞の増殖刺激とグルタミン酸放出に至る分化誘導が引き起こされることが示唆された．

では神経細胞間のシナプス形成や樹上突起発達に対し，アストロサイトはどのような寄与を示すのだろうか．アストロサイトの代表的な伝達物質 ATP は痛覚や炎症の情報分子として知られており，発達期の小脳では，プルキンエ細胞と顆粒細胞のシナプス形成と，プルキンエ細胞樹上突起の発達に関与することが知られている[11]．ATP は通常ルシフェリン–ルシフェラーゼ発光反応 (L-L 反応) によって測定されるが，この反応は高感度だが光量が少なく，ATP 放出の空間分布・時間変化を CCD で測定することが困難だった．

そこで，ATP 要求性の酸化還元酵素反応であるグリセルアルデヒド 3-リン酸脱水素酵素 (GADPH) を用い，100 μM のグルタミン酸刺激によって放出される ATP を，酵素反応で化学量論的に発生する NADH によって可視化することを試みた．

生後 8 日の小脳組織ではほとんど ATP 放出が見られないが，生後 10 日の小脳では急激な ATP 放出の増加が見られた (Fig. 4c)．ATP 放出はグルタミン酸刺激から約 50 sec 後にゆっくりと上昇し，従来の L-L 反応の結果と一致していた．各種のグルタミン酸受容体アゴニストで刺激したところ，刺激される受容体サブタイプによって ATP 放出部位が異なることが見られ，アストロサイトが直接グルタミン酸刺激を受けるだけでなく，神経細胞の刺激を増幅・制御して ATP を放出していることが示唆された[12]．

アストロサイトからの小脳発生に関わる情報分子を Fig. 5 に示す．出生直後の小脳では GABA 作動性神経細胞が未発達であるため，アストロサイトは神経細

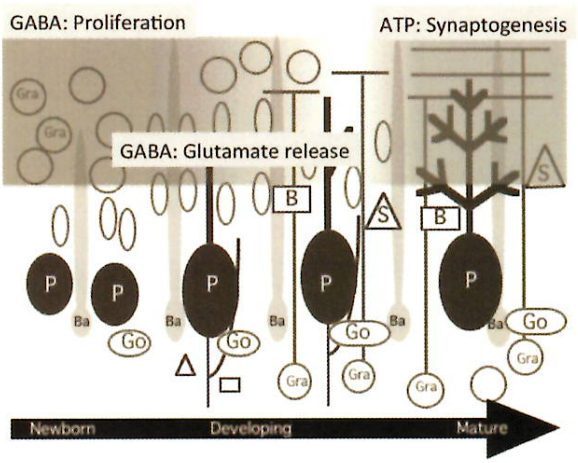

Fig. 5　Schematic diagram of glial coordination to cerebellar development. Released GABA would modulate granule cell proliferation and neuronal development, and release ATP would accelerate synaptogenesis between Purkinje cell and granule cells.

胞の代替としてGABAを放出し，顆粒細胞の増殖を促していると考えられる．さらに分化が進むと，顆粒細胞からグルタミン酸の放出を促し，同時にアストロサイトからのGABA放出は急激に消失する．代わりにグルタミン酸刺激によりATPを放出し，プルキンエ細胞と顆粒細胞のシナプス形成を誘導すると考えられる．

神経回路は，記憶することに見られるようにきわめて可塑性の高い組織である．これまで神経回路の秩序形成は，神経相互間の自己組織化から遺伝子発現による決定論的な形成まで様々の仮説で説明され，データの蓄積がなされてきた．そのなかでアストロサイトは，神経細胞が遊走する足場として，あるいは伝達物質やイオン環境の調整役として，「重要だがぼんやりした存在」と捉えられることが多かった．しかし脳皮質内で大きく広がり，細胞間の結合によって広い範囲の物質環境を制御するアストロサイトは，神経回路の発達過程で積極的に神経細胞を刺激し，構造的な秩序を作り出している．神経細胞との相互作用によりアストロサイト自身も分化し，機能を変化させながら，神経回路の機能発現にも関わっていることが示唆される．

