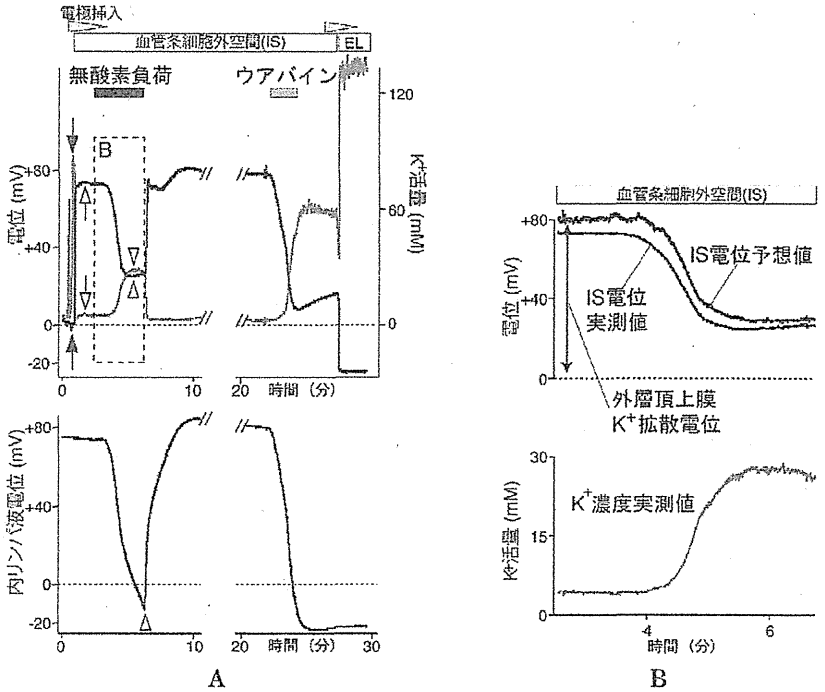

図3　Na^+, K^+－ATPase 阻害の効果（文献7）から改変）

A：黒線は電位を，灰色線は K^+ 濃度（活量）の変化を示す．無酸素負荷およびウアバインの還流で内層の Na^+, K^+－ATPase を阻害した．上段：K^+ 電極による血管条の各区分の K^+ 濃度と電位の測定．下段：別電極による内リンパ液電位の経時変化．

B：無酸素下での IS の K^+ 濃度の変化（下段：Aの点線四角内の灰色線のデータと同じ）から IS 電位の予想値を求め，その実測値（下段：A点線四角内の黒線と同じ）と比較した．EL：内リンパ液．

検討した．この電極は二連管となっており，K^+ 濃度・電位が一本で測定可能である．同時に通常の一連管電極で内リンパ液の電位変化も観察した[9]．

図3A上段は，K^+ 電極を外リンパ液から血管条・内リンパ液へと進めた際の電位（黒線）と K^+ 濃度（灰色線）の変化を示し，下段は，通常の電極で内リンパ液の電位を経時的に観察したものである．濃度は，活量という単位で表しているため，馴染み深い一般的な値より低く示される．また，血管条に薬剤を投与するため，血管条毛細血管の源流である椎骨動脈にカテーテルを留置した．外リンパ液（0mV・低 K^+）より K^+ 電極を進めると，最初に K^+ 濃度が 65～85mM と高く，電位が＋2～3mV と軽度正の値を示す地点（黒矢印）を見出した．解剖学的に（図1B），これは外層の内部を観察していると考えられた．さらに電極を進めると，電位が＋70mV と高く，K^+ 濃度が 4mM と低い IS を認めた（白矢印）．電極を留置して，無酸素負荷により血管条内層の Na^+, K^+－ATPase を阻害したところ，IS 電位は＋70mV から＋22mV へと下降して 3mV だけ上昇し，K^+ 濃度は 4mM から 28mM へと上昇して 2mM のみ下降する二層性変化を認めた（図3A点線四角と図3B）．同時に内リンパ液電位は－14mV の負値まで下降し（図3A下段），正の値でとどまった IS 電位との差が認められた（白矢頭）．無酸素を解除すると，IS の電位と K^+ 濃度，内リンパ液電位の値は，すべて元に戻った．IS 電位と内リンパ液の電位は低下の様式や度合いに差はあるが，おおむね同じように推移しているので，前者が後者の主な構成要素であるとする過去の説[7]は正しいと結論づけた．

式(a)において，もし空間Aの K^+ 濃度が一定であると過程すると，空間Bの K^+ 濃度が上昇すれば両空間の濃度差が少なくなるので，膜を介した電位差に相当する K^+ 拡散電位の絶対値は小さくなる（図2A）．空間Aを基準（0mV）とすれば，最初は高い正値をとっていた空間Bの相対的電位は K^+ 濃度上昇に従って低下する．そこで，図3Aの実験結果に当てはめて考えてみる．まず，別の個体を用いて，外層内部の電位と K^+ 濃度は，無酸素負荷によってほとんど影響されないことを見出した（データ示さず）．従って，IS 電位の変化は，外層頂上膜を介した電位差の変化を表すことになる（図1B）．

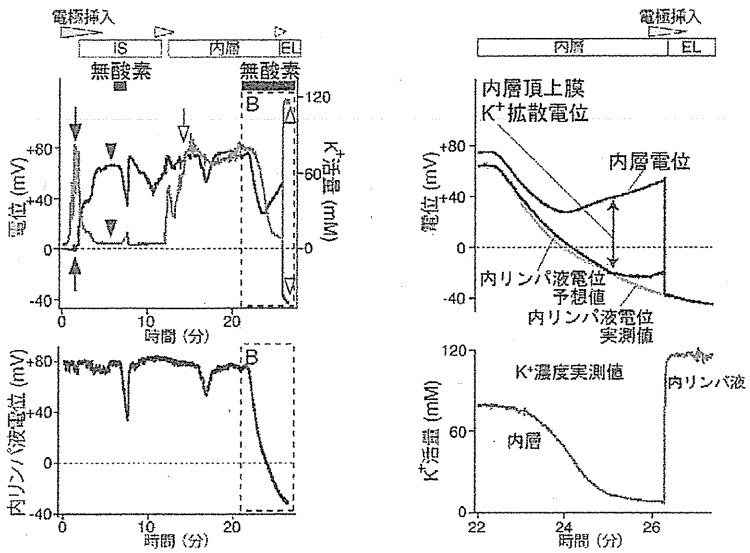

図4　内層の解析（文献7）から改変）

A：上段：K^+電極による測定．外層（黒矢印），IS（黒矢頭）を通過後，高電位・高K^+の内層の内部（白矢印）に到達．無酸素負荷の後，内リンパ液（白矢頭）へ挿入した．下段：内リンパ液電位の変化．

B：無酸素による内層K^+濃度の実測値（下段：Aの点線四角内の灰色線と同じ）と内リンパ液K^+濃度の実測値（A上段白矢頭：一定値）から内層頂上膜のK^+拡散電位を求め，内層の電位の実測値との和を内リンパ液電位の予想値（上段黒線）として示した．さらに内リンパ液電位の変化の実測値（上段灰色線：A下段点線四角内の内リンパ液電位の変化値と同じ）を重ねた．EL：内リンパ液．

ここで，無酸素負荷時のIS電位とISのK^+濃度の鏡像反応は，外層頂上膜のK^+チャネルKir4.1を介して発生するK^+拡散電位がIS電位の主要素であることを示唆する．つまり，IS電位は以下の式で求められる．

$$ISP = V_{Outer} + \frac{RT}{F} \ln\left(\frac{aK^+_{i(Outer)}}{aK^+_{IS}}\right) \quad \cdots\cdots \quad (b)$$

ISPはIS電位，V_{Outer}は外層の電位，$aK^+_{i(Outer)}$とaK^+_{IS}は外層内とISのK^+濃度（活量）である．上記のごとくV_{Outer}と$aK^+_{i(Outer)}$は常に一定であるため，図3A上段の実測値（黒矢印）を代入した．さらに(b)の式へ，無酸素時におけるISのK^+濃度変化の実測値（図3A上段点線四角と図3B下段）を代入して，IS電位の変化の予想値を求め，IS電位の実測値と比較した（図3B上段）．両者はよく一致した．以上より，IS電位は主に外層のKir4.1を介したK^+拡散電位により成立することが示された．Na^+, K^+, $2Cl^-$共輸送体の阻害薬であるブメタニドも，無酸素負荷と同じ効果を呈した（データ示さず）．

5．内層の役割

図3Aの無酸素負荷時に認められたIS電位と内リンパ液電位との差（白矢頭）から，内リンパ液電位の成立にはIS電位以外の要素がかかわると予想された．無酸素解除後，引き続きK^+電極をISにとどめ，Na^+, K^+−ATPase阻害薬のウアバインを血管灌流した（図3A）．無酸素時と同様に，IS電位の低下と軽度回復，ISのK^+濃度の上昇と軽度回復という二層性変化を認めた（図3A上段）．また，IS電位が負値を示さなかったのに対し，内リンパ液電位は約−20mVまで降下した（図3A下段）．さらにK^+電極をISから内リンパ液へ進めると，K^+濃度は100mM以上に上昇すると共に（図3A上段），電位は大きく降下し，別電極で測定されている内リンパ液電位の値とほぼ同じとなった（図3A下段）．故に，IS電位と内リンパ液電位の差は，内層で生じていることが判明した．

次に，この電位差が内層のどの部分で起こるかを検討した．電極を外リンパ液から進めていくと（図4A上段），僅かに正電位かつ高K^+濃度を示す外層（黒矢印），高電位かつ低K^+濃度を示すIS（黒矢頭）を認めた．さらにK^+電極を進めると，高電位のままでK^+濃度が急に約80mMと高くなる点を見出した（白矢印）．内リンパ液のK^+濃度は100mM以上であるため（図3A），この場所は内層の内部であると考えられた．電極

