シンポジウム3「脳性麻痺は防止できるか？」

子宮内炎症と神経細胞死

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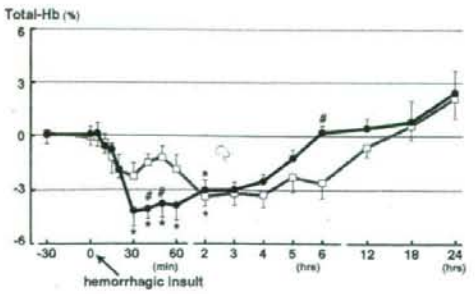
near-infrared spectroscopy
brain-sparing effect

1. はじめに

早産低出生体重児に生じる脳白質損傷（white matter injury，以下WMI）は脳性麻痺をはじめとする神経学的後遺症の責任病変として重要である[1]．そこで，われわれは巣状WMIの本質的成因が低酸素ではなく循環不全にもとづく脳虚血であることを証明するために，先ず，ヒツジ胎仔に急性脱血性低血圧を負荷する脳室周囲白質軟化（periventricular leukomalacia，以下PVL）モデルを開発し[2][3]，次いで，近赤外線分光法（NIRS）を用いてその脳血流動態を解析することによって胎生期PVL発症時における脳虚血の病態生理学的特徴を明らかにした（図1）[4]．さらに，子宮内炎症がWMI発症に与える影響を解析するために，ヒツジ胎仔を用いた子宮内炎症モデルを作成し（図2），胎仔に顆粒球コロニー刺激因子（granulocyte-colony stimulating factor，以下G-CSF）を静注して血中多核白血球（polymorphonuclear leukocytes，以下PMNL）数を増加させ，さらに羊水腔にendotoxinを注入してこれらを局所で活性化させることによって，早産陣痛を誘発することなく壊死性の臍帯炎ならびに絨毛膜羊膜炎を誘導することができた[5]．

本研究の目的は，上記のヒツジ胎仔による慢性実験系を応用して，子宮内炎症がWMIに与える影響を解析することである．

図1　NIRSで解析した胎仔脳組織中total Hb濃度の経時的変化[4]．脱血群胎仔では急性脱血による全身性低血圧から回復した後（負荷後40分以降）も脳血液量の減少が遷延し（no re-flow phenomenon），負荷後6時間には交換輸血群に比較して脳血液量が有意に増加した（虚血後再灌流障害によるうっ血）．したがって，PVL発症時に生じる脳虚血の病態生理学的特徴は「血圧回復後の再灌流不全と引き続く脳組織のうっ血」と考えられた．縦軸は胎児脳組織中total Hb濃度の相対値（%で表示）．●：脱血群，□：交換輸血群．*p＜0.05（Dunnett test）：各群内における負荷前値との比較．#p＜0.05（Wilcoxon signed-rank test）：負荷後6時間における2群間での比較．



2. 対象と方法

本研究は東北大学動物実験委員会の承認のもと（No. 15-128），東北大学医学部附属動物実験施設にて平成16年11月から平成18年3月にかけて実施された（文科省科研費課題番号16591702，18591213）．

対象は妊娠期間を確定したSuffolk種ヒツジ胎仔15頭．慢性実験系を作成するために，妊娠102-103日（満期147日）に全身麻酔下に母獣を開腹して子宮切開し，胎仔の腹部大動脈と上下大静脈，羊水腔内にカテーテルを留置，頭頂骨にNIRS用ライトガイドを固定した後，胎仔を子宮内にもどして閉腹した[4]．以後，胎仔の心拍数，血圧ならびに羊水内圧波形を連続監視した．胎仔胎盤系の循環血液量を算出するために，母仔ともに全身状態が安定した妊娠104-105日に胎仔に凍結新鮮血漿を用いた交換輸血を実施した[2]～[4]．子宮内炎症を誘導するために，妊娠105日からすべての胎仔

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図2　G-CSFの静脈内投与とendotoxinの羊水内投与によって胎仔に誘導された壊死性の臍帯炎（a and b）ならびに絨毛膜羊膜炎（c）の組織病理学的所見[5]．
大量のPMNLが血管内腔（v）から羊水腔（af）に向かって遊走浸潤しており，壊死した多量のPMNLが好塩基性沈着物とともに（矢印），臍帯では臍帯動脈壁の外周に円環状に蓄積し（a and b），卵膜では羊膜直下の絨毛膜内に集積していた（c）．いずれもhematoxylin-eosin染色で，拡大率はそれぞれa：x40，b：x400，c：x100．

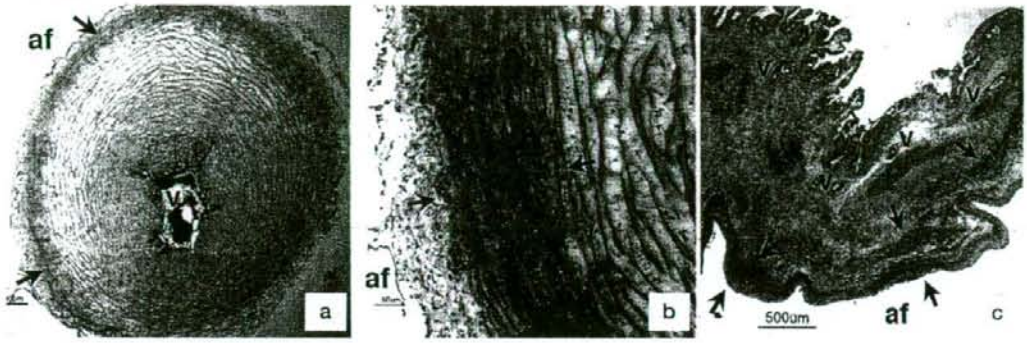
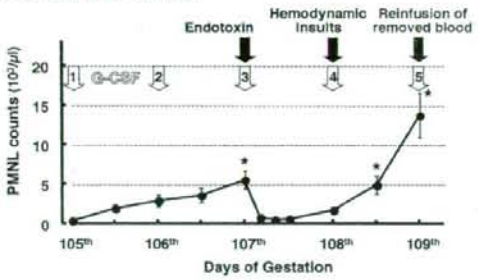


図3　胎仔腹部大動脈血中PMNL数の経時的変化．
PMNL数は羊水腔内endotoxin注入直後の24時間には一過性の減少を示したものの，妊娠107日以降にはG-CSF投与前値に比較して有意な増加が観察された．データはすべて「平均±標準誤差」で表示．*p＜0.05（Dunnett test）：妊娠105日のG-CSF投与前値との比較．



にG-CSF（ノイトロジン®，中外製薬）40 μg/日を5日間静注し，妊娠107日には羊水腔内にendotoxin（E.coli 055：B5，Sigma Chemical Co）20 mgを注入した（図3）[5]．

羊水腔内にendotoxinを注入した24時間後（妊娠108日）に胎仔を3群（各n＝5）に分け，それぞれに循環負荷実験を実施した[2]～[4]．脱血群には循環血液量の約40％を急性脱血して全身性低血圧を負荷，交換輸血群では循環血液量の約40％を凍結新鮮血漿と交換輸血して脱血にともなう貧血性低酸素のみを負荷し，それぞれ負荷後24時間から5時間かけて戻し輸血した．対照群には循環負荷は実施しなかった．羊水腔endotoxin注入前3時間から循環負荷実験終了まで，胎仔脳組織中Hb濃度の変動をNIRSにて連続解析した[4]．羊水腔endotoxin注入6日後（妊娠113日）に帝王切開し，胎仔脳を10％中性ホルマリン緩衝液で灌流固定して組織病理学的解析に供した．

統計学的検討にはDunnett test，Kruscal-Wallis test，Scheffe test，repeated-measures ANOVA，Wilcoxon signed-rank testを用い，連続データは「平均値±標準誤差」で表してp＜0.05を有意差ありとした．

3. 成績

実験期間中の胎仔血中PMNL数の経時的変化を図3に示した．PMNL数は羊水腔内endotoxin注入後24時間には一過性の減少を示したものの，妊娠107日以降にはG-CSF投与前値に比較して有意な増加が観察された．

子宮内局所に炎症が誘導され始めたendotoxin注入後の24時間には，胎仔血中oxygen contentの持続的減少とlactate濃度の一過性増加（図4，A），および脳組織中deoxy-Hbならびにtotal Hb濃度の持続的増加（図4，B）が認められた．

三群間（脱血群，交換輸血群，対照群）における胎仔基礎データ（循環血液量，脱血量，体重，脳重）の比較ではいずれも有意な差は認められず，循環負荷実験直前の生理学的パラメータ値（心拍数，平均動脈圧，血液ガス分析値，血中のPMNL数，Hbおよびlactate濃度，oxygen content，脳組織中Hb濃度）の比較でも有意な差はなかった（Kruscal-Wallis test）．

脱血群ならびに交換輸血群における循環負荷中の平均動脈圧と脳組織中total Hb濃度の経時的変化にはいずれも対照群のそれと比較して有意な差が認められた（p＜0.05，repeated-measures ANOVA）．平均動脈圧は脱血群では負荷開始後20分まで急激に減少してその後は速やかに自然回復したが（図5，A），この間に交換輸血群では一過性で軽度の頻脈が観察された．負荷実験前の脳組織中total Hb濃度は脱血群で11.3±2.8％，交換輸血群で9.7±3.9％といずれも持続的に増加して

図4　羊水腔内endotoxin注入後の24時間における胎仔血中oxygen contentならびにlactate濃度（a），および脳組織中Hb濃度（b）の経時的変化．

胎仔血中oxygen content（a：●）は羊水腔内endotoxin注入後12時間から持続的に減少し，lactate濃度（a：□）はendotoxin注入後12時間に一過性に増加した．脳組織中oxy-Hb濃度（b：■）に有意な経時的変化は認められなかったが，deoxy-Hb（b：□）ならびにtotal Hb濃度（b：●）はendotoxin注入後9時間から持続的に増加した．データはすべて「平均±標準誤差」で表示．*p＜0.05（Dunnett's test）：妊娠107日のendotoxin投与前値との比較．

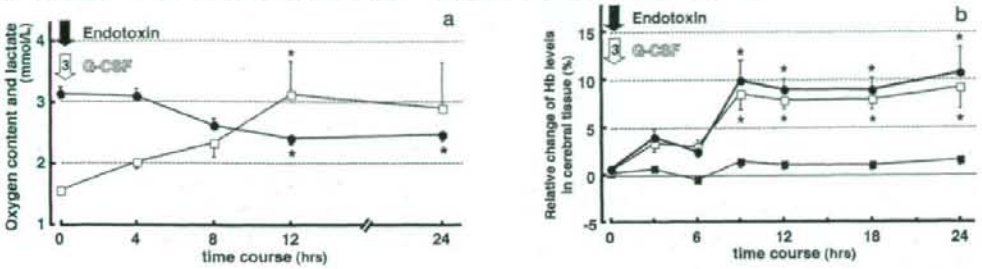
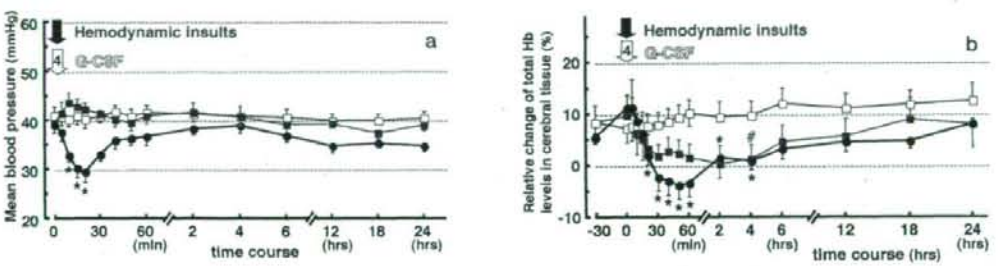


