

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 26
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ALICIA LLOREDA RICAURTE

Asunto: Radicación: 13-25485- -13
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 10513
 Folios: 26

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/043297				
Fecha de Presentación Internacional	08/07/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 13-025485-00000-0000	07/Febrero/2013
Reivindicaciones:	Radicado No. 13-025485-00000-0000	07/Febrero/2013
	Radicado No. 13-025485-00004-0000	14/Marzo/2013
	Radicado No. 13-025485-00009-0000	12/Noviembre/2013
	Radicado No. 13-025485-00012-0000	13/Febrero/2014
Secuencias:	Radicado No. 13-025485-00000-0000	07/Febrero/2013
Oposiciones:	Radicado No. 13-025485-00006-0000	Tecnoquímicas S.A. 12/Agosto/2013
	Radicado No. 13-025485-00007-0000	Lafrancol S.A.S. 13/Agosto/2013
Respuesta del solicitante:	Radicado No. 13-025485-00012-0000	13/Febrero/2014
Prioridad:	US 61/363,120	09/Julio/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Para la presente solicitud se estudió el capítulo reivindicatorio completo (Radicado No. 13-025485-00012-0000).

Título de la solicitud: “Proteínas de unión de dominio variable dual que se unen a NGF y a al menos un blanco adicional, y métodos para hacer y usar dichas proteínas de unión” (modificado Radicado N° 13-025485-00003-0000).

El problema planteado en esta solicitud consiste en la necesidad de proteínas de unión multivalentes mejoradas para el tratamiento de varias enfermedades.

Para resolver este problema técnico la solicitud en estudio proporciona proteínas multivalentes de unión capaces de unirse a NGF y simultáneamente a uno de los siguientes blanco seleccionado de IGF1,2, RON, ErbB3, IGFR, HGF, VEGF, DLL4, P1GF, CD-20, EGFR, HER2, c-MET o NRP1.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/00, C12N 5/07, C12N 5/16, C12P 21/08.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (c))

El objeto de las reivindicación 11 no es patentable de acuerdo con el Art citado, puesto que hacen referencia a células que no se encuentran definidas en términos de “una línea celular”.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio completo carece de claridad y concisión:

1. En primer lugar, se recuerda a la solicitante que una reivindicación independiente debe reclamar el objeto a partir de todas sus características técnicas esenciales. Al respecto, en la reivindicación 1 se reclama una proteína de unión que comprenden dos polipéptidos, donde cada uno de los polipéptidos está definido por la fórmula $VD1-(X1)_n-VD2-C-(X2)_n$, sin que sea explicado de forma clara cada uno de los componentes. Si bien en la descripción se afirma que C es un dominio constante que puede ser de cadena ligera o cadena pesada, en el capítulo reivindicatorio su secuencia no está caracterizada; Tampoco es claro cómo ambos dominios variables pueden estar unidos en ausencia de un enlazador (cuando n vale cero). Por último debe indicarse a que inmunoglobulina corresponde la región Fc.
2. Así mismo, se recuerda a la solicitante una proteína de unión o anticuerpo debe ser reclamando por sus seis CDRs o por la cadena variable ligera y la cadena variable pesada. En la reivindicación 1 se reclama una proteína de unión que comprende dos cadenas de polipéptido, donde cada una de las cadenas tiene dos dominios variables. De acuerdo con la descripción y la reivindicación 6 puede interpretarse que dichos dominios variables definidos como VD1 y VD2 corresponden a las cadenas variables ligeras y pesadas, sin embargo, el capítulo reivindicatorio no deja claro cuál de dichos dominios variables corresponde a la

cadena variable ligera y cual a la cadena variable pesada. Además, en la reivindicación 1 se caracteriza a los dominios variables a partir de secuencias de CDRs que en realidad no corresponden a CDRs puesto que al confrontarlo con el listado de secuencias y la descripción, corresponden a regiones VH y VL de anticuerpos (SEQ 28-39). Esa inconsistencia también se refleja en la reivindicación 3, la cual refiere dichas secuencias como sitios de unión blanco funcional para cada uno de los antígenos. Puesto que reivindicación dependiente 6 define a otras secuencias como las cadenas variables, se sugiere hacer las aclaraciones necesarias. Se sugiere tener en cuenta las recomendaciones anteriores en la reivindicación 2.