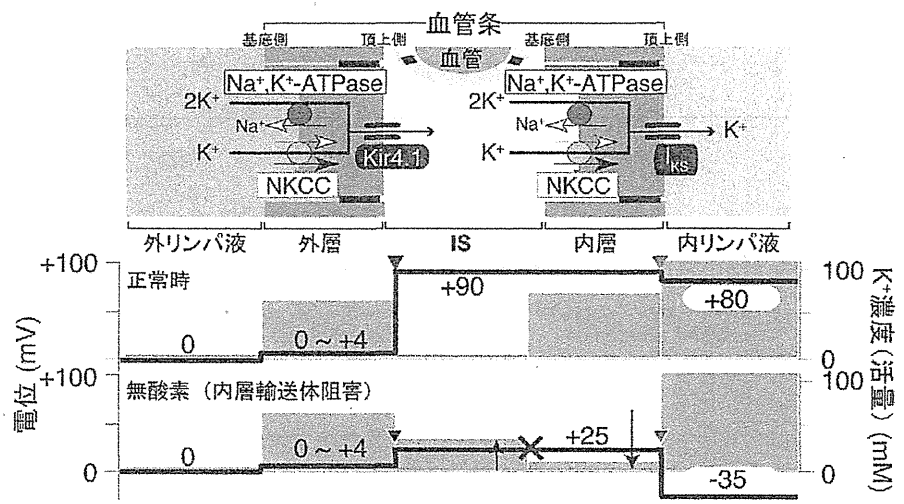

図5　2つのK^+拡散電位に依存する内リンパ液高電位

血管条の模式図（上段）と，正常時（中段）・無酸素負荷時（下段）の電位・イオン動態．中・下段において，灰色棒グラフはK^+濃度（活量）を，黒太線は電位を示す．正常（中段）では，外層と血管条細胞外空間（IS）との間のK^+濃度勾配を活用し，Kir4.1が外層頂上膜において大きなK^+拡散電位を発生する（黒矢頭）．この電位が，ISや内リンパ液の高電位の主要素となる．無酸素時や内層基底膜のK^+取り込み輸送体を阻害した際（下段）では，ISのK^+濃度が上昇することで，外層とのK^+濃度差が減少し，K^+拡散電位の絶対値とIS電位が低下する．同時に内層のK^+濃度が低下し，内リンパ液との間の濃度差が拡大する．その結果，内層頂上膜にてI_{Ks}を介したK^+拡散電位が増大し（灰色矢頭），IS電位はさらに内リンパ液で低下して負値を示す．外層と内リンパ液のK^+濃度は常に不変．

を留置し，無酸素を負荷したところ，電位は＋74mVから＋27mVへ大きく下降した後，＋52mVへ回復したが，K^+濃度はS字状に低下した（図4A上段点線四角内）．この内層の電位変化は，無酸素時・ウアバイン投与時のIS電位の二層性変化に類似している（図3A）．一方，内リンパ液の電位は低下し続け，−40mVを示した（図4A下段点線四角内）．さらにK^+電極を内層内から内リンパ液へと進めると，K^+濃度は約100mMへ上昇し，電位は大きく下降して内リンパ液電位とほぼ同じ負値を示した（図4A白矢頭）．従って，図3Aで観察されたIS電位と内リンパ液電位の差は，内層の頂上膜で生じていたことになる．

内層の基底側にはK^+チャネルが実験的に認められないことが報告されているが[10]，頂上膜にはK^+チャネルI_{Ks}が分布する（図1B）[4]．故に，図4A上段のごとく内層のK^+濃度が低下すると，内リンパ液のK^+濃度は変わらないので[11]，膜を介した濃度勾配が大きくなる．これは，内層頂上膜におけるK^+拡散電位が増大し，内リンパ液電位と内層との電位差が拡大することを意味する．従って，内リンパ液の電位は，外リンパ液を基準とした際の内層の電位と，頂上膜を介したK^+平衡電位の和として以下のように計算できる．

$$EP = V_{Inner} + \frac{RT}{F} \ln\left(\frac{aK^+_{i(Inner)}}{aK^+_{EL}}\right) \quad \cdots\cdots\cdots \quad (c)$$

EPは内リンパ液の電位，V_{Inner}・$aK^+_{i(Inner)}$はそれぞれ内層の電位・K^+濃度である．内リンパ液のK^+濃度（aK^+_{EL}）は一定であり，図4A上段の実験の場合には白矢頭の値となる．(c)式へ無酸素時の内層の電位・K^+濃度変化の実測値（図4A上段点線四角内）を代入し，内リンパ液電位の予想値を求めた（図4B上段）．これは，内リンパ液電位の変化の実測値（図4A下段点線四角内と図4B上段灰色線）とよく合致した．従って，無酸素負荷時に拡大する内層頂上膜を介した電位差は，主にI_{Ks}チャネルが発生するK^+拡散電位によることが明らかとなった．

6．実験結果のまとめとさらになる疑問点

ここまでの実験により，正常状態においては内リンパ液高電位の主な起源がIS電位であること，またこの電位の大部分はK^+チャネルKir4.1が発生するK^+拡散電位であることを示し（図3，5），従来の仮説[7]をはじめて実証した．高いIS電位は，タイトジャンクションによってISが隣接する細胞外液から電気的にバリアされていることにより保たれると考えられる（図1B）[9]．

また，内層の頂上膜を介したK^+拡散電位も内リンパ液の電位に深くかかわることを新たに見出した（図4，5）．内層のNa^+, K^+−ATPaseを阻害した際に，IS電位と内リンパ液の電位の間に差が見られた（図3）．これは，内層内のK^+濃度の低下によって拡大する内層頂上膜のK^+拡散電位により説明できた（図4，5）．また内層の基底側にはK^+チャネルが殆どないため[10]（図1B），内層の電位はISのK^+濃度変化の影響を受けず，ほぼIS電位と等価と考えられる．事実，無酸素負荷時の内層の二層性電位変化パターンは，ISのそれに類似している（図3，4）．よって，内リンパ液電位（EP）は，(b) (c)より以下の式で求められる．

$$EP = V_{Outer} + \frac{RT}{F} \ln\left(\frac{aK^+_{i(Outer)}}{aK^+_{IS}}\right) + \frac{RT}{F} \ln\left(\frac{aK^+_{i(Inner)}}{aK^+_{BL}}\right) \quad \cdots\cdots\cdots \quad (d)$$

以上より，内リンパ液の電位は，血管条の2つのK^+チャネルが発生する拡散電位に依存することが明らかとなった．

実験によって内リンパ液高電位の成立機序が解けたようであるが，さらなる疑問点もわいてくる．まず，内層基底膜のK^+取り込み輸送体が阻害された時に，どのような機序によってISのK^+が上昇し，かつ内層内のK^+が低下するのか，という疑問である．これは，正常状態において大きな拡散電位に基づくISの低いK^+濃度は，どのように維持されるのか，ということにも直結する．また，蝸牛のK^+循環と内リンパ液の電位との相関は具体的にどのようになっているか，ということも未解決である（「1．はじめに」を参照）．これらには血管条のK^+輸送分子が駆動するK^+の一方向性輸送（図1B）が関与するとも予想されるが，われわれは具体的に説明したいと考えた．そのためには，血管条の膜のイオン電流と電位の相関，そしてこれらの要素とK^+循環や内リンパ液の電位を実験で観察する必要があるが，各種電流を生きた動物において測定することは，現在の技術ではほぼ不可能である．従って，コンピューターシミュレーションを用いて検討した[12]．

7．蝸牛K^+循環の数理モデル化

図2Bに示した通り，イオンチャネルに依存して変化する膜電位と膜を介した電流の動態は，電気回路で表現できるため，計算式で表すことができる．一般に，イオン輸送体は，膜に隣り合う空間の濃度を調節する因子として，これも数式化できる．そこで，各膜上の輸送分子を回路で示し，有毛細胞の回路と合わせて直列につなぐことで，血管条のK^+輸送と蝸牛のK^+循環を数理モデル化した（図6A）．K^+が内リンパ液から有毛細胞へと透過する際の唯一の経路は，感覚毛頂部にある機械刺激感受性チャネルであるが（図1A，6A），このチャネルは無音状態でも常に少し開いているという特徴を持つ．そこで，K^+循環の電流量は機械刺激感受性チャネルを介したK^+流入の駆動力，すなわち，内リンパ液の電位と有毛細胞の細胞体との電位の差に依存するという前提を立てた．つまり，内リンパ液の電位が低下すれば，K^+循環の電流量が減ることになる．そして，無音状態においてシミュレーションを走らせた．

正常状態と無酸素負荷時の内リンパ液とISの電位，ISと内層のK^+濃度の計算結果を図6Bに示す．無酸素の条件は，内層基底膜のK^+取り込み輸送体を阻害することで再現した．2つの条件において，計算結果は実験値を上手く再現した（図6B，図3と図4も参照）．そこで，無酸素の条件下における各膜のイオン電流の動態を分析した（図6C）．すると，外リンパ液からISまでK^+で構成される循環電流が，内層基底膜ではK^+取り込み輸送体が機能しないためK^+として運搬されず，共存するCl^-チャネルと陽イオンチャネルを介してCl^-とNa^+に置き換わることが判明した．Cl^-は陰イオンであるため，電荷としては図6Cの矢印とは逆に流れていることにご留意頂きたい．ちなみに，これら2つのチャネルは，正常状態ではイオンを通す能力が弱く，膜電位に殆ど寄与しない．内層頂上膜では，I_{Ks}チャネルが健在であるため，循環電流はK^+を使って内リンパ液へと流れる．すなわち，ISではKir4.1を通ってK^+が入るが，K^+が出て行かないのでK^+濃度が増加し，内層内ではK^+が入ってこないがK^+が出て行くのでK^+濃度が減少することが分かる（図6C，6Bも参照）．その結果，血管条のイオンバランスの崩壊が徐々に起こり，内・外層のK^+拡散電位が大きく変化して内リンパ液の電位が降下する（図5）．それに呼応して，内リンパ液と有毛細胞の電位差に依存するK^+循環の量が減少していく，という現象がシミュレーションされた．以上より，イオン輸送分子を介した膜の電流と，膜電位，各空間のイオン濃度は密接な関係があり，これに立脚して内リンパ液と蝸牛K^+循環が双方向性に共役していることが示された．

8．おわりに

実験とコンピューターシミュレーションを駆使した一連の研究により，内リンパ液高電位の成立機構と，K^+循環との協関の主軸は理解された．数理モデルでは，まだ分子同定されていない輸送分子を幾つか仮定しており，今後はそれを実験によって同定する作業が必要にな