図5　脱血群，交換輸血群，対照群における循環負荷中の平均動脈圧（a）と脳組織中total Hb濃度（b）の経時的変化の比較

脱血群の平均動脈圧（a：●）は負荷開始後10～20分まで急激に減少してその後速やかに自然回復したが，交換輸血群（a：■）ならびに対照群（a：□）では有意な変動は観察されなかった．脱血群の脳組織中total Hb濃度（b：●）は負荷後20分-4時間まで最大幅-15.2±2.8％の減少が観察され，交換輸血群（b：■）では対照群（b：□）と比較して有意な減少は負荷後4時間のみであったが最大幅-10.4±4.9％の減少が観察された．データはすべて「平均±標準誤差」で表示．*p＜0.05（Dunnett test）：妊娠108日の循環負荷開始前値との比較．#p＜0.05（Scheffe test）：負荷後4時間における3群間での多重比較．



いたが，脱血群では負荷開始後20分から4時間まで最大幅-15.2±2.8％の減少が観察され，交換輸血群では対照群と比較して有意な減少は負荷後4時間のみであったが最大幅-10.4±4.9％の減少が観察された（図5，B）．

胎仔付属器における組織病理学的検索の結果，15例すべての胎仔において図2と同様の壊死性臍帯炎ならびに絨毛膜羊膜炎が観察された．胎盤分葉では羊膜上皮に炎症細胞浸潤が認められたが，絨毛血管壁には明らかな炎症性変化は認められなかった．

胎仔中枢神経系における組織病理学的検索の結果を三群間で比較して表1に示した．脱血群の4例と貧血群の4例にそれぞれPVLが観察されたが（図6，A），一頭当たりの白質軟化巣数（径＞500 μm）では両群間に明らかな差はなく，対照群では中枢神経系病変は認められなかった．また，脱血群の1例と貧血群の2例にはPVLのみならず皮質下白質を主座とする多発性小出血壊死巣が観察された（図6，B）．多くの小出血巣ではその中心に小血管に隣接する壊死病変が認められ（図6，C），壊死巣内には多核好中球と単核球が多数含まれていた．

表1　脱血群，交換輸血群，対照群における脳白質損傷所見の比較

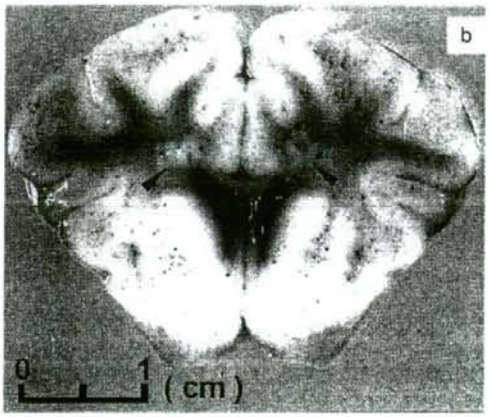
	脱血群 (n＝5)	交換輸血群 (n＝5)	対照群 (n＝5)
PVL*	4	4	0
一頭当たりの白質軟化巣数*	3.0±1.8	3.0±1.4	0.0±0.0
皮質下多発性小出血壊死	1	2	0

離散量は群内の陽性頭数で，連続量は「平均±標準誤差」で表した．「白質軟化巣数」では径500 μm以上の軟化巣を計数した．
*p＜0.05（Kruscal-Wallis test）．

4. 考察

本実験では胎仔にG-CSFを静注して血中PMNL数を

図6 脱血群ならびに交換輸血群で認められた脳白質損傷の組織病理像.
a：交換輸血群に認められたPVLの組織像．線条体レベルの冠状断において側脳室（v）外背側の深部白質に凝固壊死巣が観察され（矢頭），病変内部は空洞形成されつつあった（矢印）．**b**：交換輸血群に認められた皮質下白質を主座とする多発性小出血壊死巣の前基底核レベル冠状断面における肉眼像．PVLは小白色結節として認められた（矢頭）．**c**：多発性小出血壊死巣の組織像．大多数の小出血病変ではその中心に小血管に隣接する壊死巣が観察された（矢印）．いずれもhematoxylin-eosin染色で，拡大率はそれぞれA：x40，B：x1，C：x40.



増加させ，さらに羊水腔内にendotoxinを注入してこれらを局所で活性化させることにより，強度の子宮内炎症である壊死性の臍帯炎ならびに絨毛膜羊膜炎を誘導することができた．Endotoxin投与直後の24時間に観察された一過性の血中PMNL数減少は（図3），臍帯ならびに卵膜において大量のPMNLが血管内腔から羊水腔に向かって遊走浸潤した結果を反映したものと考えられた[5]．

同時にこの期間には胎仔血中oxygen contentの持続的減少とlactate濃度の一過性増加（図4，A）が認められ，強度の子宮内炎症は胎仔に低酸素症を誘導する可能性が示された．すなわち臍帯や卵膜に生じた広範で強い血管炎が胎盤血管まで波及して血管攣縮や間質の浮腫が生じ，その結果として胎盤血管でのガス交換が機能的可逆的に障害された可能性が示唆された[6][7]．一方，このとき全身性には低酸素症が誘導されていたにもかかわらず，NIRSによる脳血流解析上では代償性の脳血流量増加によって胎仔脳への酸素供給が維持されていた．血中oxygen contentは明らかに減少したにもかかわらず脳組織中oxy-Hb濃度が低下しないばかりか，deoxy-Hbならびにtotal Hb濃度が持続的に有意な増加を示しており（図4，B），これはbrain-sparing effect（以下，BSE）と呼ばれる臓器血流の再分配にもとづく代償性の脳血流増加によるものと推察された[8][9]．

胎仔脳にBSEが誘導されている状況において引き続き循環負荷実験を実施したところ，以前に報告した非炎症下での循環負荷実験（図1）[4]とは異なる興味深い結果が三点得られた．

第一の相違点は循環負荷によって生じた脳組織中total Hb濃度における減少の程度にあった．本実験の脱血群（-15.2±2.8％）ならびに交換輸血群（-10.4±4.9％）における循環負荷直後の最大減少幅（図5，B）はいずれも非炎症下実験における脱血群（-4.2±0.8％）ならびに交換輸血群（-3.3±0.5％）の最大減少幅（図1）に比較して有意に大きかった（$p<0.05$；Wilcoxon signed-rank test）．両実験で認められた減少幅の差は本実験において循環負荷前に観察されていたBSEによる脳組織中total Hb濃度の増加分に匹敵しており，本実験では両群ともに循環負荷を契機として急激にBSEが機能しなくなった可能性が示唆された．

第二の相違点は，交換輸血群では非炎症下においてこれまで決してPVLが誘導されず[2][4]，本実験においても脳組織中total Hb濃度は決して基線以下には減少しなかったにもかかわらず（図5，B），その80％の胎仔脳にPVLが誘導されたことである．脱血群と交換輸

血群の間でPVLの発症頻度とその程度に差が認められなかったことから（表1），両群胎仔に生じた脳虚血はおそらく同じ原因によって誘導されたものと推察された．

第三の相違点は本実験では両群ともその20～40％の胎仔脳においてPVLに加えて皮質下白質に多発性小出血壊死巣が認められたことである（図6，BとC）．大多数の出血病変が小血管に接した壊死巣を取り囲んでいたことから，胎仔脳に生じた脳虚血が髄質動脈周囲に虚血病変を誘導した後，引き続く虚血後再灌流によって小血管壁が破綻して出血したのかも知れない．さらに，皮質下白質は一般的に脳室周囲白質に比較して血流量が豊富であるため，本実験で誘導された脳虚血は非炎症下実験でPVLを誘導した脳虚血に比較してより重篤であった可能性が高い．

したがって，本実験では循環負荷そのものによる低血圧や低酸素ではなく，循環負荷を契機として突然BSEが解除されたことこそが重篤な脳虚血ならびに虚血後再灌流が誘導された原因であり，その結果，脱血群のみならず貧血のみ負荷された交換輸血群にも初めてPVLおよび皮質下白質の多発性小出血壊死巣が誘導されたものと推察された．FuとOlofssonは子宮内発育遅延と診断された胎児の中大脳動脈血流速の波形を解析して[10]，いったんBSEが確立された後では，さらなる低酸素ストレスに対して胎児が脳血流を増加させる能力には限界があり，少ない余力しか有していないことを指摘した．本実験の結果はこうした臨床報告と矛盾しないばかりか，BSE予備能を越えるような循環動態の変動が急激に生じた場合には胎児はこれに適応できず，その代償反応自体が消滅して重篤な脳虚血が誘導されるという新たな可能性を指摘できた．

以上の考察にもとづいて，胎仔へのG-CSF静注と羊水内endotoxin注入によって誘導された強度の子宮内炎症のもとでは，胎盤炎症による低酸素症に対して代償性に作動した胎仔脳血流量の増加反応は低血圧や低酸素による循環変動によって破綻しやすく，その結果生じた脳虚血によって未熟な胎仔脳に脳室周囲白質の巣状WMIのみならず皮質下白質に多巣性WMIが引き起こされる可能性が高いとわれわれは結論した．したがって，胎仔の脳循環動態に影響するほどの子宮内炎症は巣状WMI発症に対する増悪因子の一つと考えられた．

5. おしまいに

早産低出生体重児に生じるWMIの病態を解明する道程は遠く，これを予防するための有効な方法を開発するには現状はまだほど遠いレベルにあると言わざるを得ない．現在，われわれは上記の研究成果を踏まえて，その追加実験として「子宮内炎症ではなく母獣への低酸素負荷によって胎仔に誘導されたBSEにおいてWMIを検討」「母獣への酸素投与によるWMI予防効果を検討」「母獣への副腎皮質ステロイド投与によるWMI予防効果を検討」「microglia/macrophage，astrocyte，oligodendrocyte系幼若細胞の分布動態を画像解析することによって皮質下白質のびまん性WMIを検索」している．これからも本学会会員によって胎児WMIに関する多くの臨床ならびに基礎研究が発展することを期待している．