6.　情報が見えることの意味

自分が知りたい情報を得る手段があるとは限らない．そういう情報を見たいがために開発してきた我々のデバイスは，小脳発生研究の枠を超えて，現在パーキンソン病の研究やアルツハイマー症，網膜変性の基礎研究に，あるいは糖尿病の予防効果の研究へと利用が広がっている．情報分子の可視化技術によって，神経細胞の生死を左右する脳の中のリズムを見ることが可能になり，神経回路の新しいルールが顕らかになることを期待している．

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Improvements in Enzyme-Linked Photoassay Systems for Spatiotemporal Observation of Neurotransmitter Release

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Neurotransmitters and neuronal releasing molecules are not only the regulators of neuronal function but also the indicators of neuronal conditions. Glutamate and γ-amino butyric acid (GABA) play important roles in cerebellar differentiation and function. In the mature cortex, they are released from synapses and taken up by transporter molecules. We have developed enzyme-linked photoassay systems for glutamate, GABA, and adenosine triphosphate (ATP), and reported their release in the developing cerebellar cortex. Our systems showed slow transmitter release in the immature cerebellum, whereas it was hard to detect the fast synaptic release from mature neurons, because there were some limitations in time resolution and data depth derived from a charge-coupled device (CCD), and the enzyme-linked photodevice was sometimes unstable. In this study, we report the dynamic observation of neurotransmitter release in the developing cerebellar slices using improved photodevices and a high-speed 16-bit CCD. With this new system, the rapid measurement of transmitter release in a young-adult cerebellar cortex is possible. We suggest that these photoassay systems are useful for observing synaptic release in several diseases.

1. Introduction

Neurotransmitter molecules released from neurons are not only the regulators of neuronal transduction but also the indicators of neuronal conditions.[1–3] Glutamate and γ-aminobutyric acid (GABA) are known as typical transmitters in the brain's cortex, and they play important roles as stimulators and suppressors, respectively. Lack of balance in the release of glutamate and GABA may lead to autism, epilepsy, or Parkinson's disease.[4]

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To observe spatiotemporal neurotransmitter release in the cerebellar cortex, we have recently developed an enzyme-linked photoassay system, which is a device with an immobilized enzyme on a quartz glass surface. Using this system, we observed glutamate or GABA release in developing cerebellar slices using either new or authorized methods.[5] Enzyme-linked photoassay is sensitive and selective, and it can discriminate the substrates from their pharmacological analogues. Our system can detect transmitter release in the cerebral cortex,[6] hippocampus, retina, and cultured cells,[7] and made it possible to detect the release of adenosine triphosphate (ATP),[8] glucose, sucrose, and fructose. On the other hand, enzymes tend to denature and separate from the quartz. For the detection of transmitter release in mature neuronal circuits, increasing the sensitivity and stability of the device is required.

In this paper, we propose new immobilizing methods and discuss the optimization of the enzyme-linked photoassay.

2. Materials and Methods

2.1 *Substrate and enzyme reaction*

Imaging neurotransmitter release was monitored for the reaction in which oxidoreductases generate reduced nicotinamide adenine dinucleotide (NAD^+) or diphosphonucleotide ($NADP^+$). For glutamate, GABA, or adenosine triphosphate (ATP) imaging, we used glutamate dehydrogenase, GABA disassembly enzyme [GABase, Fig. 1(a)] or glyceraldehyde 3-phosphate dehydrogenase, respectively.[9–11] The NADH or NADPH, the reductants of NAD^+ or $NADP^+$, respectively, which is generated stoichiometrically, emits 480 nm fluorescence after excitation at 340–365 nm.