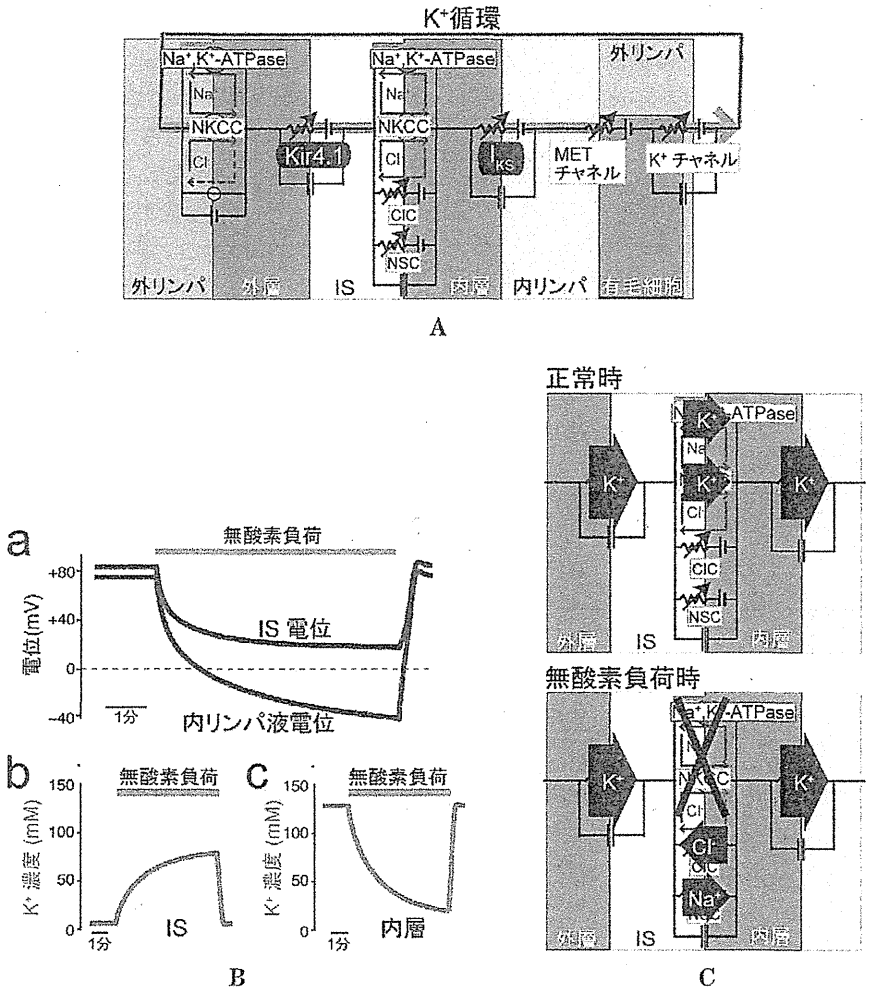

図6　数理モデル化による蝸牛 K^+ 循環のシミュレーション

A：血管条 K^+ 輸送と蝸牛 K^+ 循環を表す等価電気回路．NKCC：$Na^+, K^+, 2Cl^-$ 共輸送体，ClC：Cl^- チャネル，NSC：非選択性陽イオンチャネル，MET：機械刺激感受性．

B：正常時および無酸素負荷時における各区分の電位（a，黒線）と K^+ 濃度（b，灰色線）のシミュレーション結果．活量ではなく濃度表示であることに留意．

C：外層・IS・内層・内リンパ液を横切る K^+ 循環の動態．正常時（上段）では，K^+ のみが各空間と膜を透過する．無酸素負荷時（下段）には，内層基底膜において K^+ 取り込み輸送体が阻害されている．従って，外層頂上膜を流れてくる K^+ が，内層基底膜においては，Na^+ と Cl^- に置換されて陽イオンチャネル（NSC）と Cl^- チャネル（ClC）を透過する．Cl^- は陰イオンであるので，電荷は矢印とは反対の方向に流れることに注意．内層頂上膜では K^+ として電流が流れる．このイオン動態は，IS と内層内のイオン濃度の変化を惹起し，その結果，内リンパ液の電位が低下する（B，文中参照）．

ってくる．そして，さらに充実させたモデルを用いて，高電位の成立にかかわる機能分子を *in silico* で破綻させて生ずる病態を理論的に抽出し，疾患標的分子を予測することが可能であると考えている．その情報を元として，新しい難聴モデル動物も作製したい．実験科学と計算科学をフィードバックさせながら研究を進め，原因不明の聴覚疾患の病因究明や難聴に対する新しい治療法の開発などにも挑戦していく所存である．

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REGULAR ARTICLE

Developmental changes in the responsiveness of rat spiral ganglion neurons to neurotrophic factors in dissociated culture: differential responses for survival, neuritogenesis and neuronal morphology

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Abstract The way that the development of the inner ear innervation is regulated by various neurotrophic factors and/or their combinations at different postnatal developmental stages remains largely unclear. Moreover, survival and neuritogenesis in deafferented adult neurons is important for cochlear implant function. To address these issues, developmental changes in the responsiveness of postnatal rat spiral ganglion neurons (SGNs) to neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and leukemia inhibitory factor (LIF) were examined by using a dissociated cell culture system. SGNs at postnatal day (P) 0, P5 and P20 (young adult) were cultured with the addition of NT-3, BDNF, or LIF or of a combination of NT-3 and BDNF (N + B) or of NT-3, BDNF and LIF (ALL factors). SGNs were analyzed for three parameters: survival, longest neurite length (LNL) and neuronal morphology. At P0, SGNs required exposure to N + B or ALL factors for enhanced survival and the ALL factors combination showed a synergistic effect much greater than the sum of the individual factors. At P5, SGNs responded to a wider range of treatment conditions for enhanced survival and combinations showed only an additive improvement over individual factors. The survival percentage of untreated SGNs was highest at P20 but combinations of neurotrophic factors were no more effective than individual factors. LNL of each SGN was enhanced by LIF alone or ALL factors at P0 and P5 but was suppressed by NT-3, BDNF and N + B at P5 in a dose-dependent manner. The LNL at P20 was enhanced by ALL factors and suppressed by N + B. Treatment with ALL factors increased the proportion of SGNs that had two or more primary neurites in all age groups. These findings suggest that NT-3, BDNF, LIF and their combinations predominantly support different ontogenetic events at different developmental stages in the innervation of the inner ear.

Keywords NT-3 · BDNF · LIF · Age-related changes · Inner ear · Rat (Sprague Dawley)

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Introduction

In the auditory system, various growth factors are known to play roles in the development of the innervation of the inner

ear (Fritzsch et al. 1997b, 2004; Rubel and Fritzsch 2002). For example, studies of transgenic mice have shown that two members of the nerve growth factor (NGF) family of proteins, namely brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), play essential roles in the normal development of innervation (Agerman et al. 2003; Ernfors et al. 1995; Farinas et al. 2001; Fritzsch et al. 1997a, 2004; Tessarollo et al. 2004). In vitro studies of spiral ganglion neurons (SGNs) have revealed that both NT-3 and BDNF can promote SGN survival and neuritogenesis in organotypic SG explants (Aletsee et al. 2001; Hartnick et al. 1996; Mou et al. 1997; Mullen et al. 2012; Pirvola et al. 1992) and in dissociated cultures (Gillespie et al. 2001; Hartnick et al. 1996; Hegarty et al. 1997; Malgrange et al. 1996; Marzella et al. 1999; Pirvola et al. 1994; Vieira et al. 2007; Wei et al. 2007; Whitlon et al. 2006; Zheng et al. 1995). Recent studies have shown that neurotrophins also regulate the physiological properties of SGNs (Adamson et al. 2002; Davis and Liu 2011). Other peptide growth factors, such as ciliary-derived neurotrophic factor and leukemia inhibitory factor (LIF), also promote survival and neuritogenesis in SGNs in vitro (Gillespie et al. 2001; Hartnick et al. 1996; Marzella et al. 1997; Vieira et al. 2007; Whitlon et al. 2006).

In various peripheral nervous systems, the magnitude and manner of the neuron's response to neurotrophic factors is known to be age-dependent. For example, sympathetic neurons require NGF for their survival in early development. After they become mature, they are less dependent on NGF for survival (Easton et al. 1997; Putcha et al. 2000) but still respond to NGF with increased neurite growth (Orike et al. 2001b). Moreover, the survival dependence of trigeminal ganglion neurons has been shown to switch from BDNF and NT-3 to NGF during the early stages of target field innervation (Buchman and Davies 1993). Similarly, a subpopulation of dorsal root ganglion (DRG) neurons depends on NGF during embryonic development but switches its dependence to glial-cell-derived neurotrophic factor in early postnatal life (Molliver et al. 1997). These data suggest that various neurotrophic factors can exert their effects on the same neuronal population, either simultaneously or sequentially, at different phases of their development and further, that each factor might regulate different ontogenetic events at different developmental stages. Although a few studies have addressed this issue in the vestibular system (Chihara et al. 2011; Hashino et al. 1999), the degree to which similar age-dependent regulation of SGNs by neurotrophic factors occurs remains unclear.

Accordingly, we have performed a systematic developmental study of rat SGNs by culturing dissociated SGNs harvested at various postnatal stages from birth to the age at which the hearing function is mature. We have tested the responsiveness of these SGNs to NT-3, BDNF and LIF, alone or in various combinations, focusing mainly on SGN survival, neuritogenesis and the neuronal morphology of individual SGNs.

Materials and methods

All animal procedures were approved by the relevant local animal subjects committees (Graduate School of Medicine, The University of Tokyo, #P08-029) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of tissue culture plates

Circular coverslips (13 mm; Matsunami glass, Tokyo, Japan) were incubated with a mixed solution of fibronectin (Sigma Aldrich Japan, Tokyo, Japan; 10 μg/ml in phosphate-buffered saline [PBS; pH 7.4]) and laminin (Sigma Aldrich Japan; 10 μg/ml in PBS) at 4°C overnight and then washed three times in PBS (pH 7.4). The coverslips were then incubated with poly-L-lysine (Sigma Aldrich Japan; 20 μg/ml in Dulbecco's modified Eagle's medium [DMEM; Invitrogen life technologies, Tokyo Japan]) at 37°C for 1 h, washed three times in PBS (pH 7.4), air-dried and placed in each well of 24-well plates (BD Biosciences, Tokyo, Japan).

Animal dissection and dissociated cell culture of SGNs

Cultures of dissociated SGNs were prepared from Sprague Dawley rats (Saitama experimental animals, Saitama, Japan) at postnatal day 0 (P0), day 5 (P5) and day 20 (P20). We chose these developmental ages for the following reason: the period of P0 culture (equivalent to P0-P4 in vivo) corresponds to the time of naturally occurring SGN death (Echteler et al. 2005; Rueda et al. 1987); P5 culture (equivalent to P5–P9 in vivo) corresponds to the terminal stage of the remodeling of afferent projections within the sensory epithelium (Echteler 1992; Wiechers et al. 1999); and P20 culture (equivalent to P20–P24 in vivo) corresponds to the stage in which all of the major developmental events for SGNs are complete and the hearing function has achieved maturity (Pujol et al. 1998).