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胎児脳障害

上田恵子　池田智明

胎児脳障害の発症と分類

受精後第3週ころに，外胚葉より神経管が形成される．これは，さらに頭側から尾側にかけて前脳胞，中脳胞，菱脳胞となり，前脳は終脳と間脳に，後脳は後脳と髄脳を形成する．終脳は大脳皮質に，間脳は視床，視床下部，下垂体，松果体等を形成する．後脳は小脳や橋に，髄脳は延髄へと発展する．神経管が閉鎖する時に神経堤細胞が中胚葉側に遊走を開始し神経節の形成に関わる[1]．

胎児期に発症する脳障害はこうした脳の発生の過程におこる奇形と，周産期に関連した，虚血，低酸素等でおこる障害(アスフィキシア性脳障害)とに分類される(表1)．

胎児中枢神経系の先天異常は多くは発生過程で生じる異常であり，妊娠20週以前に形成される．代表は胎生期早期(3週まで)に生じる神経管の形成障害による髄膜瘤，脳瘤，Chiari奇形(図)や，遊走障害による滑脳症等である．原因は不明であることが多いが，遺伝的因子の他に，環境因子(放射線，薬物等)とその障害の強さとタイミングに左右される．最近の話題として，神経管異常と葉酸摂取量の低下との関係が注目されており，少なくとも妊娠1ヵ月前から1日400 μgの葉酸摂取が推奨されている．

これに対し，周産期における低酸素症に関連したアスフィキシア性脳障害も知られている．これらは虚血性低酸素性脳症，脳室周囲白質軟化症に代表され，様々な要因による子宮内低酸素症が関連する．以下にアスフィキシア性脳障害について述べる．

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いけだ ともあき　同 部長

アスフィキシア性脳障害

アスフィキシア性脳障害とは胎児アスフィキシアと関連した脳障害である．「アスフィキシア」とは，胎児の子宮内低酸素症が進行し，嫌気性代謝が亢進する状態である．また，ここでいう「低酸素症(hypoxia)」とは組織中の酸素レベルが低下している状態をいい，原因として血液中の酸素分圧の低下，つまり，低酸素血症(hypoxemia)と虚血(ischemia)がある[2]．

表2にアスフィキシア性脳障害の原因をあげる．分娩前から分娩中の原因をあわせると，およそ55%が胎児期に由来する．さらに，胎児期に脳障害をきたしたものの新生児期には回復した例を含めると，胎児期に起因するアスフィキシア性脳障害はさらに頻度が高いことが予想される．

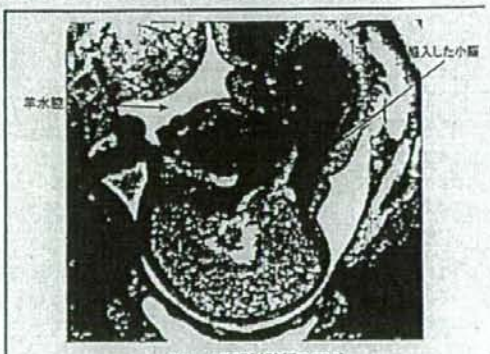
種々の動物実験の結果より胎児アスフィキシアによる神経障害のメカニズムが明らかとなってきている．

表1　胎児脳障害の分類

先天奇形
1．神経管異常(～3週)：無脳症，脊髄髄膜瘤，脳瘤，Chiari奇形など
2．先天奇形(～20週)：終脳形成異常→全前脳症など
遊走障害→滑脳症など
その他→小脳異常など
(Dandy-Walker症候群など)
(20週～)：孔脳症，裂脳症，先天性水頭症など
3．薬物，その他：喫煙，アルコール，麻薬など

周産期に関連した脳障害
アスフィキシア性脳障害(虚血性低酸素性脳症，脳室周囲白質軟化症など)

その他
1．感染：サイトメガロ，ヘルペス，パルボ，風疹，HIV，トキソプラズマなど
2．代謝異常，その他



Chiari奇形(胎児MRI)

表2 アスフィキシア性脳障害―脳に障害の及ぶ時期とneonatal encephalopathy (Volpe[7]より改変)

時期	分娩前	分娩前から分娩中	分娩中	分娩後
頻度	20%	35%	35%	10%
原因	母体の低血圧 母体の低酸素症 子宮出血(前置胎盤など) 子宮内感染 脳の発生異常 (孔脳症，水無脳症)	糖尿病 妊娠高血圧症候群 子宮内胎児発育不全 Dysmorphic syndrome	常位胎盤早期剥離 子宮破裂 臍帯脱出 臍帯卵膜付着 前置血管 分娩外傷	新生児期の心停止 重症肺疾患

胎児は低酸素症に対して，血流再分配を行うことによって，脳，心，副腎等の生命維持に関わる臓器に循環を維持する．しかし，さらに低酸素ストレスが進行すると代償が破綻し，これらの臓器の障害に至る．また，低酸素症時の臓器障害には胎児の低血圧と脳灌流低下，脳虚血が強く関連する．アスフィキシア中の脳虚血のメカニズムは胎児脳における脳血流の自動調節能の破綻と心筋ポンプ機能の障害であるといわれる．細胞分子レベルにおいては脳に低酸素刺激が加わることによって細胞内好気性代謝が障害され，ATPが減少することで，細胞浮腫と壊死を引きおこすことが知られている．さらにこれらの組織に再還流が発症すると，ハイパーオキシド，H_2O_2，ヒドロキシラジカル等の不対電子をもったフリーラジカルや活性酸素が血管内皮細胞や，神経細胞を障害する．

胎児脳障害は児の成熟度によって影響される．早産児においては脳室周囲白質軟化症に代表される白質障害が主である．これは，早産児においては成熟児に比較し，① 脳室周囲白質の血管構築の未熟性，② 脳血流自動調節能の未熟性，③ 神経調節とくにオリゴデンドログリアの脆弱性がみられるためである．これに対し成熟児では，大脳皮質，基底核，脳幹部への灰白質障害が主な病変となる．

脳障害の予防と治療

胎児脳障害のうち，先天異常は永続的な異常をきたすことが多いのに対し，周産期に関連したアスフィキシア性脳障害は，適切な妊娠分娩管理による予防，出生後の対応によって障害を最少限に止めたり，機能回復を図ることができる可能性がある．出生前胎児心拍モニタリングの向上は胎児低酸素の検出を容易にした．出生後の蘇生プログラムの適用，脳低体温療法は出生後の新生児脳障害の予防，軽症化に効果が期待される．

しかし，こうした進歩にも関わらず，いったん障害された脳を治療して再生することは未だ大きな課題である．出生前に起因する障害に対し，障害直後に治療を行うことは困難である．現段階で胎児脳障害に対する直接治療は知られていない．しかし，近年，障害神経組織の再生，あるいは機能回復を目的とした再生医療の領域に新しい知見が得られている[3]．

神経の再生(neurogenesis)は以前より，胎児新生児脳障害の分野で注目されてきた．この過程は大きく① proliferation，② migration，③ differentiationの3段階に分けられる．いくつかの動物実験はHIEモデルにおいて，①，②の増強を確認した．しかし，同動物モデルにおいて，障害部のニューロンの自己再生能には限界があることを示した動物実験もみられる[4]．

一方，HIEラットモデルにmultipotent astrocyte stem cellを直接，細胞移植し，障害部位の周囲でニューロンマーカー陽性(beta 3 tublin, NeuN)の細胞への分化を確認できたという実験[5]や，neural precorser stem cellを同様に移植し，宿主ニューロンと移植細胞由来ニューロンのネットワーク形成に成功したという実験もみられる[6]．しかし，こうした幹細胞の障害部位での再生能力の可能性と脳機能の改善を明確に確認した報告はみられない．こうした再生医療を実際の臨床に応用できるかについては，今後さらなる検討が必要である．

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特集 2

胎児循環から新生児循環を押さえる！ 隠れた異常を見逃さない！

新生児の適応生理の理解と重要観察ポイント

胎児循環から新生児循環への移行理解と観察ポイント

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医員 上田恵子　　部長 池田智明

胎児から新生児への移行において，人間は生涯で最も劇的な変化を経験します。妊娠期間中を通じて胎児を支え，養ってきた胎盤の役割は失われ，心臓，肺，脳，皮膚，腸管，腎臓など，さまざまな臓器がこの変化への適応を必要とされます。

循環器は呼吸器と共に，生後早期からの大きな変化にさらされる器官の一つです。胎児の循環から新生児の循環への移行は，胎児特有の卵円孔，動脈管といった構造が機能しなくなり，肺動脈圧が低下することで肺循環が確立し，左心室優位の循環に切り替わるダイナミックな変化です。乳児期以降，こうした変化を観察することはなく，特別な理解が必要となります。

本稿では，胎児特有の循環動態について理解を深め，出産後，胎児循環から新生児循環に移行する過程と，その過程において起こり得る異常を中心に説明します。

胎児循環から新生児循環へ

1）発達生理

胎児期には，胎盤を通じて母体と交通し，すべての栄養，排泄，ガス交換が行われます。胎児循環が新生児循環と最も大きく異なるのは，①胎盤循環から酸素を摂取し，肺循環が関与しない，②胎児に特有な血管，心構造（卵円孔，動脈管，静脈管）があるという2点です。

母体からの血流は胎盤を介して，臍帯静脈から胎児に入ります（**図1**）。臍帯静脈と下大静脈の間には静脈管がバイパスし，胎児循環の中で最も酸素化された血液が，肝臓を通過せずに心臓に直接流入します。この血流は，下大静脈血流と一緒になって卵円孔に向かいます。一方，上大静脈から右心房に注ぎ込む血液は，三尖弁方向に向かいます。

心房中隔には，そのほぼ中央，右心房側から左心房側に卵円孔が開いています。前述の酸素化された血液は，この卵円孔を介して右心房から左心房へ向かい，左心室を介して大動脈，特に上肢，頭，心臓に向かいます。これにより，酸素飽和度の高い血液が，これらの臓器に優先されることになります。また，肺循環から左心房に帰る血流はわずかであるため，左心房発達には卵円孔を介しての右心房からの血流が必要となります。

一方，上大静脈からの血液は右心室から肺動脈に向かいますが，胎児期には肺血管が強く収縮し，肺血管抵抗が高い状態である（肺高血圧）ため，肺にはわずかな血液（肺動脈に入る血液の約10%）しか流れません。よって，右心室からの血液の大部分（肺動脈に入る血液の約90%）は，

大動脈とほぼ同じ直径を持つ太い動脈管から大動脈に流入します。動脈管からの血液は下行大動脈となり，両腸骨動脈を分岐して，左右の臍帯動脈となって胎盤に戻ります。

胎児期には，自律神経系によって心拍数が決定されています。これは低酸素に影響され，胎児の一過性徐脈となって表れるため，心拍数モニタリングは胎児の低酸素状態の検出に利用されます。胎児の循環は，前述のごとく胎盤循環による物質交換がメインですので，これを担う拍出量が必要となります。しかし，胎児の１回心拍出量はある程度一定であり，これを増加させる予備能はありません。したがって，心拍の増加によってこれを補充します。そのため，胎児の心拍数は140～160回/分前後と，通常新生児より高めに設定されています。