2.2 *Surface photoexcitation*

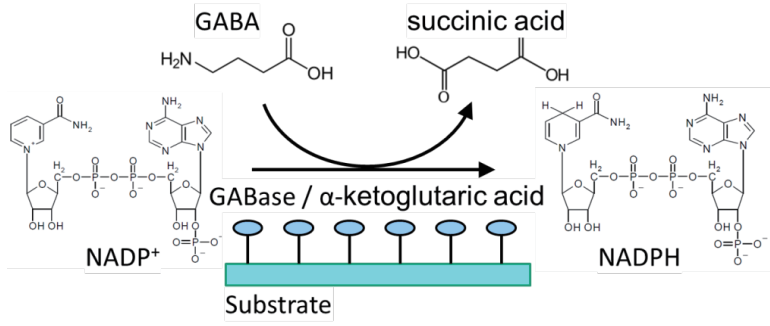
For UV excitation, a quartz glass plate illuminated with an ultraviolet light-emitting diode (UV-LED, Nichia, Tokushima, Japan) was used. Leaking UV light onto the glass surface excited fluorescent NADH or NADPH [Fig. 1(b)].

2.3 *Imaging apparatus*

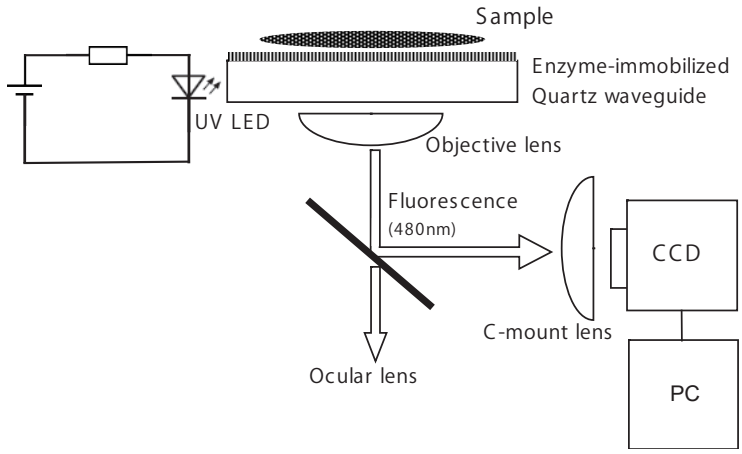
All fluorescence images through the inverted microscope (IX73, Olympus Co., Ltd., Tokyo, Japan) were observed by a cooled charge-coupled device (CCD) (ORCA-ER CCD) or a high-speed complimentary metal-oxide semiconductor (CMOS) (ORCA-Flash 4.0) camera, supplied by Hamamatsu Photonics Co., Ltd., Hamamatsu, Japan. Imaging data were analyzed by iVision software (BD Biosciences, San Jose, CA, USA).

2.4 *Enzyme immobilization and sample preparation*

Enzymes were typically covalently immobilized on the quartz glass surface using a silane coupling agent and a crosslinking agent, 3-aminopropyltriethoxy silane (3-APTS) and glutaraldehyde, respectively [Fig. 2(a)].[12] These surface modifications determine both the stability of the enzyme reaction and the distance between the sample and the glass surface.



(a)



(b)

Fig. 1. (Color online) (a) Released neurotransmitters are oxidized by the oxidoreductase, and NAD(P)H is formed stoichiometrically. In GABA oxidization, released GABA is converted to succinic acid with NADPH formation by GABase. (b) Schematic diagram of the enzyme-linked photoassay system. The oxidoreductases are immobilized on the quartz glass surface, and the excitation light radiating from UV-LED passes through the quartz waveguide. Fluorescent images are obtained by CCD or CMOS, and analyzed using a computer system.

In some cases, glass surfaces were treated with either aromatic crosslinkers, 1,4-phenylene diisothiocyanate (1,4-DIC), or 1,3-phenylene diisothiocyanate [1,3-DIC, Fig. 2(b)], and glutaraldehyde (GA).[13] Others were treated with a phosphonic acid, 11-aminoundecylphosphonic acid [11-AUPA, Fig. 2(c)], as a replacement for 3-APTS.[14]

Cerebellar acute slices were treated from postnatal day 3 (P3) to P15 in rats, sliced sagittally to a thickness of 400 μm with a rotor slicer (Dohan EM, Kyoto, Japan),