3. Sumado a lo anterior, en la reivindicación 1 se mencionan valores de EC50 y de KD, lo cual corresponde a un resultado a alcanzar que no es limitativo al objeto reclamado; por lo tanto es información que debe mencionarse en la memoria descriptiva, pero no debe hacer parte de las reivindicaciones.
4. La reivindicaciones 2, 4 y 6 reclaman las mismas características técnicas de la reivindicación 1 sin adicionar características, por lo tanto son redundantes y le restan concisión al capítulo reivindicatorio. Se sugiere restringir el capítulo
5. La reivindicación 3 no es clara puesto que reclama una proteína de unión con cuatro sitios funcionales, sin que estos estén caracterizados.
6. La reivindicación 5 tampoco es clara ni concisa pues indica que X1 puede ser cualquiera de las secuencias 1 a 27 y que X1 no es CL, debido a que no se explica en la descripción qué es CL y por el hecho de que seleccionar entre veintisiete secuencias le resta concisión a la invención. Además, el hecho de poder seleccionar entre cualquier isotipo o clase de inmunoglobulinas de la región Fc le resta concisión a la invención. Además, deben indicarse las características químicas de la proteína de unión que permiten que sea cristalizada. Se sugiere restringir a las realizaciones de la solicitud.
7. En las reivindicaciones no se debe hacer mención a códigos o nomenclatura propia. En su lugar, deben indicarse bien sea las secuencias de sus seis CDRs o las secuencias de las cadenas variables etiquetadas por su número de identificación SEQ ID N°.
8. La reivindicación 6 no es concisa ya que reclama una proteína de unión donde las cadenas pueden seleccionarse de una lista de 8 secuencias para los primeros tres grupos inventivos, y una lista de 4 secuencias para el cuarto grupo inventivo. Si se trata de variantes de la proteína de unión reclamada en la reivindicación 1, dicha reivindicación debe indicarlo claramente. O de lo contrario parece que se estuvieran reclamando 28 anticuerpos duales diferentes.
9. Se debe aclarar si la composición farmacéutica de las reivindicaciones 13 y 14 es la misma del conjugado de la reivindicación 8, pero se están reclamando como dos objetos diferentes. En el caso de conjugado se debe indicar el nombre y estructura del agente. En el caso de la composición esta debe definirse por sus ingredientes activos y excipientes y por las proporciones de los mismos.
10. La molécula de ácido nucleico debe ser caracterizada a partir de la secuencia nucleotídica de la misma y hacer referencia a la(s) secuencia(s) del anticuerpo completo (reivindicación 9);

11. La célula huésped transformada, reclamada en la reivindicación 13, debe ser definida como una línea celular, y debe ser restringida, pues de lo contrario se considerará materia exceptuada de patentabilidad.
12. Las reivindicaciones de procedimiento deben referirse a unas actividades organizadas en una serie de pasos de manera determinada. El método de producción del anticuerpo de la reivindicación 12 corresponde a un resultado a alcanzar, ya que se busca producir una proteína, sin indicar las condiciones específicas en que debiera realizarse el cultivo, ni las características del medio que le permitieran a la célula producir la proteína de interés. Por otra parte, el método para determinar la presencia, cantidad o concentración de al menos un blanco en una muestra de la reivindicación 15 carece de claridad puesto que no menciona cada una de las etapas, y éstas están mencionadas en reivindicaciones dependientes (reivindicaciones 16 y 17); y se refiere a un blanco de una muestra, lo cual no está caracterizado. Se deben caracterizar también las condiciones en que debe estar la muestra y la naturaleza del marcador detectable. Tampoco se indica el instrumento de detección de la señal.
13. Las reivindicaciones 18 aunque encabecen como kit, no cumplen con los criterios definidos para la caracterización de éste. Se recuerda a la solicitante que un kit-de-partes debe estar caracterizado por una preparación farmacéutica o medicamento A y una preparación farmacéutica o medicamento B indicándose las cantidades o proporciones definidas de ambas preparaciones, donde tales componentes forman una unidad funcional (combinación verdadera) dirigida a un único propósito con efecto sinérgico. Sin embargo, en el kit reclamado en la presente solicitud no se indican las preparaciones farmacéuticas A y B, pues sólo se menciona que comprende la proteína de unión de acuerdo a las reivindicaciones 1 a 7 e instrucciones de uso. Dichas instrucciones de uso se entienden como un método de tratamiento en el que se emplea la proteína dual y de hecho correspondería a materia no patentable. Según lo anterior, el objeto reclamado en la reivindicación 18 no corresponde a un kit pues comprende un solo componente, el cual es una proteína de unión, además no existe en la descripción el sustento para el kit. Se sugiere hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 13 grupos inventivos.