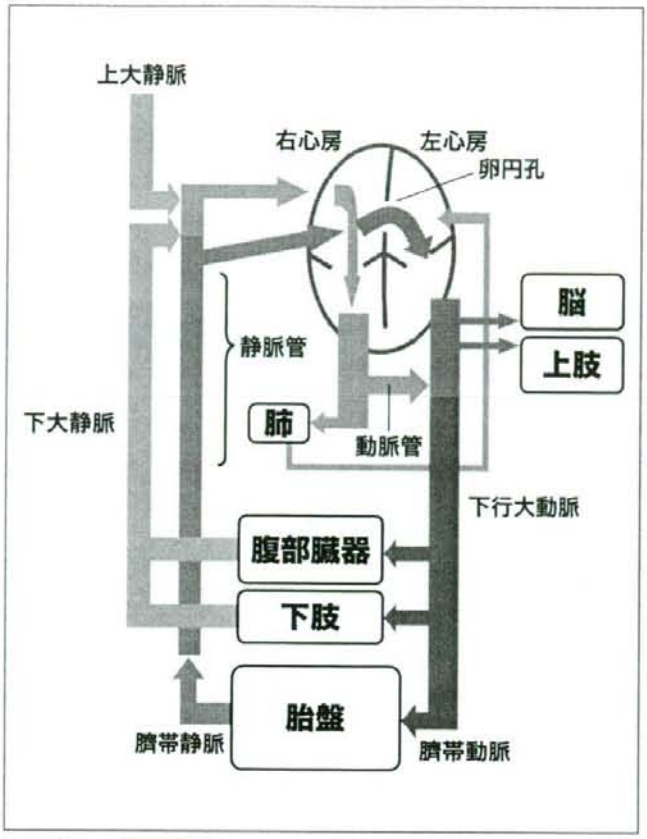

図１　胎児循環

２）適応生理

出産と同時に胎児は胎盤系と分離され，また，第一啼泣と共に肺でのガス交換が開始します。この変化に伴い，新生児は劇的な循環動態の移行を経験します。その第一歩が，肺循環の確立であると言えます。

胎児期に収縮した肺血管は，出生後に急激に拡張し，肺血管抵抗が約半分に低下します。肺血管抵抗は，生後１週間で急速な低下が続き，その後１カ月くらいで成人レベルに達します。肺血流量は肺血管抵抗の低下に伴い，胎児期の10倍にも増加します。

こうした肺循環の確立は，右心室メインであった胎児の循環から，左心室メインの新生児循環（**図２**）への切り替わりを意味します。元気に啼泣する新生児では，酸素化が十分なされるため，全身の動脈血酸素飽和度が上がります。また，肺血流が増えることによって，左心房への還流も増加します。

動脈管は，動脈管を流れる血流の酸素飽和度の上昇とプロスタグランディンの増加によって，平滑筋を収縮させ閉鎖傾向をつくります。生後３～４時間では，まだ両短絡性の血流を認めますが，次第に連続性の左右両方向血流になり，生後10～15時間で血流が認められなくなります。しかし，完全閉鎖までは時間を要し，３日ほどかかります。

また卵円孔は，肺血流の増加に伴う還流血流により，左心房圧が右心房圧より高くなる過程で，機能的に閉鎖されます。しかし，器質的閉鎖には１カ月ほどを要することが多いと言われます。

このような変化によって，胎児期には並列状態で全身に血液を送っていた右心室・左心室が，完全に独立した直列状態となるため，体循環の酸素飽和度は96％以上に上昇します。

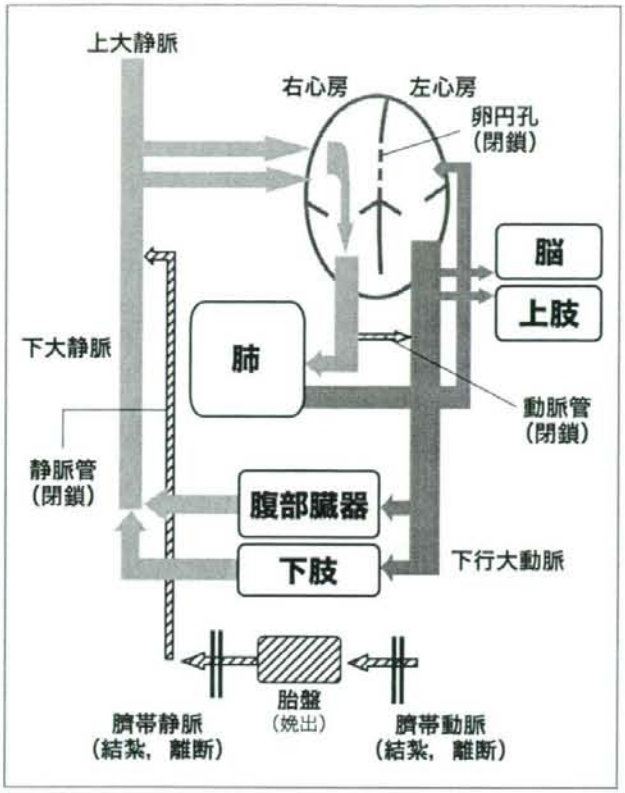

図2　新生児循環

循環に関して新生児を観察する上で観察すべきポイント

新生児に見られる疾患は，胎内環境から胎外環境への変化の過程において，何らかの支障が生じた際に発症することが多いです。

循環器の疾患に限らず新生児の疾患のほとんどは，元気がない，哺乳が悪いなどの非特異的症状で発症します。しかし，こうした非特異的症状の中に重篤な疾患が隠されていることも多く，さらなる観察によってチアノーゼ，多呼吸，頻脈，心雑音といった所見を発見し，診断の糸口になることがあります。

胎児循環と新生児循環，そしてその移行についての生理学を理解し，一般的な症状に潜む特徴的な病態を考えた観察が重要です。

1）動脈管開存（PDA）

胎児期に肺動脈と大動脈を交通していた動脈管は，出生後2，3日以内にほとんど自然閉鎖します。しかし，通常よりも残ることがあります。

新生児期には，肺動脈圧が下がり体血圧が上昇するため，血流は大動脈から肺動脈となることが多く，こうしたシャント血流が増えると，左心室の容量負荷が起こり，肺血流も増加します。これにより，肺に過剰な血流がうっ滞するうっ血性心不全という状態に陥るため，多呼吸，頻脈といった症状が現れます。また，下肢への血流が低下するため，腸管虚血や尿量低下といった症状も現れます。さらに，生後2，3日を超えても，連続性雑音が聴取され，bounding pulse，心尖部拍動なども認められます。血圧の検査では，拡張期血圧の低下が見られます。

動脈管開存は，未熟な早産児において問題となることが多く，特に1,500g以下の未熟児の10～15％に症候性PDAが見られると言われています。超音波法による診断後，水分制限，インドメタシン投与や外科的手術による治療が行われます。

2）卵円孔開存

前述のように，胎児期に開存し，心房間交通を担っていた卵円孔は，生後自然閉鎖しますが，この閉鎖が遅延する病態が見られます。急激に心不全症状が進むことは少ないですが，心雑音（特に収縮期雑音）で気付くことがあります。

3）遷延性児循環遺残（PFC）

胎児に特徴的な循環動態は，生後劇的に変化することで新生児循環が確立します。しかし，この過程において移行がスムーズにいかず，胎児循環が新生児期にも残ってしまうことがあります。この病態を「遷延性児循環遺残（PFC）」または，「新

生児遷延性肺高血圧症（PPHN）」と呼びます。

これは，何らかの原因で生後の肺動脈圧低下が妨げられるために起こります。多くは胎児仮死，新生児仮死に引き続いて発症しますが，横隔膜ヘルニア，重症胸水，肺低形成など，肺血管床に異常を持つ場合にも発症の危険性があります。

通常，元気に啼泣して酸素化と換気が十分にできている児においては，肺血管抵抗が低下し，肺循環が増加するのでみるみる全身がピンク色になります。しかしPFCの場合，肺血管抵抗が下がらないために，動脈管や卵円孔を介して肺動脈側から大動脈側への右左シャントが残るため，酸素化はさらに悪くなり，全身チアノーゼが出現します。特に，動脈管を通った下肢への血流で酸素飽和度（SpO_2）の低下が著しいため，下肢のSpO_2が上肢よりも5～10%低くなるという特徴があります。

同時に，体血圧の低下，肺の換気不全による高炭酸ガス血症も伴うため，集中的な呼吸管理や循環管理を必要とします。具体的には，高頻度振動換気法（HFO）などを用いた人工換気と，昇圧剤や血漿製剤，輸血などを用いた体血圧上昇のための治療が必要となります。また，肺血管拡張や心機能の改善を目的として，ニトログリセリン（ミリスロール），PDE阻害剤（アムリノン，ミルリーラ）なども使用されます。ほかにも，積極的に肺血管抵抗を下げるためのNO吸入療法も効果が知られています。

4）先天性心疾患

生後数日間には，胎児循環から新生児循環への循環動態の変化に伴い，先天性心疾患の症状が顕在化することがあります。先天性心疾患の中でも特に，肺血流または体血流を動脈管に依存する疾患（動脈管依存性先天性心疾患）と，肺動脈，肺静脈の異常を伴う疾患が新生児期に発症します。

それぞれの疾患についての各論は書に譲りますが，動脈管依存性先天性心疾患には肺動脈閉鎖や狭窄を伴い，肺血流を動脈管に依存する疾患群（純型肺動脈閉鎖，ファローの4徴，肺動脈狭窄，両大血管右室起始，多脾症候群，無脾症候群など）と，体血流を動脈管に依存する疾患群（左心低形成，大動脈弓離断，大動脈縮窄）が知られています。

肺血流を動脈管に依存する疾患群では，生後1，2日で動脈管が閉鎖するのと同時に，高度のチアノーゼが出現します。また，体血流を動脈管に依存する疾患群では，新生児期に動脈管閉鎖と共に心不全を来します。出生前診断の向上により，これらの重篤な心疾患が生後初めて指摘される例は少なくなったものの，こうした知識は生後早期の新生児に起こり得る異常の発見に重要と言えます。

5）その他

新生児，特に未熟児の重篤な合併症として知られる肺出血，脳室内出血，脳室周囲白質軟化症，壊死性腸炎，腎不全などは，新生児循環の破綻によって起こります。

したがって，循環動態の適応生理を理解することは，これら未熟児の合併症を予防，あるいは軽症化するために役立ちます。

まとめ

新生児の循環，特に胎児循環から新生児循環への移行と適応，またそれに基づいた臨床現場での異常の考え方について解説しました。適切な循環管理は，新生児の胎外生活の確立に非常に重要です。この安全性を高め，異常をいち早く発見するためだけでなく，元気な成長発達の理解のためにも，正確な知識と病態を理解したベッドサイドでの観察が不可欠と言えます。