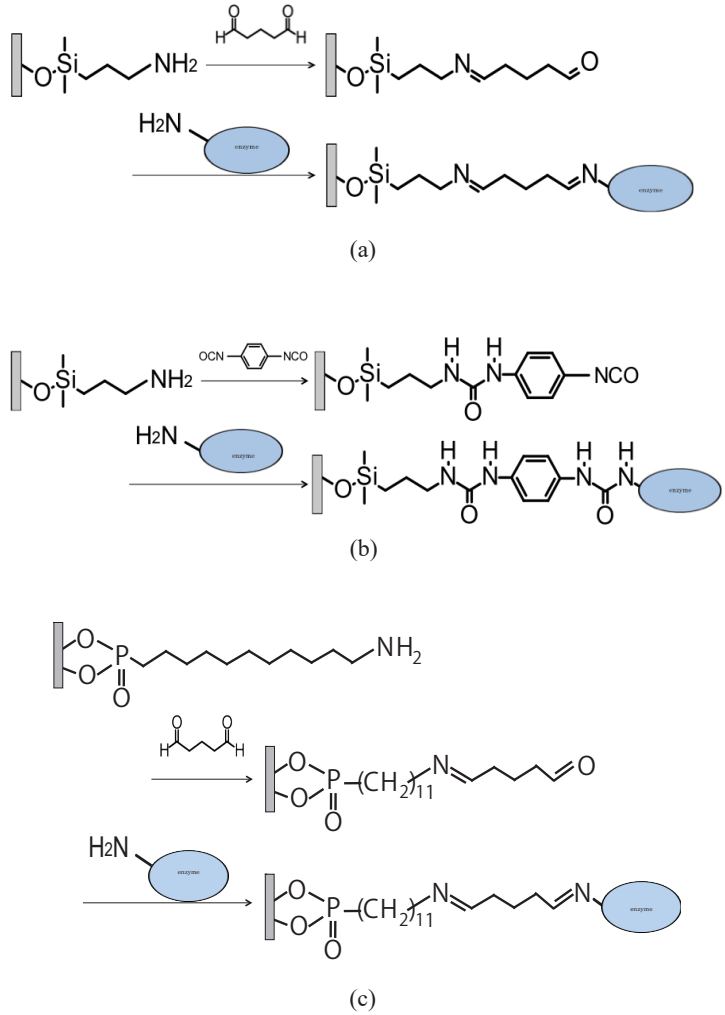


Fig. 2. (Color online) (a) Method of enzyme immobilization using 3-APTS and glutaraldehyde. Surface treatment and crosslinking between waveguide quartz and enzyme. (b) Crosslinking using 1,3-DIC. (c) Surface treatment with 11-AUPA.

and incubated in oxygen-aerated PBS for 45 min. All experimental procedures were approved by the committee for the use of animals at Toyohashi University of Technology and by the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

3. Results

3.1 *Spatiotemporal observation of glutamate release*

Figure 3(a) shows an illustration of rat cerebellar development. In the developing cerebellum, neuronal arrangement and circuit formation progress after birth. Granule cells, small input neurons, proliferate and migrate down from the external granular layer (EGL) to the internal granular layer (IGL). Purkinje cells, major output neurons, develop their dendrites and associate neuronal connections between granule cells and other interneurons. The layer of Purkinje cell somas is identified to be the Purkinje layer (PL). A neuronal circuit layer forms the molecular layer (ML).[15,16] To understand the roles of neurotransmitters in the cerebellar development, we have developed a new visualizing device and, with it, we have observed spatiotemporal molecular dynamics.

Using the enzyme-linked photoassay system, we have observed many kinds of transmitter release in several developmental stages and organs. Our system has visualized both spontaneous and responsive transmitter release processes with 0.5 s time resolution. Figures 3(b)–3(k) show the transitions in glutamate release in response to 100 μM GABA application in developing cerebellar slices.[17] Glutamate was released in both the EGL and the IGL, whereas the PL was indicated by a negative line. In the developing cerebellum, the granule cells that distributed in the EGL and IGL are the only neurons that release glutamate, so both layers showed fluorescence activities. Glutamate release in P3 cerebellar slices appeared in both layers slowly but continuously, whereas it started rapidly in the lower EGL and then spread to the IGL within a short time in P7 celebellar slices.[18] Granule cells in the P3 cerebellum did not develop sufficiently to react to GABA stimulation nor release the transmitter actively, but they still proliferated. On the other hand, the granule cells in the P7 cerebellum developed sufficiently to react to GABA stimulation, so they released glutamate rapidly.