For the dissociation of SGNs, rats were deeply anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (9 mg/kg) and then decapitated. The mandible was removed and skulls were opened mid-sagitally. Under a dissecting microscope, the brain was removed and the temporal bones were harvested and transferred to Petri dishes containing sterile PBS (pH 7.4). The membranous labyrinth was exposed by removal of the bony or cartilaginous cochlear capsule. After

the removal of the spiral ligament, stria vascularis and organ of Corti, the SG was carefully dissected from the modiolus by using fine forceps and placed in calcium/magnesium-free Hank's balanced salt solution (HBSS, Invitrogen life technologies). Pooled ganglia (n=10–16) were then enzymatically digested in HBSS with 0.1% collagenese and then in HBSS with 0.25% trypsin (both from Invitrogen life technologies) in a gently shaking 37°C water bath. Incubation times varied depending on the age of the tissue (see Table 1). Enzymatic digestion was terminated by the addition of fetal bovine serum (Invitrogen life technologies) to a final concentration of 10% and the digested ganglia were centrifuged at 1,500 rpm for 5 min at room temperature (RT). The supernatant was discarded and fresh primary growth medium consisting in DMEM (Invitrogen life technologies), fetal bovine serum (10%; Invitrogen life technologies), HEPES buffer (10 mM; Invitrogen life technologies) and penicillin (300 U/ml; Sigma Aldrich Japan) was then added, following which the ganglia were dissociated by mechanical trituration through a plastic pipette tip. In this procedure, a maximum of four triturations were carried out before the cells were removed by decanting the supernatant medium. Fresh medium was added, a further four triturations were carried out and the cells were again removed. These sequential triturations were repeated until the majority of the neurons were removed, with the number of repeats depending on the age of the donor. This method of avoiding excess mechanical damage to the dissociated SGNs enhanced the survival of SGNs several fold, especially in P20 SGNs, as demonstrated in the dissociation of superior cervical ganglion neurons (Orike et al. 2001a).

Each cell suspension was again spun at 1,500 rpm for 5 min and the resultant pellet was resuspended in fresh primary growth medium. Cell counts were made by using a hemocytometer and dissociated cells were plated onto the center of the glass coverslips at a density of 20,000 cells/100 μl per well. The cultures were maintained at 37°C in a humidified atmosphere containing 8% CO_2 for 2 h to promote the attachment of the neurons to the bottom of the coverslips. The cultures were then incubated in 300 μl primary growth medium for 10 h for the complete attachment of the SGNs, followed by incubation in a serum-free maintenance medium consisting in DMEM, N2 supplement (10 μl/ml; Invitrogen life technologies), HEPES buffer (10 mM), penicillin (300 U/ml), glucose (at a final concentration of 6 g/l; Sigma Aldrich Japan) for an additional 72 h. To compare the effects of neurotrophic factors, cultures in each age group were further divided into six subgroups and supplemented with the following factors: (1) recombinant human NT-3 (catalog number 480875; EMD biosciences, La Jolla, Calif., USA; 50 ng/ml), (2) recombinant human BDNF (catalog number 01-194; Upstate Biotechnology, Lake Placid, N.Y., USA; 50 ng/ml), (3) LIF (catalog number LIF3005; Chemicon, Temecula, Calif., USA; 50 ng/ml), (4) a combination of NT-3 and BDNF (50 ng/ml each; referred to as the N + B group), (5) a combination of NT-3, BDNF and LIF (50 ng/ml each; referred to as the ALL factor group). One group was cultured without addition of any of the factors to serve as an untreated control. The concentration of each factor was determined based on previous studies that had examined the responses of SGNs from postnatal mice and rats in vitro (Gillespie et al. 2001; Hegarty et al. 1997; Malgrange et al. 1996; Marzella et al. 1999; Vieira et al. 2007; Wei et al. 2007; Whitlon et al. 2006). To assess the effects of concentration on SGN neurite extension (see Results), additional P5 cultures were treated with NT-3 (10 ng/ml), BDNF (10 ng/ml), or N + B (10 ng/ml each).

To determine the initial yields of plated SGNs, cultures (n=4 in each age group) were maintained in primary growth medium for 4 h after plating, followed by fixation for immunostaining.

Table 1 Conditions for dissociation of spiral ganglion neurons (SGNs) at postnatal day 0 (P0), P5 and P20

Treatment	P0	P5	P20
Duration of collagenase incubation (min)	15	20	30
Duration of trypsin incubation (min)	20	30	40

Immunohistochemistry

Cultured cells were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at RT. After several PBS (pH7.4) washes, endogeneous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 min at RT. The cells were then incubated with blocking solution (PBS, pH 7.4, containing 4% fetal bovine serum [Invitrogen life technologies], 0.2% Triton X-100 and 0.1% sodium azide) for 30 min each at RT to reduce non-specific antibody binding, followed by incubation with anti-neurofilament (NF) 200 mouse monoclonal antibody (clone NR52, Sigma; 1:500 in blocking solution) at 4°C overnight. After three washes in PBS (pH 7.4), the tissues were incubated with biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories Japan, Tokyo, Japan) for 1 h at RT. Following three washes in PBS (pH 7.4), the tissues were then reacted with ABC solution (Vectastain Elite kit; Vector Laboratories Japan) for 30 min at RT, according to the manufacturer's instructions. After further washing steps in PBS (pH 7.4), immunoreactivity was visualized by using the diaminobenzidine (Vector Laboratories Japan) reaction. After additional washes in distilled water, the cells were dehydrated through graded ethanol and xylene and then mounted on glass slides. The primary antibody was omitted from the procedure as a negative control; this gave no labeling corresponding to the immunostaining of the primary antibody.

Image presentation

Photomicrographic images of the immunostained SGNs were taken on a Nikon E800 microscope (Nikon, Tokyo, Japan) under brightfield illumination and phase-contrast optics with a digital microscope camera (AxioCam, Carl Zeiss Japan, Tokyo, Japan). Digital images were edited with Adobe Photoshop CS1 software (Adobe Systems Incorporated, Tokyo, Japan). The images were not modified except for minor adjustments of size, orientation, brightness, contrast and conversion to grayscale consistent with analysis.

Data analysis

We used the following indices for evaluation of the effects of neurotrophic factors on SGNs: the number of surviving neurons in each culture, the number of surviving SGNs relative to the initial seed number, the length of the longest neurite extending from each SGN and neuronal morphology. Data were collected from at least four independent experiments with at least duplicate wells for each of the experimental conditions. Quantitative analyses described below were performed by independent observers in a blinded manner.

To evaluate SGN survival, we counted all neurons present on each coverslip. Every cell that was labeled with the anti-NF200 antibody and also had a visible nucleus was counted as a surviving neuron, without regard to morphology or the number of processes.

To evaluate the length of the longest neurite on SGNs, neurons were sampled from the center field of view (4× objective) within each coverslip. Two additional sampling fields adjacent to the center field were employed in P20 cultures, because of the small number of SGNs in each field. The neurons within each 4× microscopic field were individually photographed and every SGN inside each field was included for analysis. All processes that emanated from the cell body of the SGNs were traced and their lengths were measured by using image analysis software (Microanalyzer, Polaroid Japan). The length of the longest neurite from each SGN was defined as the longest neurite length (LNL). The length of the neurite in SGNs without a neurite was listed as 0. When measuring neurite length, only those neurons whose processes could be clearly traced were included. This might have biased our results toward cells with shorter neurites, as some neurites exited the microscopic field. A total of 1247, 1355 and 953 neurons were analyzed from P0, P5 and P20 cultures, respectively.

Neuronal morphology was assessed for the same SGNs evaluated for the neurite extension. Each neuron analyzed was categorized into monopolar, bipolar, multipolar, or no processes. Neurons extending only neurite(s) shorter than the diameter of their cell body were categorized as having no processes. The percentage of the various morphologies was calculated from the total number of neurons analyzed.

Statistical analysis

Results were presented as the mean ± SE of samples in each experimental group. Statistical analyses for the number of surviving neurons and LNL were performed by using one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test with GraphPad Prism 5 (GraphPad Software, La Jolla, Calif., USA) to compare each neurotrophin subgroup with the untreated control group. For the statistical analysis of the effect of neurotrophic factors on neuronal morphology, neurons were divided into two categories, i.e., SGN without or one neurite (referred to as the 0-1 neurite group) and SGN with two or more neurites (referred to as the ≥2 neurite group) and a chi-square test was used. Differences associated with P values of <0.05 were considered as statistically significant.

Results

General appearance of cultures

Immunostaining of the cultured cells at 4 h after plating revealed that the mean seed number of SGNs per well was 2387±88 in the P0 group, 889±65 in the P5 group and 164±18 in the P20 group.

Figure 1a illustrates an example of cultured SGNs after 12 h in primary growth medium containing serum and a further 72 h in serum-free maintenance medium. The surviving SGNs were identified as NF200-positive cells. As described in previous studies of murine dissociated SGN cultures (Vieira et al. 2007; Whitlon et al. 2007), cultured neurons either had no neurite (Fig. 1b) or showed monopolar (with one neurite emanating from the cell body; Fig. 1c), bipolar (with two neurites emanating from the cell body; Fig. 1d), or multipolar (with three or more neurites emanating from the cell body; Fig. 1e) morphologies.

We also examined the cultures under phase-contrast optics to evaluate the appearance of SGNs and non-neuronal cells (possibly consisting in glial cells and fibroblasts that were unlabeled). As shown in Fig. 2, non-neuronal cells in each culture exhibited two morphologies, i.e., flat-shaped cells with large nuclei and spindle-shaped cells with small nuclei and processes that extended from the cell body. This difference was most readily observed in P20 cultures (Fig. 2c). The density of non-neuronal cells decreased with increasing age of the donor animals; in P0 and P5 cultures, the non-neuronal cells formed a confluent layer on the glass surface (Fig. 2a, b), whereas the glass surface was partially free of non-neural cells in P20 cultures (Fig. 2c).