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INVOLVEMENT OF CYCLOOXYGENASE-2 IN LIPOPOLYSACCHARIDE-INDUCED IMPAIRMENT OF THE NEWBORN CELL SURVIVAL IN THE ADULT MOUSE DENTATE GYRUS

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Abstract—There is growing evidence indicating that neurogenesis in adulthood is influenced by certain types of the central diseases such as neuroinflammation, however, its mechanism is not fully understood. This study was, therefore, designed to examine the effects of lipopolysaccharide (LPS), a bacterial endotoxin known to cause the neuroinflammation, on the neurogenesis in the dentate gyrus of adult mice using the bromodeoxyuridine (BrdU) –pulse chase method. LPS failed to affect the number of BrdU-labeled cells in the dentate gyrus 2 h after BrdU injection, indicating no effects of LPS on the proliferation of the neural stem cells (NSCs). On the other hand, we found that LPS dose-dependently (0.1, 0.5, 1 mg/kg) decreased the number of BrdU-labeled cells 7 and 21 days after BrdU injection. We also observed that LPS increased cell death in the dentate gyrus using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, suggesting that LPS impaired the survival of newborn cells derived from the NSCs. The double-immunostaining for BrdU and specific cell type markers revealed that LPS did not alter the commitment of the NSCs to the neurons and astrocytes. The systemic injection of indomethacin, a non-selective cyclooxygenase (COX) inhibitor, and NS398, a selective COX-2 inhibitor, but not SC560, a selective COX-1 inhibitor, did not only ameliorate LPS-induced suppression of the newborn cell survival, they fully protected against the LPS effect. Furthermore, the central injection of NS398 also ameliorated LPS-induced suppression of the newborn cell survival in the dentate gyrus. The treatment with LPS increased the expression of COX-2 protein 7 h and 7 days after the injection in the dentate gyrus. These results suggest that LPS impairs the survival of newly generated cells derived from the NSCs in the dentate gyrus without affecting the differentiation fate, and these effects of LPS were mediated presumably by COX-2 expression in the dentate gyrus. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: dentate gyrus, lipopolysaccharide, neural stem cells, survival, bromodeoxyuridine.

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Abbreviations: BrdU, bromodeoxyuridine; COX, cyclooxygenase; DCX, doublecortin; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide; ML, molecular layer; NeuN, neuronal nuclei protein; NSCs, neural stem cells; PB, phosphate buffer; PBS, phosphate-buffered saline; PBSGT, phosphate-buffered saline containing 1% normal goat serum and 0.3% Triton X-100; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; 15d-PGJ_2, 15-deoxy-prostaglandin J_2.

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Recent studies have revealed that the neural stem cells (NSCs), which possess the ability of proliferation and differentiation into neurons and glial cells (Ono et al., 2001), exist in the mammalian adult brain, including the anterior subventricular zone (aSVZ) and the hippocampal dentate gyrus (Gage, 2002). The NSCs in the dentate gyrus are known to lie along the border between the hilus and the granule cell layer (GCL), so called subgranular zone (SGZ), and to migrate into the GCL and to differentiate into the glial cells and the granule cells, resulting in the generation of several thousands of newborn cells each day (Cameron and McKay, 2001; Christie and Cameron, 2006). Furthermore, the small portion of newly generated neurons is reported to be integrated into existing neuronal circuitries (Mandyam et al., 2007). The role of neurogenesis in the dentate gyrus is still unclear, however, several lines of evidence suggest its involvement in the learning and memory (Gould et al., 1999, 2000; Shors et al., 2001; Aimone et al., 2006; Dalla et al., 2007) and neuropsychiatric disorders (Elder et al., 2006). Interestingly, the proliferation and differentiation of the NSCs as well as the survival of newborn cells are dynamically influenced by a certain type of brain injuries such as ischemia (Sasaki et al., 2003), epilepsy (Takemiya et al., 2006) or neuroinflammation (Quin et al., 2007). Thus, the identification of the signaling molecules regulating NSC activity may contribute not only to the understanding of the neurogenesis mechanisms but also toward the development of new therapy against neural death.

Recently, lipopolysaccharide (LPS), a bacterial endotoxin, is reported to modulate the neurogenesis in the dentate gyrus of mammalian brain by causing the neuroinflammation including the activation of microglial cells. Ekdahl et al. (2003) and Monje et al. (2003) independently demonstrated that the peripheral administration of LPS diminishes the neurogenesis in the hippocampal dentate gyrus via the activation of microglial cells in the brain. The neurogenesis consists of the proliferation and the neural differentiation of the NSCs and the survival of newborn cells. However, it is not clear which processes of the neurogenesis are diminished by LPS. Also, the molecular mechanisms underlying the impairment of the neurogenesis elicited by systemic treatment with LPS are not fully understood. In general, the central and the peripheral actions of LPS are mediated or enhanced by the arachidonic acid cascade. In this cascade, two types of cyclooxygenase (COX), COX-1 and COX-2, act as rate-limiting enzymes catalyzing the conversion of arachidonic acid to prostaglandin H_2 (Bazan, 2001) and several prostanoids

such as prostaglandin E_2 (PGE_2), prostaglandin$F_{2\alpha}$, prostaglandin I_2, prostaglandin D_2 (PGD_2) and thromboxane A_2 are known to be produced from prostaglandin H2 by specific synthetase (Nakahata, 2008). In addition, LPS is known to increase the expression level of COX-2 not only in the peripheral tissues but also in the brain (Bazan, 2001). On the other hand, COX and some prostanoids are reported to involve the modulation of the neurogenesis in the dentate gyrus by the ischemia (Sasaki et al., 2003) and the epilepsy (Jung et al., 2006). However, little is known about the involvement of COX in the modulatory actions of LPS on the neurogenesis in adult brains. This study was, therefore, designed to determine which processes of the neurogenesis in the adult mouse dentate gyrus are affected by LPS treatment and to clarify the involvement of COX in the LPS actions using the bromodeoxyuridine (BrdU) –pulse chase method and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining.

EXPERIMENTAL PROCEDURES

Animals

Adult male ICR mice (SLC, Shizuoka, Japan), 6 to 8-weeks-old at beginning of experiment, were used. All mice were housed in polypropylene cages (31×22×14 cm) with wood shavings, and were kept in an environment with a controlled temperature (23±2 °C) and light (12-h light/dark). Food and water were available *ad libitum*. All procedures were conducted in accordance with the guidelines of the Institution for Animal Care and the Use Committee of the Tohoku University. All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Drug treatment and sampling schedule

Mice received vehicle or various doses (0.1, 0.5 and 1 mg/kg i.p.) of LPS from *Escherichia coli* O111:B4 (Wako, Osaka, Japan). Five hours after LPS treatment, mice were intraperitoneally injected with BrdU (50 mg/kg; Nacalai Tesque, Kyoto, Japan) to label dividing cells. Two hours after BrdU injection, some mice were deeply anesthetized with diethyl ether and then, were perfused intracardially with 25 ml of chilled saline followed by 25 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and thereafter their brains were quickly removed. The remaining mice were returned to their home cages and were maintained for 1, 7 or 21 days. Thereafter, animals were anesthetized and perfused as described above and their brains were quickly removed. In some experiments, mice were treated with the non-selective COX inhibitor indomethacin (10 mg/kg i.p., Wako), the selective COX-1 inhibitor SC-560 (12 mg/kg i.p., Cayman Chemical, Ann Arbor, MI, USA) or the selective COX-2 inhibitor NS-398 [10 mg/kg i.p. or 1 μg/10 μl saline/mouse i.c.v. injection, Wako] 1 h (i.p.) or 15 min (i.c.v.) before LPS injection. In one experiment, mice were first injected with BrdU (50 mg/kg i.p.) and received daily injection of LPS (1 mg/kg) for consecutive 7 days, which started 24 h after BrdU injection. Twenty-four hours after last LPS injection, their brains were sampled as described above.

Immunohistochemistry

The brains were post-fixed in 4% paraformaldehyde in 0.1 M PB overnight at 4 °C, followed by immersion in 20% sucrose in 0.1 M PB for 48 h. The brains were cut into 40 μm sections from the rostral to caudal edge of the dentate gyrus using a cryostat (MICROM HM560, Mikron Instrument, Inc., CA, USA) and the sections were alternately divided into two groups.

For BrdU-immunohistochemistry, one group of sections was treated with HCl (2 N) at 37 °C for 20 min, followed by neutralization with sodium borate buffer (0.15 M, pH 8.5) at room temperature for 10 min. After three washes with phosphate-buffered saline (PBS), the sections were incubated with rat anti-BrdU antibody (1:200; Oxford Biotechnology, Oxford, UK) diluted with phosphate-buffered saline containing 1% normal goat serum and 0.3% Triton X-100 (PBSGT) at 4 °C overnight, followed by Alexa Fluor® 568–conjugated goat anti-rat IgG (1:200; Molecular Probes, Eugene, OR, USA) and 1 μg/ml of Hoechst33258 for a nuclear counter staining, diluted with PBSGT at room temperature for 2 h. For the double-labeling of dentate gyrus, another group of the sections was processed for sequential immuno-staining using the following combination of primary antibody and appropriate second antibody; mouse anti–neuronal nuclei protein (NeuN) antibody (1:500, Chemicon, Temecula, CA, USA) followed by Alexa Fluor® 488–conjugated goat anti-mouse IgG (1:200; Molecular Probes), rabbit anti–glial fibrillary acidic protein (GFAP) antibody (1:150, Sigma-Aldrich, St. Louis, MO, USA) followed by Alexa Fluor® 488–conjugated goat anti-rabbit IgG (1:200; Molecular Probes), goat anti-doublecortin (DCX) antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by Alexa Fluor® 488–conjugated donkey anti-goat IgG (1:200; Molecular Probes), rabbit anti-COX-2 antibody (1:100, Cayman Chemical) followed by Alexa Fluor® 568–conjugated goat anti-rabbit IgG (1:200; Molecular Probes). Thereafter, the sections were mounted on slide glasses, dried and coverslipped with Gel/MountTM, aqueous mounting gel (Biomeda Corporation, Burlingame, CA, USA).

TUNEL staining

The effects of LPS on the cell death in the dentate gyrus were evaluated by TUNEL In Situ Cell Death Detection Kit, Fluorescein (Roche, Penzberg, Germany). The brains were post-fixed in 4% paraformaldehyde in 0.1 M PB overnight at 4 °C, followed by immersion in 20% sucrose in 0.1 M PB for 48 h and the hippocampus block was dissected out using scissors. The blocks were cut into 40 μm sections from the rostral to caudal edge of the dentate gyrus using a cryostat (MICROM HM560, Mikron Instrument, Inc.) and the sections were alternately divided into two groups. The sections were treated in Tris-buffered saline containing 0.15% Triton X-100 for 5 min and dehydrated in an ascending ethanol/distilled water series (30%, 70% and 95%, 5 min each), incubated in 100% ethanol (15 min), and gradually rehydrated (95%, 70% and 30%, 5 min each). The sections were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After being washed with PBS three times, they were incubated with 100 μl TUNEL reaction mixture for 5 h at 37 °C. After being washed with PBS three times, the sections were incubated in PBS containing 1.25 μg/ml of Hoechst 33258 for 10 min at room temperature. After sequential washing with PBS and water, the slides were mounted on slide glasses, dried and coverslipped with Gel/MountTM, aqueous mounting gel (Biomeda Corporation).