Although spatiotemporal observation could give us dynamic information about neuronal reaction, our system needs to be improved in terms of stability, sensitivity and time resolution for us to observe fast synaptic transmissions. The targets of our improvements were the (1) sensing CCD, (2) excitation waveguide, and (3) manner of enzyme immobilization shown in Fig. 2.

3.2 *Effects of new crosslinkers and surface treatment*

Two types of glass devices with either aromatic crosslinkers, 1,3-DIC or 1,4-DIC, and GA were examined to observe spontaneous GABA release with 500 ms time resolution using ORCA ER CCD. The device formed using 1,3-DIC and GA gave images with a better contrast of GABA release than the GA crosslinked device in the P10 cerebellar slice [Fig. 4(a)], whereas it showed no difference in the P6 cerebellar slice. The 1,4-DIC crosslinked device yielded no good images.

The aromatic crosslinkers make the glass surface hydrophobic. Because mature brain tissues become hydrophobic as the myelin structure develops, 1,3-DIC crosslinking should increase the affinity of the enzyme for the tissues.

The binding between the glass and the acceptor molecules has been weak, because the silane coupling agents tend to undergo hydrolysis under biological conditions. The

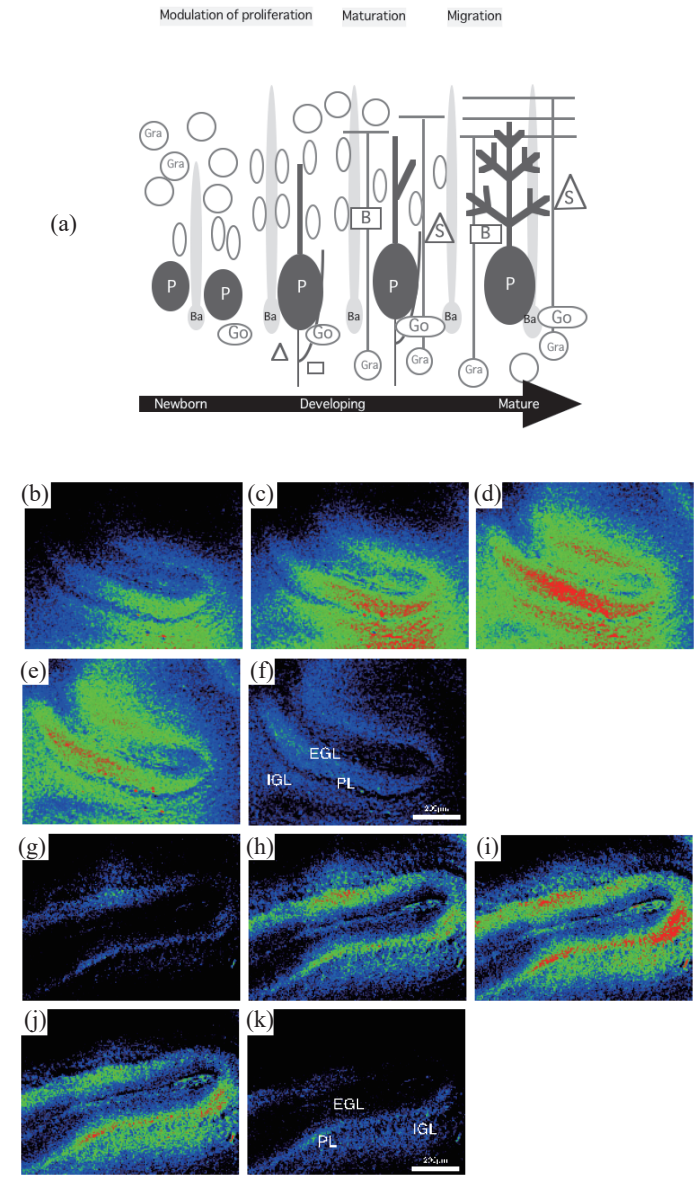