Los grupos inventivos están definidos por las siguientes características esenciales:

Grupo No. 1: Anticuerpo de dominio dual contra NGF y IGF1,2.

Grupo No. 2: Anticuerpo de dominio dual contra NGF y RON.

Grupo No. 3: Anticuerpo de dominio dual contra NGF y ErbB3.

Grupo No. 4: Anticuerpo de dominio dual contra NGF y IGFR.

Grupo No. 5: Anticuerpo de dominio dual contra NGF y HGF.

Grupo No. 6: Anticuerpo de dominio dual contra NGF y VEGF.

Grupo No. 7: Anticuerpo de dominio dual contra NGF y DLL4.

Grupo No. 8: Anticuerpo de dominio dual contra NGF y P1GF.

Grupo No. 9: Anticuerpo de dominio dual contra NGF y CD-20.

Grupo No. 10: Anticuerpo de dominio dual contra NGF y EGFR.

Grupo No. 11: Anticuerpo de dominio dual contra NGF y HER2.

Grupo No. 12: Anticuerpo de dominio dual contra NGF y c-MET.

Grupo No. 13: Anticuerpo de dominio dual contra NGF y NRP1.

No se guarda un concepto común inventivo porque los anticuerpos no comparten una estructura esencial para la actividad común. Así mismo, el único concepto común, un anticuerpo de dominio dual donde una de los antígenos sea NGF, ya había sido divulgado con anterioridad en el estado de la técnica (Documento D1).

Además, también existe un problema de unidad de invención dentro de cada uno de los grupos anteriormente mencionados puesto que en cada uno de dichos grupos se da a elegir entre dos regiones VH de secuencias 60 o 86, las cuales tienen un porcentaje de identidad menor al 80%, como se muestra a continuación, clasificándolas como dos cadenas diferentes, y en consecuencia, un anticuerpo anti-NGF que tenga la secuencia 60 como cadena VH será diferente a un anticuerpo que contenga la secuencia 86 como cadena VH. En consecuencia, se trata de grupos inventivos diferentes.

Score	Expect	Method	Identities	Positives	Gaps
156 bits(395)	2e-54	Compositional matrix adjust.	77/122(63%)	93/122(76%)	1/122(0%)
Query 1	QVQLQESGPGGLVKPSETLSLTCTVSGFSLIGYDLNWIWIRQPPGKGLEWIGIIWGDGTTDYN				60
	+VQL ESG GLV+P +L L+C SGFSL ++NW+RQ PGKGLEW+G +W G TDYN				
Sbjct 1	EVQLVESGGGLVQPGGSLRLSCAASGFSLTNNNVNWVRQAPGKGLEWVGGVWAGGATDYN				60
Query 61	SAVKSRVTISKDTSKNQFSLKLSSTAAADTAVYYCAR-GGYWYATSYFYFDYWGQGTLLTV				119
	SA+KSR TIS+D SKN L+++S+ A DTAVYYCAR GGY +T Y D WGQGTLLTV				
Sbjct 61	SALKSRFTISRDNSTAYLQMNSLRAEDTAVYYCARDGGYSSSTLYAMDAWGQGTLLTV				120
Query 120	SS	121			
	SS				
Sbjct 121	SS	122			

Figura 1. Porcentaje de identidad del 63% entre la secuencia 60 (query) y la secuencia 86 (sbjct) de la presente solicitud. Alineamiento realizado con Blastp (NCBI).

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

7. Análisis de las Oposiciones

Argumentaciones de Tecnoquímicas S.A.

1. Dice el opositor que esta solicitud carece de claridad, concisión, soporte de la solicitud y unidad de invención, toda vez que reclama una fórmula general conocida de anticuerpos duales sin indicación de la estructura de los anticuerpos, las reivindicaciones carecen de sustento y menciona un número elevado de posibles invenciones al reclamar múltiples proteínas de unión.
2. Afirma el opositor que esta solicitud pretende obtener patente para materia no patentable al reclamar usos de las proteínas duales.
3. El opositor presenta el documento US2009/0304693, con el cual indica la falta de novedad de la solicitud.
4. El opositor objeta la falta de nivel inventivo de la solicitud a partir de los documentos US2009/0304693, US2007/0092507 y US2009/0208490.