Quantification of the number of BrdU-labeled cells and TUNEL-positive cells and the proportion of BrdU-labeled cells expressing cell type markers

BrdU-labeled cells and TUNEL-positive cells were counted using a 40× objective (IX70, Olympus, Tokyo, Japan) throughout the rostrocaudal extent of the dentate gyrus. For brains sampled 2 h after BrdU injection, we counted the number of BrdU-labeled cells in the SGZ, which was defined as a two-cell-body-wide zone (approximately 10 μm) along the border of the GCL and the hilus. For brains sampled 7 and 21 days after BrdU injection, we counted the number of BrdU-labeled cells in the SGZ and the

GCL. In TUNEL assay, we counted the number of positive cells in the SGZ and the GCL. Since we stained one of two section groups, resulting numbers were then multiplied by two to obtain the estimated total number of BrdU-labeled cells or TUNEL-positive cells per dentate gyrus. The counting was performed by an observer without any knowledge of the experimental groups.

The phenotype of the newly generated BrdU-labeled cells was analyzed by confocal scanning microscopy (TCS-NT, Leica Microsystems, Tokyo, Japan) and was expressed as the proportion, which was calculated by dividing the number of double-labeled cells by total number of BrdU-labeled cells in the SGZ and GCL.

Statistical analysis

The data in the present study were statistically analyzed by one-way ANOVA followed by Dunnett's test, Fisher's PLSD test or unpaired Student's *t*-test.

RESULTS

The effect of LPS treatment on the proliferation of the NSCs in the SGZ of the dentate gyrus

To determine whether LPS treatment affects the proliferation of the NSCs in the SGZ of the dentate gyrus, we quantified the number of BrdU-labeled cells in the SGZ of mice sampled 2 h after BrdU injection, since this procedure has been shown to be adequate to distinguish the proliferation from the survival of newborn cells (Mandyam et al., 2007). Fig. 1A shows the representative immunofluorescence images of BrdU-labeled cells in the SGZ of control (saline) and LPS-treated mice. LPS at doses of 0.1, 0.5 and 1 mg/kg did not affect the number of BrdU-labeled cells in the SGZ. There was no statistical difference in the number of BrdU-labeled cells in the SGZ between control and LPS-treated mice (Fig. 1B).

The effect of LPS treatment on the survival of the newborn cells and the differentiation of the NSCs

We next investigated whether the treatment with LPS alters the survival of newborn cells in the dentate gyrus. We, therefore, quantified the number of BrdU-labeled cells in the SGZ as well as the GCL of control and LPS-treated mice at 7 and 21 days after the LPS treatment followed by BrdU injection, since the death of newly generated cells was reported to occur between 7 and 21 days after the terminal differentiation (Gould et al., 1999; Prickaerts et al., 2004; Mandyam et al., 2007). As shown in Fig. 2, LPS reduced the number of BrdU-labeled cell in the dentate gyrus 7 days after BrdU injection in a dose-dependent manner. The significant reduction in BrdU-labeled cell number was observed at 1.0 mg/kg of LPS (Fig. 2B). Twenty-one days after BrdU injection, the absolute number of BrdU in the dentate gyrus was lower than that at 7 days after BrdU injection (Figs. 2 and 3). The number of BrdU-labeled cells in the dentate gyrus 21 days after BrdU injection was dose-dependently reduced by LPS (Fig. 3B). The significant decrement in BrdU-labeled cell number was observed at 1.0 mg/kg of LPS (Fig. 3B). When we utilized a "pure" protocol to investigate the survival of newborn cells (Prickaerts et al., 2004) in which mice were first

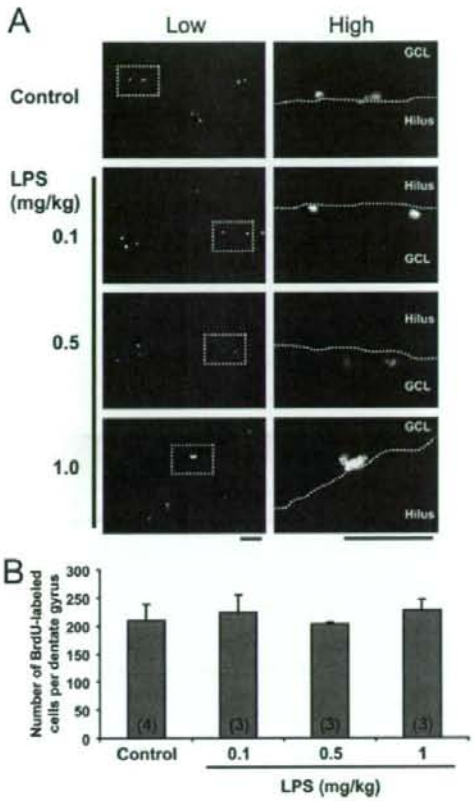

Fig. 1. The effects of LPS treatment on the number of BrdU-labeled cells in the SGZ 2 h after BrdU injection. (A) The representative immunofluorescence images of BrdU-labeled cells in the SGZ of mice treated with or without LPS. Mice were injected with BrdU 5 h after LPS treatment and their brains were sampled 2 h after BrdU injection. The left images represent entire dentate gyrus and right images are the enlargement of the SGZ enclosed by dashed rectangle in corresponding left images. Scale bar=50 μm. (B) The number of BrdU-labeled cells in the SGZ with or without LPS treatment (0.1, 0.5 and 1 mg/kg). Data represent the mean±S.E.M, and the number in parentheses indicates the number of animals.

injected with BrdU (50 mg/kg i.p.) and then received daily injection of LPS (1 mg/kg) starting 24 h after BrdU injection for consecutive 7 days, LPS caused the significant decrease in the number of BrdU-labeled cells in the dentate gyrus [Control; 234.5±23.2 (n=4), LPS; 108.2±38.5 (n=4), P<0.05 (Student's *t*-test)].

As shown in Fig. 4, we observed that the systemic injection of LPS at 1 mg/kg significantly increased the number of TUNEL-positive cells in the dentate gyrus 5 days after the treatment, suggesting the LPS causes the cell death in the dentate gyrus.

To examine the effect of LPS on the fate of the NSCs, we next quantified the proportion of BrdU-labeled cells co-expressing the immature neuronal marker DCX, the mature neuronal marker NeuN or the astrocyte marker

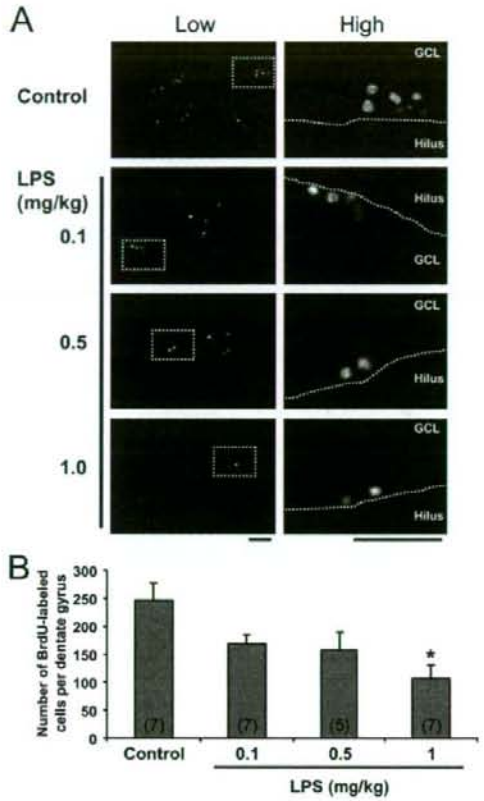

Fig. 2. The effects of LPS treatment on the number of BrdU-labeled cells in the dentate gyrus 7 days after BrdU injection. (A) The representative immunofluorescence images of BrdU-labeled cells in the dentate gyrus of mice treated with or without LPS. Mice were injected with BrdU 5 h after LPS treatment and their brains were sampled 7 days after BrdU injection. The left images represent entire dentate gyrus and right images are the enlargement enclosed by dashed rectangle in corresponding left images. Scale bar=50 μm. (B) The number of BrdU-labeled cells in the dentate gyrus. Data represent the mean±S.E.M, and the number in parentheses indicates the number of animals. The asterisk indicates a significant difference ($P<0.01$ vs. Control group, one-way ANOVA followed by Dunnett's test).

GFAP in the dentate gyrus 7 and 21 days after BrdU injection, respectively. Fig. 5A exhibits the representative confocal images double-immunostained with DCX and BrdU in the SGZ of mice sampled 7 days after BrdU injection. The majority of BrdU-labeled cells co-expressed DCX, and the proportion of DCX-positive cells among BrdU-labeled cells for control mice was 81.06±10.90%. LPS at doses up to 1.0 mg/kg failed to affect the proportion of DCX-positive cells (Fig. 5B). Fig. 5C and 5E shows the representative confocal images showing double-immunostaining of BrdU and NeuN, or BrdU and GFAP in the GCL of the dentate gyrus of mice sampled 21 days after BrdU injection, respectively. The majority of the BrdU-labeled cells co-expressed NeuN, while the BrdU-labeled cells with GFAP expression were sparsely observed. Fig. 5D and 5F shows the proportion of the cells double-labeled with NeuN and GFAP in the GCL, respectively. No difference was observed in the proportion of the BrdU-labeled cells co-expressing either NeuN or GFAP between groups.

The effects of COX inhibitors on LPS-induced impairment of the newborn cell survival in the dentate gyrus

We examined the effects of the pretreatment with COX inhibitors on LPS-induced impairment of the newborn cell survival in the dentate gyrus by quantifying the number of BrdU-labeled cells in the SGZ and the GCL 7 days after

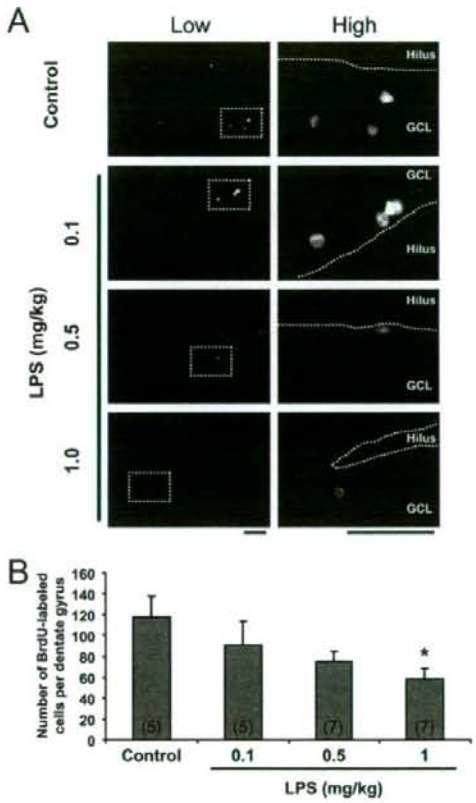

Fig. 3. The effects of LPS treatment on the number of BrdU-labeled cells in the dentate gyrus 21 days after BrdU injection. (A) The representative immunofluorescence images of BrdU-labeled cells in the dentate gyrus of mice treated with or without LPS. Mice were injected with BrdU 5 h after LPS treatment and their brains were sampled 21 days after BrdU injection. The left images represent the entire dentate gyrus and right images are the enlargement enclosed by a dashed rectangle in the corresponding left images. Scale bar=50 μm. (B) The number of BrdU-labeled cells in the dentate gyrus. Data represent the mean±S.E.M, and the number in parentheses indicates the number of animals. The asterisk indicates a significant difference ($P<0.01$ vs. Control group, one-way ANOVA followed by Dunnett's test).