Fig. 3. (a) Diagram of cerebellar development. In the newborn cerebellum (1 to 3 days after birth), granule cell progenitors (Gra) proliferate in the EGL, while immature Purkinje cells (P) form the PL with Golgi cells (Go) and some Bergmann glia (Ba). During the developement, 5 to 7 days after birth, Gra-cells elongate their axon and migrate inside, and P-cells spread their dendrites and connect to other neurons within two weeks. B denotes basket cells, and S, satellite cells. Evoked glutamate wave with GABA application in developing cerebellar cortex. (b)–(f): 2.0, 4.5, 7.0, 12.5, and 23.5 s after stimulation in P3 cerebellar cortex, respectively. (g)–(k): 0.5, 2.5, 4.0, 11.0, and 16.0 s after stimulation in P7 cerebellar cortex, respectively.

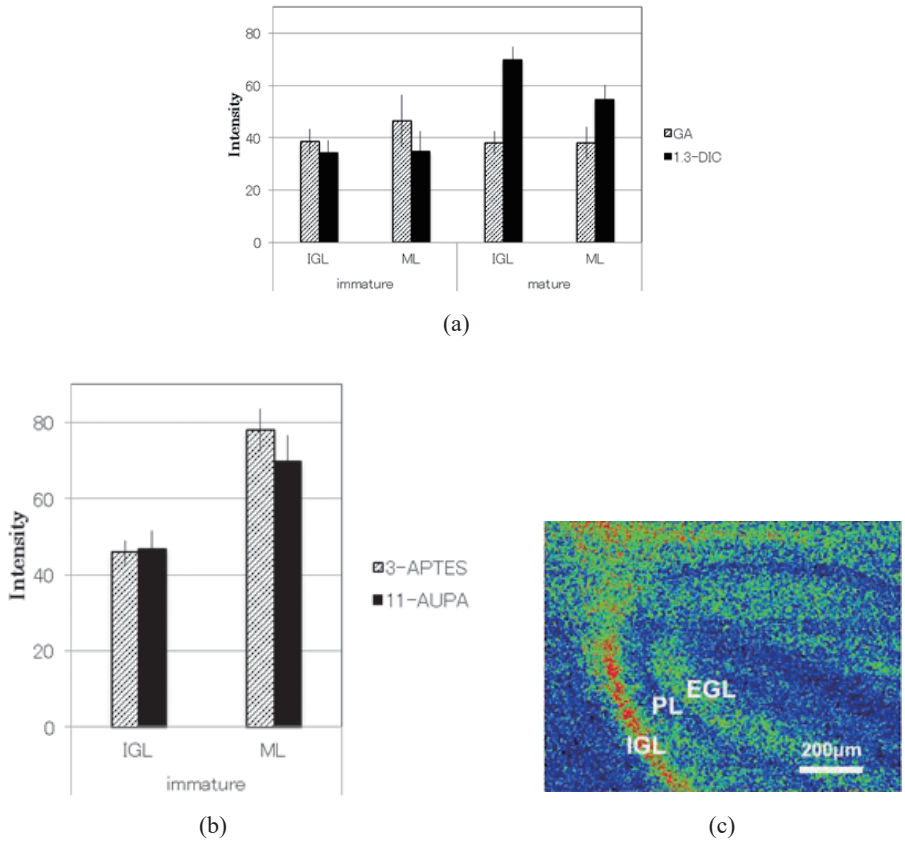


Fig. 4. (Color online) (a) New crosslinker, 1,3-DIC, gave us better contrast images than GA in mature cerebellar observation, while it made little difference from GA in immature organ. (b) The 11-AUPA-GA treatment showed the same result as the APTS-GA treatment in the immature organ. (c) Spontaneous GABA release image in P12 cerebellar slice using 1,3-DIC crosslinking glass device.

surface treatment by 11-AUPA, as a replacement for 3-APTS, was expected to inhibit hydrolysis, but it had low affinity for the glass. We constituted a new glass device with 11-AUPA-coupling enzymes and examined its sensitivity and stability. Figure 4(b) shows that the new device performed with the same sensitivity and stability as the device with APTS.