Argumentaciones de Lafrancol

1. Afirma el opositor que esta solicitud carece de novedad puesto que combinaciones farmacéuticas que se refieren a proteínas de unión multivalentes y multiespecíficas son prácticas conocidas en la industria farmacéutica.
2. Dice el opositor que la solicitud está dirigida implícitamente a extender protección a agentes conocidos en el estado del arte, como son los principios activos Bevacizumab, Tiruximab y Cetuximab.
3. El opositor objeta la falta de nivel inventivo a la luz de los documentos US2009/0304693, US2007/0092507 y US2009/0208490.
4. El opositor afirma que la solicitud carece de aplicación industrial.

Respuestas del Solicitante

En respuesta a las oposiciones interpuestas, la solicitante presentó un nuevo capítulo reivindicatorio cuyo fin es que las modificaciones realizadas restrinjan la invención.

Frente a la objeción por falta de claridad y concisión de la reivindicación 1, la solicitante afirma que el nuevo capítulo reivindicatorio especifica claramente las secuencias de los dominios variables que forman los sitios de unión NGF y otro objetivo. Frente a la objeción por falta de soporte en la descripción, la solicitante afirma que la materia reclamada en las reivindicaciones se encuentra totalmente soportada en la memoria descriptiva de manera que la persona medianamente versada en la materia pueda desarrollar la invención. Respecto a la falta de unidad de invención, la solicitante resalta que las proteínas de unión reclamadas no solo comprenden una estructura de dominio variable, sino también de unión a NGF y otros objetivos específicos y que de esta forma comparten un concepto común inventivo.

Por otra parte, las reivindicaciones referidas a usos fueron eliminadas.

Respecto al documento US2009/0304693, trasladado por la sociedad opositora Tecnoquímicas S.A., la solicitante aclara que el capítulo reivindicatorio modificado no hace referencia a mezclas de anticuerpos, sino por el contrario la invención considera pares de blancos específicos y secuencias de dominio variable que forman los sitios de unión a NGF y a segundos objetivos, los cuales están definidos a partir de la estructura de los CDRs y/o dominios variables. En consecuencia, dicho documento no anticipa la presente invención.

Frente a la objeción de falta de novedad interpuesta por la sociedad opositora Lafrancol, la solicitante aclara que el objeto de la presente no se centra en composiciones y combinaciones farmacéuticas, y por el contrario se refiere a proteínas de unión. Adicionalmente, aclara que la presente solicitud no se relaciona de ninguna manera a los principios activos ya patentados sino con proteínas de unión que comprenden secuencias específicas capaces de unirse a NGF y otro objeto.

Frente a la objeción de nivel inventivo interpuesta por las dos sociedades opositoras, la solicitante afirma que secuencias de dominios variables conocidas en el estado de la técnica no aportan la motivación para su selección y que los documentos presentados no proporcionan una expectativa razonable de que el efecto técnico inesperado podría alcanzarse mediante la incorporación de secuencias en una proteína de dominio dual. De modo que a partir de las enseñanzas de los documentos trasladados no salta a la vista de mera clara y directa la realización de proteínas de unión de la presente solicitud.

Respuesta del Examinador Técnico

Pese a que la solicitante realizó algunas modificaciones al capítulo reivindicatorio, todavía subsiste la falta de claridad de la materia reclamada, y por tal razón se hacen recomendaciones en cuanto a la claridad, concisión de las reivindicaciones y falta de unidad de invención en el presente examen.

Por otra parte, el presente despacho concuerda con lo expresado por la solicitante al negar que la presente invención pretenda obtener derecho de patente de composiciones farmacéuticas conocidas como Bevacizumab, Tiruximab y Cetuximab.

El documento US2007/0092507 (26 de abril de 2007), presentado por ambas sociedades opositoras, divulga anticuerpos anti-FcRn para el tratamiento de enfermedades autoinmunes y aloinmunes. Este documento no desvirtúa la novedad o el nivel inventivo de esta solicitud puesto que no se refiere a alguno de los anticuerpos comprendidos en el objeto de la solicitud en estudio, motivo por el cual no será tenido en cuenta en el presente examen de patentabilidad.

El documento US2009/0208490 (20 de agosto de 2009), presentado por ambas sociedades opositoras, divulga anticuerpos NGF para la prevención y el tratamiento de dolor crónico. Si bien este documento enseña anticuerpos contra NGF, así como la presente solicitud, la estructura de los anticuerpos es diferente y no se hace mención a anticuerpos duales. Por lo tanto, este documento no desvirtúa la novedad o el nivel inventivo de esta solicitud, y no será tenido en cuenta en el presente examen de patentabilidad.