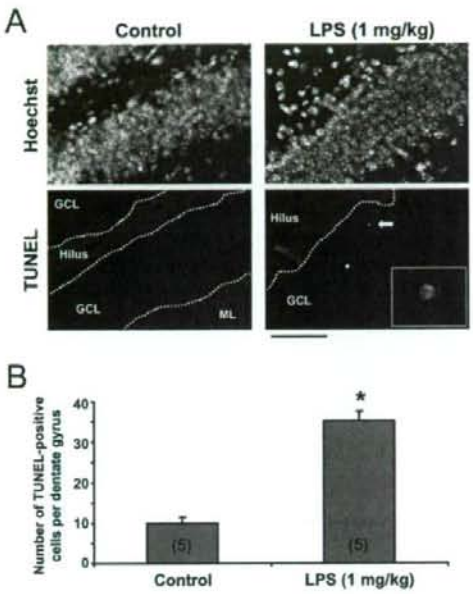

Fig. 4. The effects of LPS treatment on the number of TUNEL-positive cells in the dentate gyrus 5 days after LPS injection. (A) The representative immunofluorescence images of Hoechst33258-staining and TUNEL-positive cells in the dentate gyrus of mice treated with or without LPS (1 mg/kg). Mice were injected with LPS (1 mg/kg) and their brains were sampled 5 days after. The inset image in TUNEL staining of LPS group is an enlargement of TUNEL-positive cells indicated by an arrow. Scale bar=50 μm. (B) The number of TUNEL-positive cells in the GCL. Data represent the mean±S.E.M, and the number in parentheses indicates the number of animals. The asterisk indicates a significant difference ($P<0.05$ vs. Control group, Student's *t*-test).

BrdU injection. One-way ANOVA revealed the significant effects of treatment [$F(4, 26)=6.155$, $P<0.01$] (Fig. 6A). The systemic injection of LPS (1 mg/kg) decreased the number of BrdU-labeled cells compared with control group. The systemic injection of indomethacin (10 mg/kg), a non-selective COX inhibitor, completely protected LPS-induced reduction in BrdU-labeled cell number in the dentate gyrus (Fig. 6A). The systemic injection of NS-398 (10 mg/kg), a selective COX-2 inhibitor, also completely blocked the decrement in BrdU-labeled cell number elicited by LPS (Fig. 6A). These protective effects of inhibitors were confirmed by post hoc Fisher's PLSD test revealing that there was significant difference between control and LPS+saline group, but not between control and LPS+indomethacin group, and not between control and LPS+NS-398 group. Indomethacin or NS-398 did not affect the basal number of BrdU-labeled cells in mice without LPS treatment (data not shown). In contrast, LPS-induced reduction in BrdU-labeled cell number was unaffected by the pretreatment with SC-560 (12 mg/kg), a selective COX-1 inhibitor. Furthermore, as shown in Fig. 6B, i.c.v. injection of NS-398 (1 μg/10 μl/mouse) also completely protected against LPS-induced decrement in BrdU-labeled cell number, since there was significant difference between control and LPS+saline group, but not between control and LPS+NS-398 group (post hoc Fisher's PLSD test) (Fig. 6B). I.c.v. injection of NS-398 itself did not affect the basal number of BrdU-labeled cells in mice without LPS treatment (Fig. 6B).

Expression of COX-2 in the dentate gyrus after LPS treatment

Finally, we investigated the expression of COX-2 in the dentate gyrus of mice with or without LPS treatment. Seven hours after LPS injection (1 mg/kg), it significantly increased the number of COX-2-positive cells in the GCL and the molecular layer (ML) (Fig. 7A). Even 7 days after LPS injection (1 mg/kg), it caused the moderate, but significant increase in the number of COX-2-positive cells in the GCL, but in the ML (Fig. 7A). The immunohistochemistry of double-immunostaining for COX-2 and specific cell type markers revealed that the majority of COX-2-positive cells in the GCL co-expressed NeuN and the small portion of COX-2-positive cells in the GCL co-expressed DCX (Fig. 7C and 7D). It was noteworthy that DCX- and COX-2-double positive cells exist only in the GCL, but not in the SGZ (Fig. 7D). In control mice, COX-2 expression is not detectable in vascular associated cells in the dentate gyrus, however, numerous COX-2 immunoreactive cells were found 7 h and 7 days after LPS administration (data not shown). There were no COX-2-positive cells in the SGZ and the hilus of mice with or without LPS treatment (data not shown).

DISCUSSION

The present study was designed to examine the effects of LPS, a bacterial endotoxin, on the neurogenesis in the dentate gyrus of adult mice and to clarify the involvement of COX in the LPS actions. Using the BrdU–pulse chase method and TUNEL method, we found that LPS diminished the neurogenesis in the dentate gyrus of adult mice by impairing the survival of newborn cells and these effects of LPS were mediated by COX-2 in the brain. To our knowledge, this is the first report to show the involvement of COX-2 in the brain in the impairment of the neurogenesis in the hippocampal dentate gyrus by LPS.

The neurogenesis in the adult dentate gyrus is a complex of multi-step process, in which the immature NSCs proliferate and then differentiate into three neural cell lineages and the limited number of newborn cells survive thereafter (Reynolds et al., 1992; Kempermann et al., 2004). Since the cell cycle length of NSCs in the dentate gyrus of adult mice is reported to be approximately 14 h (Mandyam et al., 2007), the proliferation of the NSCs should be investigated using a protocol in which mice are treated with a tracer (BrdU) and killed maximally within 24 h (Prickaerts et al., 2004). Thus, our protocol of the sampling 2 h after BrdU injection enables us to exclude the contribution of the newborn cell survival and to examine the proliferation itself. On the other hand, the number of BrdU-labeled cells in the dentate gyrus was reported to decline between 1 and 2 weeks in rats (Gould et al., 1999) and between 24 h and 4 weeks in mice (Mandyam et al.,

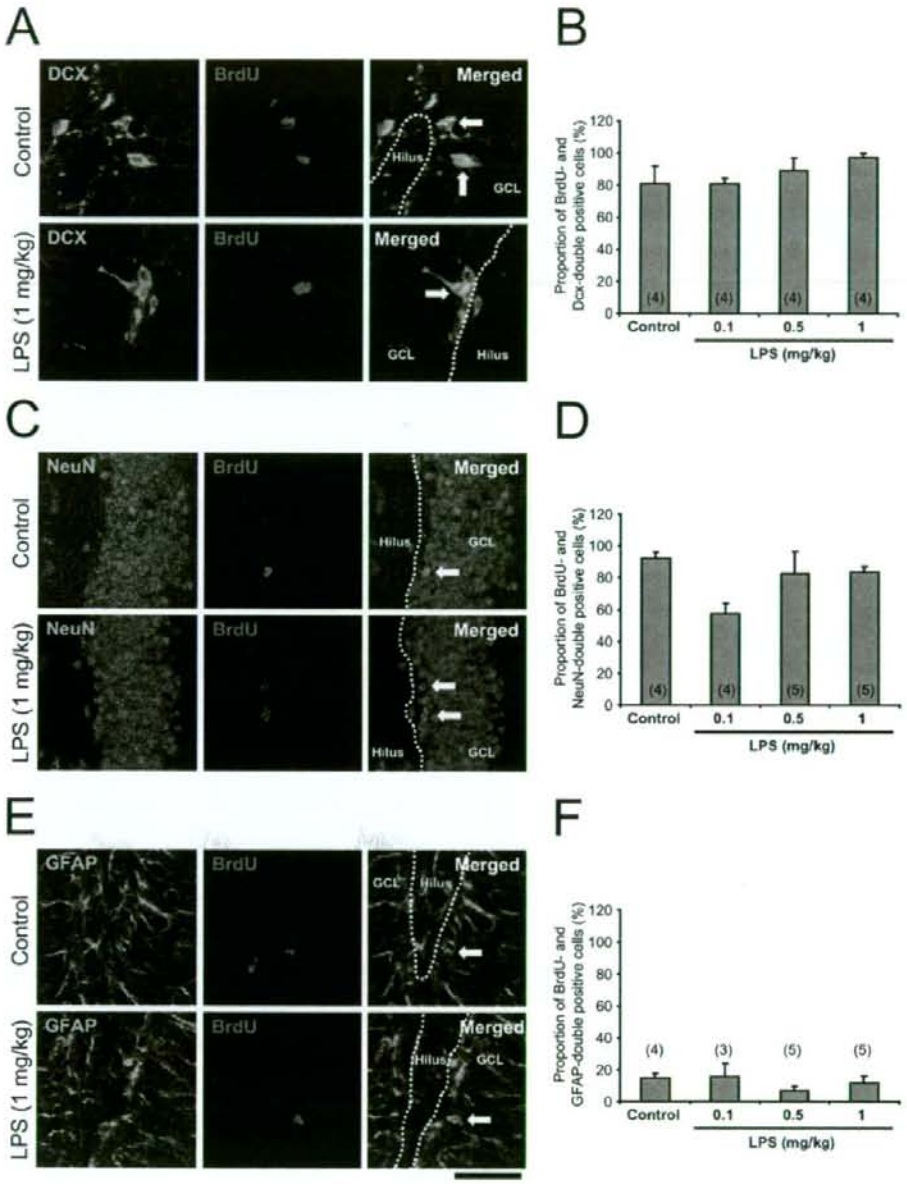

Fig. 5. The effects of LPS treatment on the proportion of DCX-, NeuN- or GFAP-positive cells among BrdU-labeled cells in the dentate gyrus. Representative confocal images of the double labeling of BrdU-labeled cells with the immature neural marker DCX (A), the mature neural marker NeuN (C) and the astrocyte marker GFAP (E) in the dentate gyrus of mice treated with or without LPS, 7 days (A; DCX) and 21 days (C; NeuN, E; GFAP) after BrdU injection. The arrows in the merged image indicate double-positive cells. Scale bar=50 μm. The proportion of BrdU and DCX (B), NeuN (D) or GFAP (F) double-positive cells in the dentate gyrus, 7 days (B; DCX) and 21 days (D; NeuN, F; GFAP) after BrdU injection. Data represent the mean ± S.E.M, and the number in parentheses indicates the number of animals.