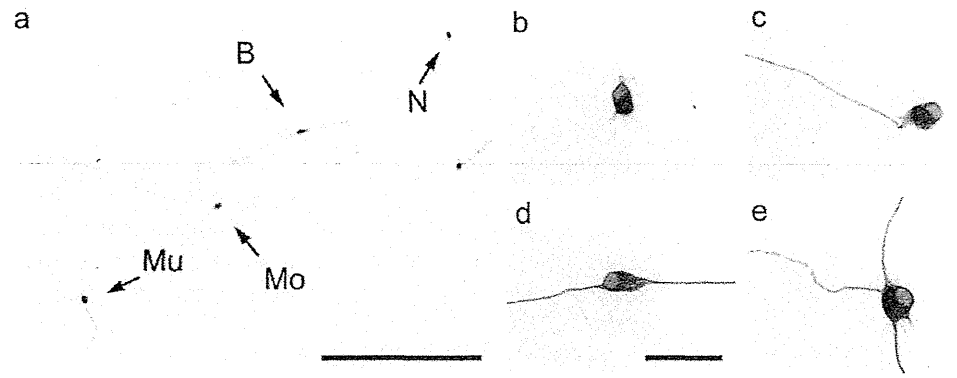

Fig. 1 **a** Example of cultured spiral ganglion neurons (SGNs) after 12 h in primary growth medium containing serum and a further 72 h in serum-free maintenance medium. SGNs were then fixed and immunostained with anti-NF200 antibody. Surviving SGNs are identified as NF200-positive cells. Cultured neurons either have no neurite (*N*) or show monopolar (*Mo*; with one neurite emanating from the cell body), bipolar (*B*; with two neurites emanating from the cell body), or multipolar (*Mu*; with three or more neurites emanating from the cell body) morphologies. High magnification views of the *N*, *Mo*, *B* and *Mu* SGNs shown in **a** are presented in **b–e**, respectively. *Bars* 0.5 mm (**a**), 50 μm (**b–e**)

Effects of NT-3, BDNF and LIF on SGN survival

Figure 3 shows representative photomicrographs of cultured SGNs in P0, P5 and P20 groups, with or without neurotrophic factor supplementation. As illustrated, increases in SGN numbers as a result of neurotrophic support are apparent for SGNs harvested at younger ages.

Figure 4 provides a quantitative analysis of SGN numbers across ages and treatment conditions. Figure 4a shows the average number of SGNs/well, uncorrected for initial seed number. In the absence of any neurotrophic factor treatment, P5 cultures obviously showed the greatest yield of surviving neurons (8.7±0.7/well), followed by P20 cultures (7.0±1.5/well) and then by P0 cultures (0.6 ±0.2/well). Moreover, the greatest sensitivity to neurotrophic treatment was observed for P5 SGNs.

Because of the difference in the initial yield of SGNs among age groups, we normalized the number of surviving SGNs/well by the initial seeding level. These data are presented in Fig. 4b, which demonstrate that the survival rate of untreated SGNs in culture increases dramatically with increasing age. In control cultures, surviving SGNs at 72 h corresponded to 0.027%, 0.98%, or 4.2% of those seeded initially in the P0, P5 and P20 groups, respectively. The normalized survival rate of SGNs in each treatment group also tended to increase for older SGNs (Fig. 4b).

In P0 cultures, neurotrophic factors had a significant effect on the survival of SGNs ($P<0.0001$; ANOVA) and this effect was additive/synergistic. When the culture was treated with N + B, the number of surviving neurons per culture (10.5±0.8) increased to 17-fold that of untreated cultures (0.6±0.2). This number almost corresponded to that of the sum of the number of surviving neurons in NT-3 (0.9±0.2) and BDNF cultures (5.3±0.5). When the culture was treated with ALL factors, the number of surviving neurons further increased to 74-fold that of untreated cultures (74.0±6.7), which is approximately 9.1-fold that of the sum of the number of surviving SGNs in NT-3-treated (0.9±0.2), BDNF-treated (5.3±0.5) and LIF-treated (1.9±0.4) cultures. The post-hoc test revealed that the treatment with N + B and ALL factors had statistically significant survival-promoting effects compared with the untreated control ($P<0.05$, $P<0.001$, respectively).

In P5 cultures, SGN survival responded to the widest range of treatments amongst the age groups; ANOVA revealed significant effect on survival ($P<0.0001$) and the post-hoc test showed that each of NT-3, BDNF, N + B and ALL factors significantly enhanced the survival of SGNs ($P<0.001$ for each; Fig. 3). In this age group, the additive/synergistic effect seen in P0 cultures was decreased under our culture conditions. When the culture was treated with N + B, the number of surviving neurons per culture (63.4±7.1), which was 6.3-fold that of untreated cultures (10.0±1.1), almost corresponded to the number of surviving neurons in BDNF cultures (5.9-fold, 59.0±4.7). When the culture was treated with ALL factors, the number of surviving neurons (nine-fold, 90.3±6.0) was slightly less than the sum of the number of SGNs treated with the individual factors: BDNF, NT-3 (3.2-fold, 31.5±2.0) and LIF(1.9-fold, 19.4±1.6).

In P20 cultures, the survival-promoting effect of each treatment was the smallest of any of the age groups ($P=0.0097$; ANOVA). The post-hoc test revealed that only treatment with NT-3 or with BDNF significantly promoted the survival of SGNs compared with the untreated control ($P<0.01$, $P<0.001$, respectively). Moreover, cultures treated with N + B or ALL factors

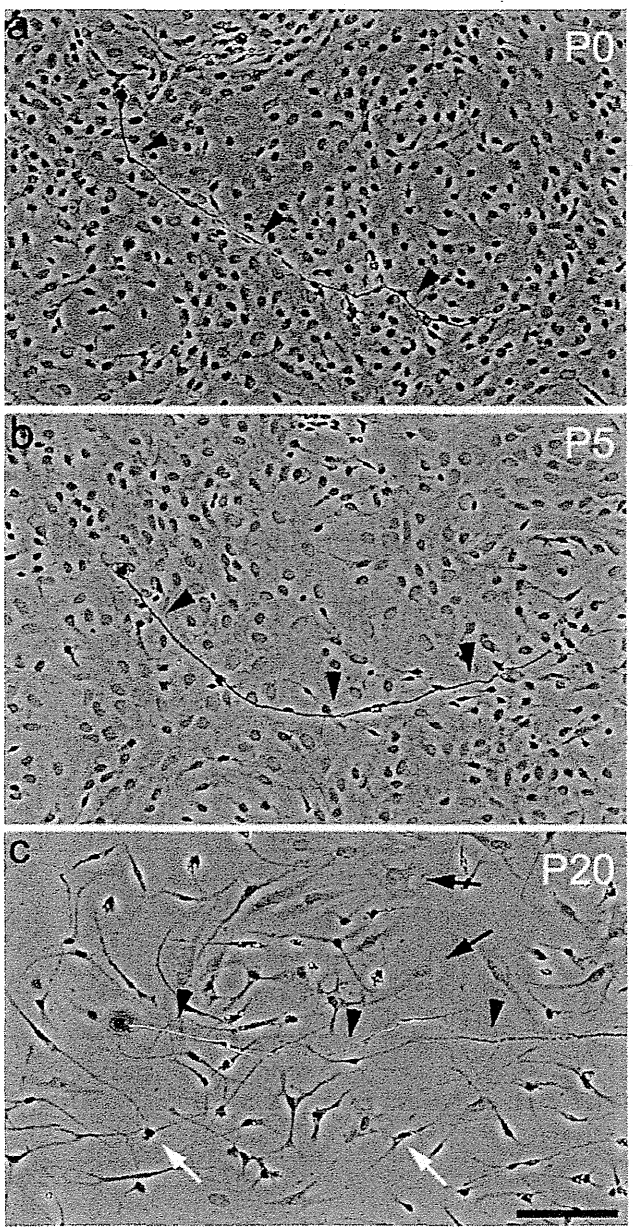

Fig. 2 Representative phase-contrast images of postnatal day 0 (P0; **a**), P5 (**b**) and P20 (**c**) control cultures maintained for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium (*arrowheads* neurites of SGNs). Non-neuronal cells in each culture can be morphologically differentiated into two cell types, i.e., *flat-shaped* cells with large nuclei and *spindle-shaped* cells with small nuclei and prominent processes. This differentiation is most readily observed in P20 culture (*black arrows* flat-shaped cells, *white arrows* spindle-shaped cells). The density of these non-neuronal cells decreases with the increasing age of the animals. In P0 and P5 cultures, the non-neuronal cells form a confluent layer on the glass surface, whereas the glass surface is partially free of cellular covering in P20 culture. *Bar* 0.1 mm

exhibited survival no greater than that observed in controls.

Effects of NT-3, BDNF and LIF on neurite extension

The LNL of untreated SGNs was shortest for neurons harvested at P0, slightly longer for P5 neurons and longest for P20 neurons.

In P0 cultures, the overall effect of treatment on the LNL was significant (Fig. 5a; $P<0.0001$; ANOVA). However, when factors were delivered alone, only LIF elicited an enhancement of LNL compared with the control group ($P<0.001$; Dunnet post hoc test). ALL factors also showed a significant increase in LNL ($P<0.01$) but this effect was lower than that observed with LIF alone. LNL was unaffected by the presence of NT-3, BDNF, or both factors combined ($P>0.05$).

LNL of P5 SGNs responded to the widest range of treatment with neurotrophic factors among the age groups (Fig. 5b; $P<0.0001$; ANOVA). Again, LIF and ALL factors showed significantly increased LNL ($P<0.001$), although ALL factors once more produced a lower increase than LIF alone. In contrast, treatment with NT-3, BDNF, or N + B inhibited neurite extension compared with that in the control group ($P<0.05$, $P<0.001$, $P<0.001$, respectively). To test whether this inhibitory effect depended on the concentration of neurotrophic factors, we also treated P5 SGNs with BDNF and/or NT-3 at 10 ng/ml. Although a survival-promoting effect was observed at this lower concentration, as seen at 50 ng/ml (Fig. 6a), LNL in the treated groups was not significantly different from that in the untreated controls (Fig. 6b). However, the addition of LIF (ALL factors) produced a significant enhancement of both survival and LNL at this age.

In P20 cultures, the effect of neurotrophic factors on LNL was the smallest among the age groups, although it remained significant (Fig. 5c; $P=0.0008$) by ANOVA. The post-hoc test revealed that treatment with ALL factors weakly enhanced LNL ($P<0.05$), whereas N + B had a strong inhibitory effect ($P<0.001$).

Effects of NT-3, BDNF and LIF on neuronal morphology

For all age groups, monopolar neurons were the most prevalent morphological type in untreated cultures (Fig. 7). In general, neurotrophic factor treatment had modest effects on neuronal morphology. An exception was ALL factors, which enhanced the proportion of bipolar and multipolar neurons, while decreasing the monopolar and especially the "no" neurites phenotypes.