3.3 *Observation using high-speed CMOS camera*

The fluorescence intensity of NADH is very low and is only a few thousands of the intensity of typical artificial fluorescence. To collect data with sufficient time resolution, a highly sensitive and rapid data transferring camera is required. The time resolution

shown in Fig. 3 is 0.5 s for the 12-bit ORCA CCD, which is too low to detect the synaptic transmitter reaction.

A 16-bit CMOS camera, Flash 4.0, could detect weak light and transfer data in less than a microsecond. Using this camera, transient glutamate release could be detected with a 20 ms time resolution (Fig. 5). In developing the P7 cerebellum, glutamate release was increased in the EGL by applying a glutamate receptor-stimulating agent, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Even in the premature P14 cerebellum, the increment in the rate of glutamate release was observed. It was not strong and noiseless, but the AMPA stimulation-induced glutamate release was observed in the ML and IGL where the glutamatergic neurons are distributed.

4. Discussion

The detection of neurotransmitter release gives us important information about developmental conditions and diseases. Parkinson's disease, a degenerative disorder of the central nervous system, is caused by the alteration of the release of neurotransmitters. The detection of the spatial or temporal alteration of the release would require early diagnosis and treatment of Parkinson's disease. In immature or lesioned neuronal organs, transmitters are released and taken up slowly, so the time resolution required is from 0.5 to 1 s. In young-adult stages, the release speed becomes higher than that in the immature stage within 20 ms.

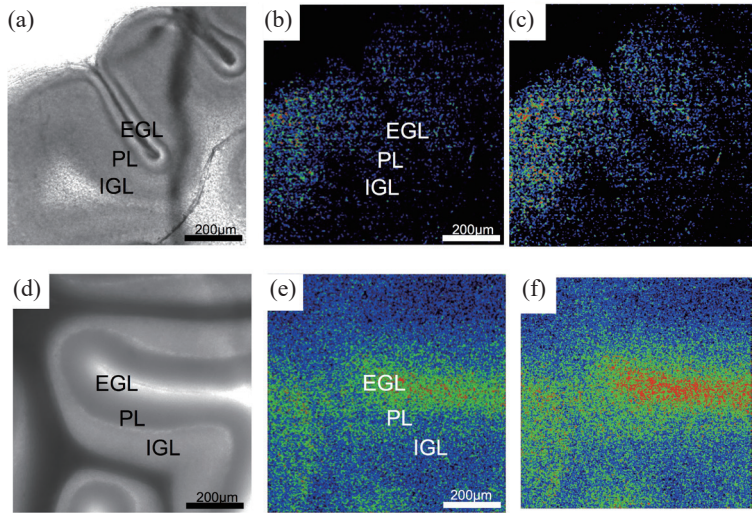


Fig. 5 (Color online) Evoked glutamate release images to AMPA stimulation for 20 ms time resolution using Flash 4.0 CMOS system. (a)–(c) P7 cerebellar slice; (d)–(f) P14 cerebellar slice. (a) and (d) Phase contrast light images. (b) and (e) Fluorescence images before stimulation and (c) and (f) just after AMPA stimulation.

Enzyme-linked assays were applied previously for chemical detection because of their specificity. In spatial observations, however, their fluorescence intensity is too weak to detect. Our enzyme-linked photodevice was developed to detect spatiotemporal neurotransmitter release, and it was improved to observe rapid synaptic release. New crosslinkers could contribute to a more sensitive detection, and the phosphonic surface treatment would expand the range of applications. In order to detect a high-speed transmitter release, both the light accumulation system for weak fluorescence and the close contact between the specimen and the enzyme are required. At present, our photodetection system detects several ms releases from neuronal synapses in the presence of noise, and in the future, it could give us more noiseless observations using an optimal image processing system.

5. Conclusions

The newly developed enzyme-linked photoassay is useful for the visualization of neurotransmitter release in brain slices. In the immature cerebellum, the granule cells release glutamate slowly or rapidly at their stage of neuronal development and synaptogenesis.

Using a fast new system, the rapid measurement of transmitter release in a young-adult cerebellar cortex became possible. Crosslinkers and other device techniques are required for stable observations. We suggest that the photoassay systems have advantages for the observation of synaptic release in several diseases.

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