2007) without the change in the labeling intensity per cell, suggesting that the death of newborn cells in the rodent dentate gyrus occurs during this period. These reports taken together with our finding that LPS decreased the

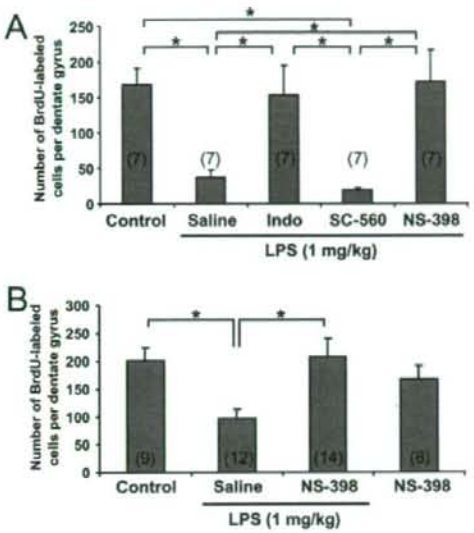

Fig. 6. The effects of COX inhibitors on LPS-induced impairment of the newborn cell survival in the dentate gyrus. (A) The effect of systemic injection of COX inhibitors on LPS-induced decrement in BrdU-labeled cell number in the dentate gyrus 7 days after BrdU injection. Indo (10 mg/kg), SC-560 (12 mg/kg) or NS-398 (10 mg/kg) was injected to mice 1 h before LPS treatment and BrdU was given 5 h after LPS injection. Their brains were sampled 7 days after BrdU injection. Indo, indomethacin. (B) The effect of central injection of a selective COX-2 inhibitor, NS-398, on LPS-induced decrement in BrdU-labeled cell number in the dentate gyrus 7 days after BrdU injection. The inhibitors were injected to mice 15 min before LPS treatment and their brains were sampled 7 days after BrdU injection. Data represent the mean±S.E.M. and the number in parentheses indicates the number of animals. * Significant difference ($P<0.05$, one-way ANOVA followed by Fisher's PLSD test).

number of BrdU-labeled cells 7 and 21 days, but not 2 h, after BrdU injection suggest that the survival of newborn cells in the dentate gyrus is negatively regulated by the systemic treatment with LPS. Our results of TUNEL staining also suggested that LPS decreased the cell survival in the dentate gyrus. The significant decrease in the number of BrdU-labeled cells in the dentate gyrus observed using a "pure" protocol investigating the newborn cell survival (Prickaerts et al., 2004) also support this idea. We also found that the proportion of cells committed to neural lineage (DCX- and NeuN-positive) and astroglial lineage (GFAP-positive) was not changed by LPS treatment, suggesting that LPS does not affect the differentiation process itself. LPS, therefore, might reduce the survival of immature cells that do not undergo the fate-determination. Alternatively, we cannot rule out the possibility that LPS decreases the survival of neuron-restricted progenitor cells and astrocyte-restricted progenitor cells in the same degree. Since recent studies revealed that neurogenesis in the adult dentate gyrus is the sequential maturation from several subtypes of dividing cells (nestin-positive type-1 and type-2 cells and DCX-positive type-3 cells) into several subtypes of postmitotic cells (calretinin- or NeuN-positive cells) (Fukuda et al., 2003; Kronenberg et al., 2003; Jessberger et al., 2005), further experiments will be required to confirm which types of newly generated cells are affected by LPS treatment.

Recently, it was demonstrated that the neural stem/progenitor cells in the dentate gyrus abundantly express Toll-like receptor four (TLR4) known as a LPS receptor and the treatment with LPS directly diminished the proliferation

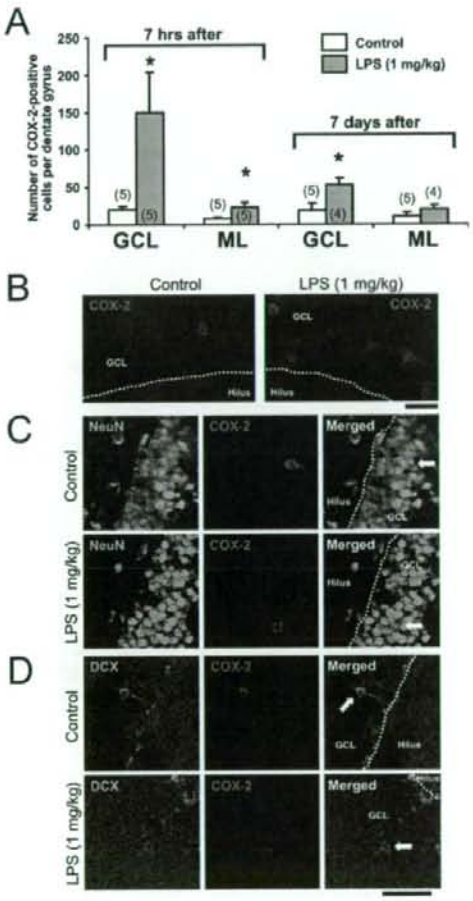

Fig. 7. The expression of COX-2 in the dentate gyrus. (A) The number of COX-2-positive cells in the GCL and ML of the dentate gyrus of mice 7 h and 7 days after the treatment with or without LPS. The number in parentheses indicates the number of animals. The asterisk indicates a significant difference ($P<0.01$ vs. Saline group, Student's t-test). (B) Representative immunofluorescence images of COX-2 positive cells in the dentate gyrus of mice 7 days after the treatment with or without LPS. The arrows in the merged image indicate double-positive cells. Scale bar=50 μm. Representative confocal images of the double labeling of COX-2 positive cells with the neural marker NeuN (C) and the immature neural marker DCX (D) in the dentate gyrus of mice 7 days after treatment with or without LPS. The arrows in the merged image indicate double-positive cells. Scale bar=50 μm.

of cultured neural stem/progenitor cells in an NF-κB-dependent mechanism (Rolls et al., 2007). It has been demonstrated, however, that LPS (up to 0.1 μg/ml) failed to affect the survival of cultured neural stem/progenitor cells under *in vitro* conditions (Rolls et al., 2007). In marked contrast, it is reported that treatment with LPS (0.1 μg/ml) induced apoptosis in hippocampus-derived NSCs (Chiou et al., 2006; Huang et al., 2007). Because it is unknown whether LPS injected intraperitoneally at doses up to 1 mg/kg would pass through the brain–blood barrier and reach the plasma membrane of the NSCs, the impairment of newborn cell survival observed in the present study might be caused by indirect actions of LPS via COX-2 as described below.

One of the novel findings in the present study is that LPS-induced impairment of the newborn cell survival was ameliorated not only by the systemic injection of either a non-selective COX inhibitor (indomethacin) or a COX-2 selective inhibitor (NS-398), but also by the central injection of NS-398, suggesting the implication of brain COX-2 in the LPS actions. There were some previous reports indicating the roles of COX-2 in the modulation of the neurogenesis in the dentate gyrus by a certain types of brain injury. Sasaki et al. (2003) demonstrated that ischemia-induced enhancement of the NSC proliferation in the dentate gyrus was blunted by treatment with indomethacin or NS-398 or in COX-2-deficient mice (Sasaki et al., 2003). Furthermore, ectopic neurogenesis in the hippocampus elicited by the epilepsy was shown to be inhibited by a COX-2 inhibitor, celecoxib (Jung et al., 2006). These facilitatory roles of COX-2 in injury-induced neurogenesis are quite opposite to the suppressive roles of COX-2 in LPS-induced diminishment of the neurogenesis in the dentate gyrus. The difference in the spatial pattern and the levels of COX-2 expression between brain injury (ischemia or epilepsy) and LPS treatment may account for these opposite roles of COX-2. Under our experimental condition, COX-2 expression in control mice was seen in the GCL, but not in the SGZ containing the immature NSCs. These expression patterns of COX-2 in the dentate gyrus are consistent with previous reports (Sasaki et al., 2003; Takemiya et al., 2006). We also demonstrated that the systemic injection of LPS (1 mg/kg) increased the expression of COX-2 in the GCL and the blood vessels, but not in the hilus. In contrast, ischemia has been shown to increase COX-2 expression in the reactive astrocytes located in the hilus (Sasaki et al., 2003). Thus, the difference in COX-2-expressing cell type as well as the expression levels may explain the opposite actions of brain injury (ischemia or epilepsy) and LPS on neurogenesis in the dentate gyrus.

Although COX-2 may modulate the neurogenesis in the dentate gyrus through generation of prostaglandins and PGE_2, and is already reported to modulate neurogenesis in the dentate gyrus (Uchida et al., 2002), it remained unclear which type(s) of prostaglandins plays a critical role in LPS-induced impairment of neurogenesis. Interestingly, the central infusion of LPS drastically increases the brain levels of PGD_2, a major metabolite of arachidonic acid in the CNS (Rosenberger et al., 2004). In addition, it is suggested that the cyclopentenone 15-deoxy-prostaglandin J_2 (15d-PGJ_2), which is non-enzymatically converted from PGD_2 (Shibata et al., 2002), induces apoptosis of a variety of cell types including the oligodendrocyte precursor cells in a peroxisome proliferators-activated receptor γ-dependent and -independent mechanism (Xiang et al., 2007). Thus, PGD_2, as well as its metabolite 15d-PGJ_2, is a feasible candidate mediating LPS action in neurogenesis.

LPS is generally known as a phlogistic agent to induce neuroinflammation (Ekdahl et al., 2003; Rosenberger et al., 2004; Rolls et al., 2007) and the activation of microglial cells is one of typical phenomena elicited by LPS treatment (Quin et al., 2007). It has been reported that the activation of microglial cells mediates LPS-induced diminishment of neurogenesis in the dentate gyrus via IL-6 (Monje et al., 2003) or TNF-α (Liu et al., 2005) secretion from the activated microglial cells (Ekdahl et al., 2003). In addition, LPS is known to increase the blood levels of interleukin-1β, which in turn increased the gene expression of COX-2 in the blood vessel cells in the brain (Fantuzzi and Dinarello, 1999; Bazan, 2001; Blais et al., 2005; Quan and Banks, 2007). Furthermore, LPS is known to increase the brain levels of interleukin-1β (Fantuzzi and Dinarello, 1999), which is shown to reduce neurogenesis in the dentate gyrus (Goshen et al., 2008; Koo and Duman, 2008; Spulber et al., 2008). Since COX-2 activation is known to be directly or indirectly involved in a wide variety of these neuroinflammation processes elicited by LPS (Minghetti, 2004; Quin et al., 2007), the ameliorating effects of COX-2 inhibitor against LPS action might be exerted by suppressing the cytokine production involved in neuroinflammation. Further experiments will be required to confirm this possibility.

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