To assess these effects statistically, neurons in each age group were divided into two categories, i.e., SGN without neurites or with one neurite versus SGN with two or more neurites. In all age groups, ALL factors significantly increased the proportion of ≥2 neurite SGNs compared with the control group (Table 2; $P<0.05$ in P0,

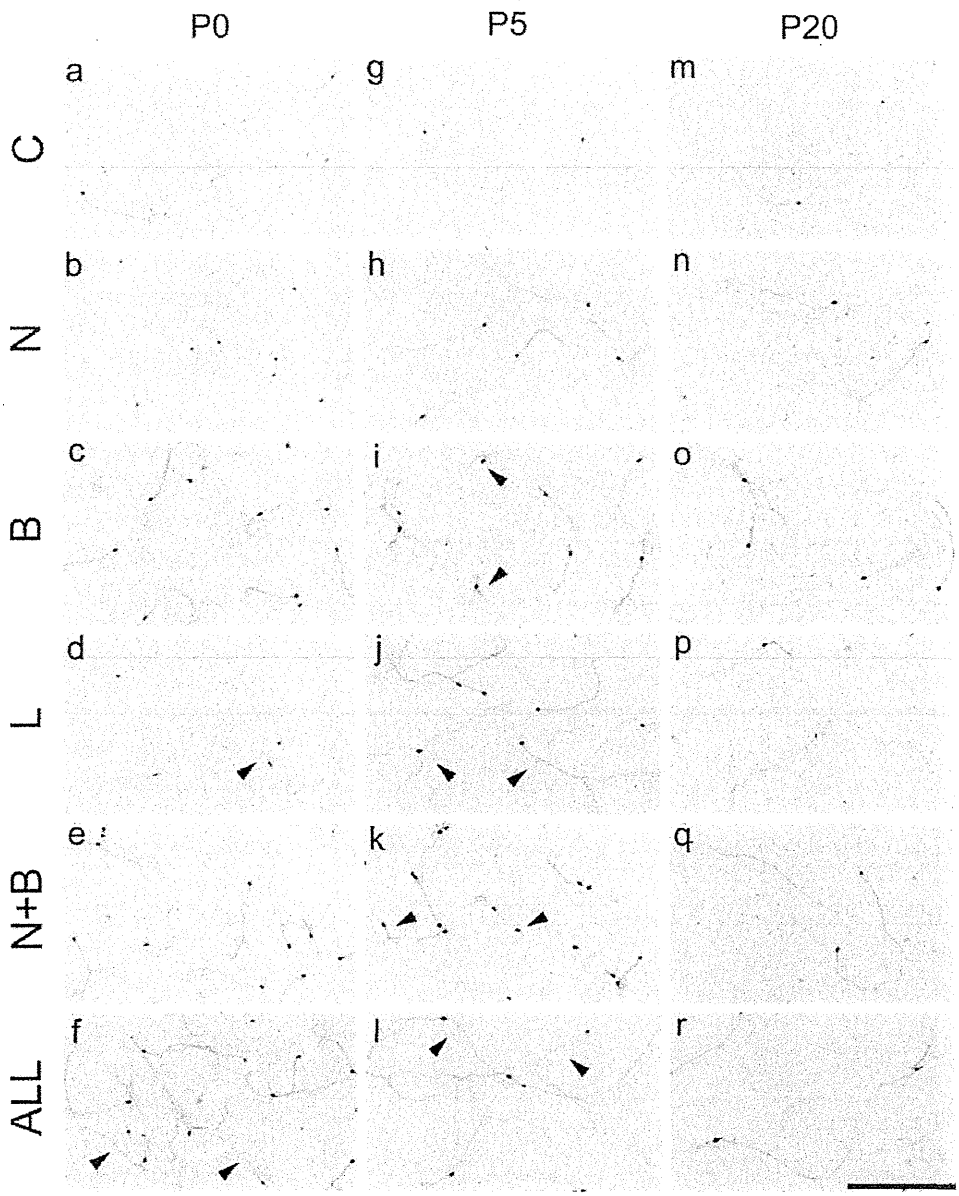

Fig. 3 Representative photomicrographs of cultured SGNs in each age group. Neurons at P0 (**a–f**), P5 (**g–l**) and P20 (**m–r**) were cultured for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium without any neurotrophic factors (**a**, **g**, **m**) or supplemented with 50 ng/ml neurotrophin-3 (NT-3; *N*, **b**, **h**, **n**), brain-derived neurotrophic factor (BDNF; *B*, **c**, **i**, **o**), leukemia inhibitory factor (LIF; *L*, **d**, **j**, **p**), a combination of NT-3 and BDNF (50 ng/ml each; *N+B*, **e**, **k**, **q**), or a combination of NT-3, BDNF and LIF (50 ng/ml each; *ALL*, **f**, **l**, **r**) and then fixed and immunostained with anti-NF200 antibody. Survival effects of NT-3, BDNF, LIF and their combinations compared with the untreated control varied depending upon the ages of SGNs. At P0, the additive/synergistic effect of treatment in the N + B and ALL factors groups is clearly apparent, whereas this effect appears to be decreased in P5 and is not obvious in P20 cultures. The length of neurites seems to be increased by treatment with LIF or ALL factors in P0 and P5 cultures (*arrowheads* in **d**, **f**, **j**, **l**). In contrast, neurite length appears to be decreased by supplemention with BDNF or N + B in P5 cultures (*arrowheads* in **i**, **k**). *Bar* 0.5 mm

$P<0.001$ in P5 and P20; chi-square test). In the P20 group, BDNF alone and LIF alone also more modestly enhanced the proportion of ≥2 neurite SGNs compared with the control group (Table 2; $P<0.05$; chi-square test).

Discussion

The present study was designed to extend our knowledge regarding age-dependent changes in the responsiveness of SGNs to NT-3, BDNF and LIF by using dissociated cultures. Our study appears to be the first systematic in vitro study to compare the regulation of survival, neurite extension and neuronal morphology of rat SGNs among different age groups under the same culture conditions. The results demonstrate that each of these indices of SGN maturation are differentially regulated by NT-3, BDNF and/or LIF in an age-dependent manner. Our data further suggest that each of these neurotrophic factors predominantly support different ontogenetic events at different developmental stages in the innervation of the inner ear.

Effects of neurotrophic factors on SGN survival

A striking feature of our results was the dramatic age-dependent increase in the proportion of neurons that survived in culture in the absence of neurotrophic factor treatment. This finding suggests that SGNs are highly dependent for their survival on exogenous neurotrophic factors at early developmental stages but become more neurotrophic-factor-

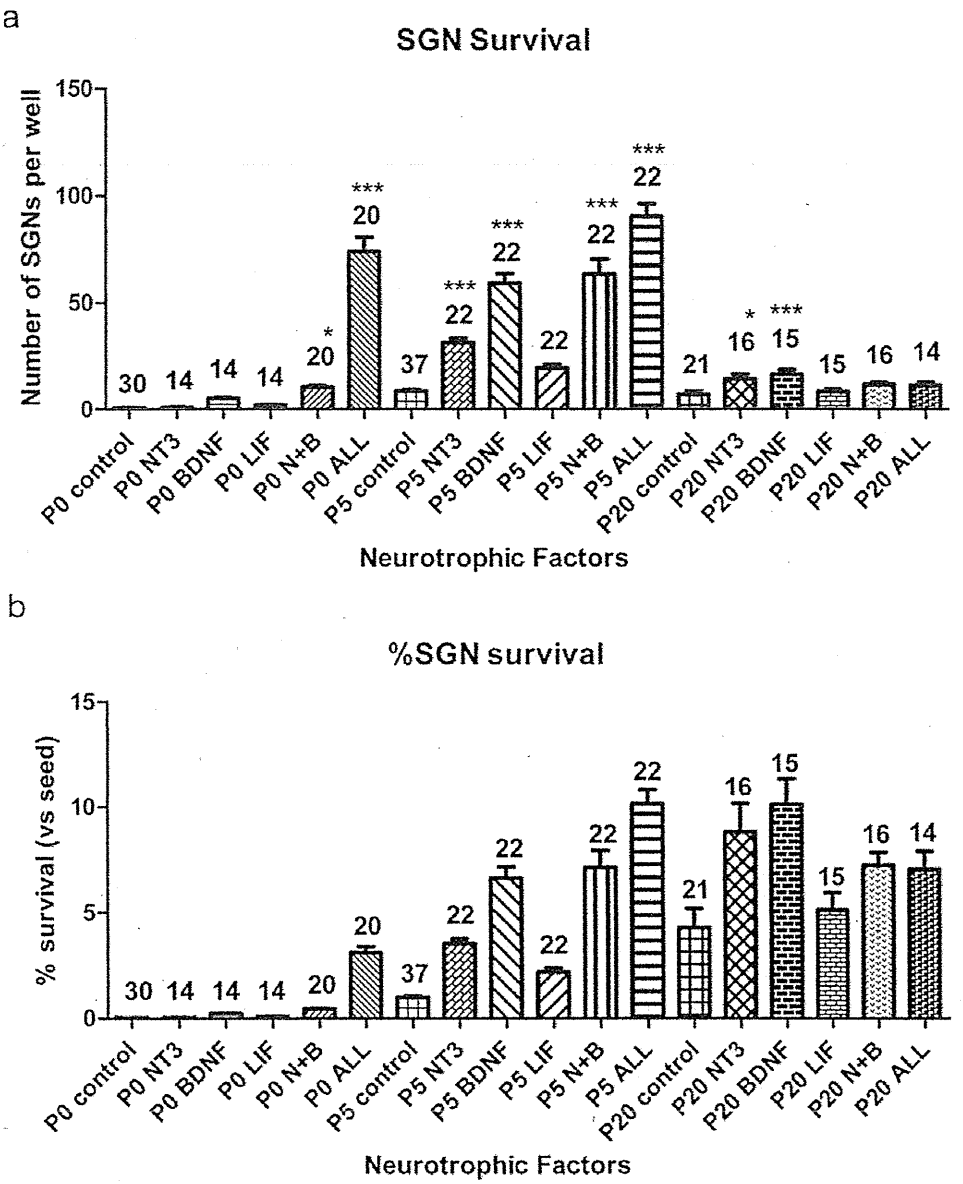

Fig. 4 **a** Effects of neurotrophic factors on SGN survival. Dissociated SG cells at P0, P5 and P20 were plated at 20,000 cells/culture well and cultured for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium without any neurotrophic factors (*control*) or supplemented with 50 ng/ml NT-3, BDNF, LIF, or N + B at 50 ng/ml each or with ALL factors at 50 ng/ml each. The data are presented as an average number ± SE of surviving SGNs per culture well at each developmental age. Significant differences compared with control cultures are indicated: **P*<0.05, ***P*<0.01, ****P*<0.001 (ANOVA followed by Dunnett's post-hoc test). The *number above* each *bar* indicates the number of cultures analyzed. **b** Percentage of surviving SGNs divided by initial number of neurons seeded. Data are presented as an average percentage ± SE of surviving SGNs compared with the initial seed number of SGNs at each developmental stage. The *number above* each *bar* indicates the number of cultures analyzed. The survival rate of SGNs in each treatment group tended to increase with increasing age of the donor animals

independent as they approach adulthood. A similar tendency has been reported for the trigeminal ganglion (Scott and Davies 1993) and sympathetic neurons (Easton et al. 1997; Orike et al. 2001b; Putcha et al. 2000). Because the period of our P0 culture (equivalent to P0–P4 in vivo) corresponds to a period of naturally occurring SGN cell death (Echteler et al. 2005; Rueda et al. 1987), we can speculate that the higher trophic factor dependence of P0 and P5 SGNs contributes to the elimination of SGNs that fail to establish connection with a factor-producing target, as has been suggested for gerbil SGN (Mou et al. 1998).

Arguing against this interpretation is the limited survival response of P0 SGN to neurotrophin treatment, since none of the factors tested were effective in promoting the survival of SGN when applied individually. However, combinations of factors were highly effective, especially when LIF was added to BDNF plus NT-3. BDNF or NT-3 alone only became effective at older ages, whereas the synergistic effects observed at P0 declined at P5 and disappeared at P20. These data suggest that more than one factor is required to rescue SGN from apoptosis during early developmental target cell interactions, thereby serving to promote the survival of neurons that make contacts with separate sources of trophic factors, such as the peripheral and central contacts of SGN. This possibility is supported by the finding that both NT-3 and BDNF are expressed in the developing cochlea and cochlear nucleus (Sugawara et al. 2007; Tierney et al. 2001; Wiechers et al. 1999). The elements of the LIF receptor-gp130 heterodimer (Auernhammer and Melmed 2000) are expressed in the P1 mouse spiral ganglion (Oshima et al. 2007) and LIF is strongly expressed in the adult cochlear sensory epithelium after noise injury (Cho et al. 2004).

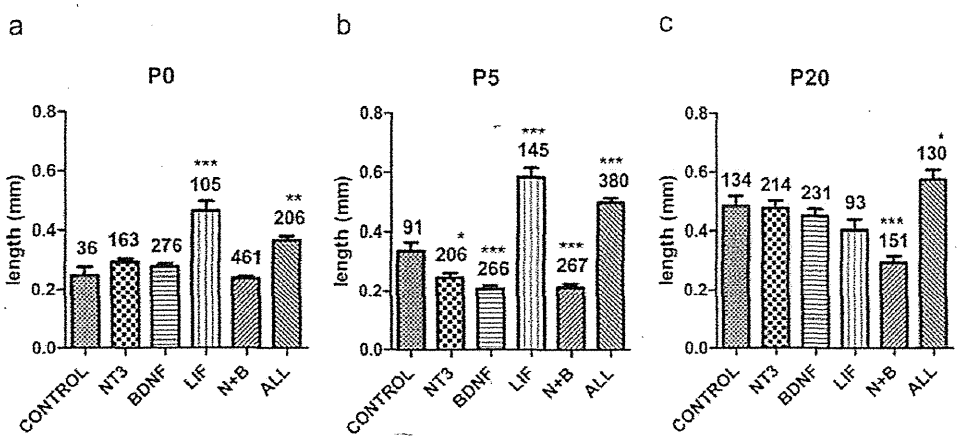

Fig. 5 Effects of neurotrophic factors on the longest neurite length (LNL) in P0 (**a**), P5 (**b**) and P20 (**c**) cultures. Data are presented as an average length ± SE of the longest neurite of each neuron at each developmental age. Significant differences compared with control cultures (*CONTROL*) are indicated: **P*<0.05, ***P*<0.01, ****P*<0.001 (ANOVA followed by Dunnett's post-hoc test). The *number above* each *bar* indicates the number of neurons analyzed. LIF supplementation of P0 and P5 cultures and ALL factors added to P0, P5 and P20 cultures significantly increased LNL compared with control SGNs. In contrast, treatment with NT-3, BDNF, or N + B in P5 cultures and with N + B in P20 cultures inhibited neurite extension

Although we could locate no published information regarding LIF expression in developing cochlear nucleus, LIF is expressed by many neurons in the brain (Lemke et al. 1996).

Notably, the initial seeding number of SGNs/well decreased dramatically with increasing age. Since the number of cells seeded/well was constant, this means that a greater proportion of the culture consisted in non-neuronal cochlear cells in older cultures. Therefore, we cannot exclude the possibility that the neurotrophic factor independence of older SGN reflects trophic support from these non-neuronal cells.

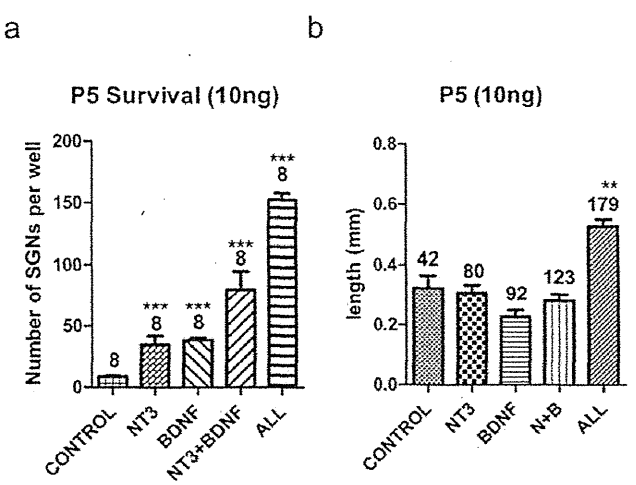

Fig. 6 Effects of NT-3 and BDNF at 10 ng/ml on survival (**a**) and LNL (**b**) in P5 cultures. Significant differences compared with control cultures are indicated: **P*<0.05, ***P*<0.01, ****P*<0.001 (ANOVA followed by Dunnett's post-hoc test). The *number above* each *bar* indicates the number of cultures (**a**) and neurons (**b**) analyzed. At 10 ng/ml, NT-3, BDNF, or N + B enhanced SGN survival, as also seen at 50 ng/ml, whereas inhibitory effects on neurite extension were not observed at this concentration

Although the density of non-neuronal cells decreased with the increasing age of the animals at the endpoint of culture period, probably reflecting the high proliferation rate of younger non-neuronal cells, the possibility that the initial support of non-neuronal cells at the early culture periods might have been associated with the higher survivial rate of SGNs at P20 cannot be excluded. The mature glial cells in P20 cultures might also have provided stronger trophic support than those in younger animals.

At P5 and P20, BDNF was more potent than NT-3 or LIF for survival. This observation is in good agreement with previous studies of dissociated early postnatal rat SGNs (Hegarty et al. 1997; Marzella et al. 1999; Zheng et al. 1995) and with the enhancement of the survival of adult SGNs by BDNF in vivo (Leake et al. 2011; Miller et al. 2007; Wise et al. 2005) and in vitro (Vieira et al. 2007; Wei et al. 2007). Although BDNF expression is almost absent in the peripheral target field of the SGNs at the later stages of development (Wheeler et al. 1994; Wiechers et al. 1999; Ylikoski et al. 1993), it is expressed by neurons in the ventral cochlear nucleus beginning on P3 (Tierney et al. 2001). BDNF is also observed in SGNs themselves until the adult stage (Ruttiger et al. 2007; Schimmang et al. 2003; Singer et al. 2008), raising the possibility of an autocrine mechanism (Schimmang et al. 2003).

With regard to the survival-promoting effects of LIF, previous in vitro studies have documented that LIF has survival-promoting effects on early postnatal rat and mouse SGNs (Gillespie et al. 2001; Whitlon et al. 2006) and adult mouse SGNs (Vieira et al. 2007). Our results suggest that the survival-promoting effects of LIF alone are not as strong as those of BDNF and NT-3. It is more potent as a synergistic enhancer of neurotrophins for the survival of P0 and, to a lesser extent, P5 rat SGNs.

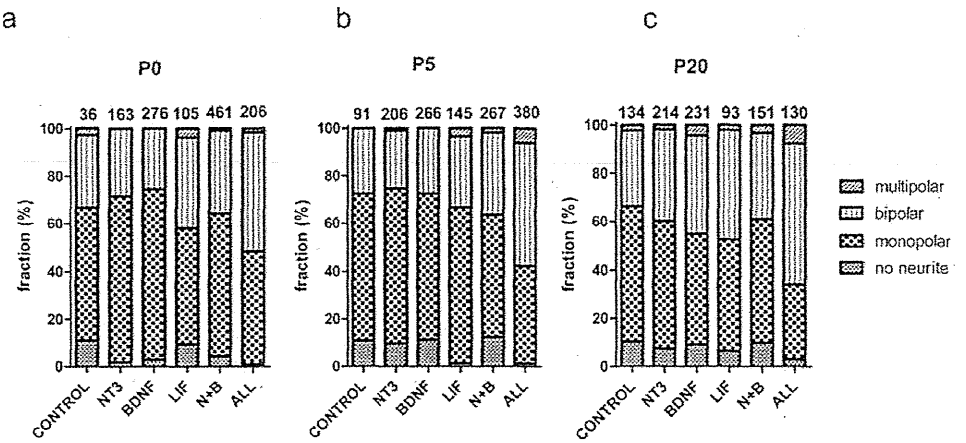

Fig. 7 Effects of NT-3, BDNF, LIF, N + B, or ALL factors on neuronal morphology. The fraction of SGNs that were without a neurite or that were monopolar, bipolar and multipolar under each culture condition are indicated. ALL factors enhanced the proportion of bipolar neurons and reduced the number of neurons without neurites at all ages. The *number above* each *bar* indicates the number of neurons analyzed

Effects of neurotrophic factors on neurite extension

The effect of neurotrophic factors on neurite extension in SGNs in vitro has been less extensively studied compared with neuronal survival or neurite number from explants and remains a relatively controversial issue. We have demonstrated that, although LNL in untreated SGNs is maximal at P20, the effect of neurotrophic factors on neurite length is greatest at P5. The period of our P5 culture (equivalent to P5–P9 in vivo) corresponds to a stage of late remodeling of afferent projections to the sensory epithelium (Echteler 1992; Wiechers et al. 1999) and cochlear nucleus (Limb and Ryugo 2000). The strong regulation of neurite extension by neurotrophic factors at P5 might be necessary to achieve the rearrangement of afferent innervation mediated by target-derived neurotrophic factors in the organ of Corti and/or brainstem.

Our study has revealed that LIF has a strong effect on neurite extension on P0 and P5 SGNs. At P0 and P5, treatment with LIF alone or with ALL factors enhanced neurite extension, whereas treatment with NT-3 and/or BDNF showed no significant effect at P0 and an inhibitory effect at P5. We can reasonably conclude that the effect of treatment with ALL factors is primarily attributable to the effect of LIF at these ages. LIF has been reported to enhance neurite extension in a variety of other neuronal types (Cafferty et al. 2001; Leibinger et al. 2009). In particular, recent studies have demonstrated that LIF mediates the enhanced intrinsic growth status after a conditioning lesion (Cafferty et al. 2001; Hyatt Sachs et al. 2010) suggesting that LIF plays a role in the regeneration of injured neurites. Therefore, our results, together with those of previous reports (Gillespie et al. 2001; Vieira et al. 2007; Whitlon et al. 2007) indicate that the application of exogenous LIF, alone or together with neurotrophins, should be clinically valuable as a treatment for central axon injury from trauma or the surgical removal of acoustic tumors and for peripheral dendrites to improve the efficacy of cochlear implants.

In contrast to LIF, NT-3 and BDNF at 50 ng/ml showed inhibitory effects for neurite extension on P5 SGNs. NT-3 and BDNF showed a survival-promoting effect on the same neuronal population at this concentration, whereas NT-3 and BDNF at 10 ng/ml did not show this inhibitory effect, suggesting that this is not a toxic effect but a specific suppression of neuritogenesis by high concentrations of NT-3 and BDNF. This finding contrasts with the extension-promoting effects of NT-3 and BDNF during in vivo and in vitro studies (Brors et al. 2008; Leake et al. 2011; Miller et al. 2007; Wise et al. 2005). The reason underlying this discrepancy is unclear but several possibilities can be proposed.

Table 2 Fraction of SGN without or with one neurite (referred to as the 0-1 neurite group) and SGN with two or more neuritis (referred to as the ≥2 neurite group). Significant differences in the fraction of ≥2 neurite group compared with control culture are indicated: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (chi-square test)

Group	Control	NT-3	BDNF	LIF	NT-3 + BDNF	ALL
P0						
0-1 neurite group	24	115	205	61	296	100
≥2 neurite group	12	46	70	44	165	106*
P5						
0-1 neurite group	66	154	193	96	170	161
≥2 neurite group	25	52	73	48	97	219**
P20						
0-1 neurite group	89	129	127	49	92	45
≥2 neurite group	45	85	104*	44*	59	87***

One possibility is that the production of other growth factors such as LIF by SGNs themselves or by adjacent tissue is higher in explants or in vivo situations. Alternatively, the discrepancy could be attributable to the difference in the mode of exposure of SGNs to the factors: in vivo administration of neurotrophic factors by osmotic pump through the scala tympani (Leake et al. 2011; Miller et al. 2007; Wise et al. 2005) might primarily expose the SGN neurites. In contrast, in our dissociated culture system, the entire surface of the SGNs is presumably exposed to the factors and so the factors might not drive the neurites to extend. Indeed, a study of rat DRG neurons in compartmented cultures has revealed that NGF promotes neurite extension when applied to the neurite alone but suppresses neurite elongation when the cell body is exposed (Kimpinski et al. 1997). Another study of rat DRG neurons has demonstrated a dose-dependent inhibitory effect on neurite extention for NGF (≥50 ng/ml; Conti et al. 1997, 2004). The authors (Conti et al. 2004) speculate that this might be necessary to terminate axon growth when the growth cone reaches its target. Therefore, the finding that 50 ng/ml NT-3 and BDNF show greater inhibitory effects on P5 SGNs than other age groups might be associated with the observation that the period of P5 culture corresponds to the stage of afferent rearrangement. The molecular mechanisms underlying this inhibition are unknown but a higher concentration of neurotrophins might change the balance of signaling mediated through Trks and p75, the latter of which has been shown to suppress neurite extension in SGNs (Brors et al. 2008).

Effects of neurotrophic factors on neuronal morphology

SGNs are bipolar neurons possessing two neuronal processes (an axon and a dendrite), which are anatomically and functionally distinct. Previous studies have indicated that not all SGNs show this in vivo morphology in culture (Vieira et al. 2007; Whitlon et al. 2007) suggesting that the initiation of neuritogenesis is regulated differentially for SGN axons and dendrites.

In the current study, the fraction of monopolar neurons was highest for P0 SGNs, whereas the fraction showing bipolar morphology was highest at P20, especially after treatment with ALL factors. These findings suggest that the capacity of SGNs to initiate regrowth of neurites changes in an age-dependent manner and that the SGNs retain the capacity to respond to exogenous neurotrophic factors for neurite regrowth, even when the neurons are functionally mature.

A few studies have addressed the regulation of neurite morphology as modulated by growth factors. When dissociated DRG neurons are cultured in the absence of NGF, most of the neurons exhibit unipolar morphology, whereas NGF or NT-3 treatment dramatically increases the percentage of bipolar neurons (Lentz et al. 1999). NGF and NT-3 have also been demsonstrated to produce differential effects on central and peripheral neurite growth patterns of trigeminal ganglion neurons in whole-mount explant cultures (Ulupinar et al. 2000). Neonatal mouse SGNs tend to show increased bipolar morphology after LIF, ciliary neurotrophic factor, or oncostatin treatment (Whitlon et al. 2007). These observations clearly show that neurotrophic factors are involved in the initiation of neuritogenesis, although molecular mechanisms regulating this morphology remain largely unknown. The findings in our study suggest that our cocktail of neurotrophic factors should be useful in studies of the transplantation of neural stem cells into spiral ganglia, in which the development of neurons with bipolar morphology is necessary to restore the functional connection between the organ of Corti and the brainstem.

Potential limitations of the study

Several limitations need to be acknowledged with regard to the present study. First, we harvested and dissociated SGNs from along the entire length of the cochlea. Therefore, our results reflect the average response of SGNs from all cochlear turns at each developmental age. Recent studies have demonstrated that SGNs present at different cochlear positions can vary in their physiological properties in response to neurotrophins (Adamson et al. 2002; Davis and Liu 2011). SGNs located in the apex show NT-3-specific patterns in terms of their electrophysiological properties and their molecular expression in response to the higher expression of NT-3 in the apical region, whereas SGNs in the base show BDNF-specific patterns in response to the higher expression of BDNF in the basal region. This finding raises the possibility that the SGNs of different turn origin might respond to neurotrophins differently with respect to survival and neuritogenesis.

A second limitation is that our study did not differentiate the responses of type I versus type II SGNs, since the reliable differentiation of these two neuronal types is difficult based purely on their morphology in culture. Moreover, rat type I SGNs unfortunately up-regulate the type II marker peripherin in culture. Although the majority of SGNs harvested should have been type I, a recent study has demonstrated that the proportion of type II/type I in mice decreases with age (Barclay et al. 2011). In addition, BDNF more strongly supports the survival and neuritogenesis of type II SGNs in explants (Barclay et al. 2011). Therefore, similar neuronal-type-specific differences in trophic dependence might have influenced the data of our study.

A third limitation concerns the possible influence of other experimental factors that could affect the survival and neuritogenesis of SGNs in dissociated cell culture. Our data could have been influenced by mechanical and chemical stresses during dissociation, the specific coating of the glass

culture surface and the type of culture media, in addition to the supplemention of neurotrophic factors. The response of SGNs to these factors might also be age-dependent and might have influenced our results. These issues need to be addressed in further investigations.

Concluding remarks

The present study demonstrates that the responsiveness of rat postnatal SGNs to neurotrophic factors with regard to several indices of growth changes in an age-dependent manner, that various factors can exert influences that are quite distinct from one another and that strong synergistic effects are observable between factors. Improved knowledge of the processes that occur during the development of SGNs should be valuable to facilitate the maintenance of SGNs and their dendrites and also the development of regenerative therapies to improve the efficacy of cochlear implants.

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Changes in responsiveness of rat spiral ganglion neurons to neurotrophins across age: differential regulation of survival and neuritogenesis

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Developmental changes in responsiveness of rat spiral ganglion neurons (SGNs) to neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) were examined using an explant culture system. Spiral ganglion (SG) explants at embryonic Day 18 (E18), postnatal Day 0 (P0), P5, P10 and P20 were cultured with the addition of either NT-3 or BDNF at various concentrations (0.1–100 ng/ml) and analyzed the dose-response characteristics of three parameters: SGN survival, the number of neurites emanating from the explants and the length of neurite extension. In E18 cultures, SGN survival and neurite number were enhanced more strongly by NT-3 than by the BDNF. As the explants became more mature, the effects of NT-3 decreased, whereas those of BDNF increased, peaking at P0. Although the intrinsic capacity of SGNs to produce and extend neurites declined considerably by P20, they still retained the capacity to respond to both NT-3 and BDNF. These temporal patterns in responsiveness of SGNs to neurotrophins correspond well to the expression pattern of the two neurotrophins in cochlear sensory epithelium *in vivo* and also correlate with the time course of developmental events in SGNs such as cell death and the establishment of mature hair cell innervation patterns.

KEYWORDS: NT-3, BDNF, age-related, neurite growth, neurite extension

Introduction

Spiral ganglion neurons (SGNs) are primary afferent bipolar neurons, with a peripheral dendrite receiving synaptic input from hair cells in organ of Corti, and a central axon projecting to the cochlear nucleus. During development, immature SGNs derived from the otic placode extend neurites toward the presumptive sensory epithelium and brainstem, then establish functional connections [1,2]. Initially, overproduced SGNs are then eliminated by programmed cell death [3–5]. The surviving neurons undergo further maturational processes including remodeling of the dendritic projection to hair cells [6–10].

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Studies of the developing auditory system have shown that the neurotrophic factors neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) are essential for the development of innervation in the inner ear [see 2,11,12 for reviews], including the response properties of SGNs [e.g. 13,14]. In various peripheral nervous systems, it is known that neuronal responses to neurotrophins can be age dependent. For example, trigeminal neuron survival dependence switches from BDNF and NT-3 to nerve growth factor (NGF) during the early stages of target field innervation [15]. Similarly, some dorsal root ganglion (DRG) neurons depend on NGF in the embryonic stage and switch to glial cell line-derived neurotrophic factor (GDNF) in early postnatal life [16]. These data indicate that neurotrophic factors can support different events in different developmental stages. Although some studies have addressed this issue in avian auditory and vestibular end organs [17,18], the extent to which neurotrophin effects in the mammalian inner ear vary across age remains unclear.