En cambio, el documento US2009/0304693 (10 de diciembre de 2009), presentado en las dos oposiciones, el cual, contrario a lo afirmado por la solicitante, considera pares de blancos específicos y secuencias de dominio variable que forman los sitios de unión a dos objetivos definidos a partir de la estructura de sus dominios variables, donde uno de ellos es NGF, desvirtúa el nivel inventivo del objeto de esta solicitud; por lo cual se considerará dentro del examen de patentabilidad.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: julio 09 de 2010, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US 20090304693 A1	"Dual Variable Domain Immunoglobulins and Uses Thereof"	10/Diciembre/2009
D2	Streppel et al. 2002	"Focal application of neutralizing antibodies to soluble neurotrophic factors reduces collateral axonal branching after peripheral nerve lesion"	06/Marzo/2002

El documento D1¹ divulga proteínas de unión multivalentes y multiespecíficas, métodos de fabricación, y su empleo en la prevención, diagnóstico y / o tratamiento de enfermedad (Resumen).

El documento D2² revela la aplicación de anticuerpos neutralizantes frente a varios agentes neurotróficos y su efecto en la reducción de ramificación y mejora de la exactitud de reinervación de los axones (Resumen)

9. Examen de Patentabilidad (Art 14 D486)

Grupo inventivo 1

1 <http://www.google.co.in/patents/US20090304693>

2 M. Streppel, N. Azzolin, S. Dohm, O. Guntinas-Lichius, C. Haas, C. Grothe, A. Wevers, W. F. Neiss and D. N. Angelov. Focal application of neutralizing antibodies to soluble neurotrophic factors reduces collateral axonal branching after peripheral nerve lesion European Journal of Neuroscience, Vol. 15, pp. 1327-1342, 2002.

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “Una proteína de unión que comprende una primera y una segunda cadena de polipéptidos, cada una de las cuales comprende de manera independiente VD1-(X1)n-VD2-C-(X2)n...”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Una proteína de unión que comprende una primera y una segunda cadena de polipéptidos, cada una de las cuales comprende de manera independiente VD1-(X1)n-VD2-C-(X2)n, en donde VD1 es un primer dominio variable;VD2 es un segundo dominio variable; C es un dominio constante; X1 es un conector, X2 es una región Fc; n es 0 o 1;	Una proteína de unión que comprende Dos polipéptidos, donde la primera y la segunda cadena polipeptídica comprende VD1-(X1)n-VD2-C-(X2)n, en donde VD1 es un primer dominio variable;VD2 es un segundo dominio variable; C es un dominio constante; X1 es un conector con la condición que no sea CH1, X2 es una región Fc; n es 0 o 1 (Párr. [0012], [0018]).
En donde los dominios VD1 en dicha primera y dicha segunda cadena de polipéptidos forman un primer sitio de unión blanco funcional y los dominios VD2 en dicha primera y dicha segunda cadena de polipéptidos forman un segundo sitio de unión funcional,	En donde los dominios VD1 en dicha primera y dicha segunda cadena de polipéptidos forman un primer sitio de unión blanco funcional y los dominios VD2 en dicha primera y dicha segunda cadena de polipéptidos forman un segundo sitio de unión funcionalDonde dichos VD1 y VD2 son capaces de unirse a antígenos (Párr. [0012] - [0018]).
En donde la proteína se une a NGF e IGF1,2	En donde la proteína de unión se une a dos blancos seleccionados de Abeta, TNF-α, (IL-1β), IGF1, IGF2 , IL-18, IL-6, RAGE, NGF , EGFRCD-20 y RGMA
i) En donde los dominios variables que forman un sitio de unión blanco funcional para NGF comprenden CDRs 1 -3 de la SEQ ID N°60 y CDRs 1 - 3 de la SEQ ID N°61, o CDRs 1 -3 de la SEQ ID N°86 y CDRs 1 - 3 de la SEQ ID N°87, y	SEQ ID N°36 y 37 (tabla 2).
ii) Los dominios variables que forman un sitio de unión blanco funcional para IGF1,2 comprenden CDRs 1 -3 de la SEQ ID N°46 y CDRs 1 - 3 de la SEQ ID N°47	SEQ ID N°32 y 33 (tabla 2).

El documento D1 se considera el estado de la técnica cercano a la invención reclamada en la reivindicación 1 puesto que divulga una proteína de unión a IGF1, 2 y NGF (Párr. [0019])que comprende dos polipéptidos, donde la primera y la segunda cadena polipeptídica comprende VD1-(X1)n-VD2-C-(X2)n, en donde VD1 es un primer dominio variable;VD2 es un segundo dominio variable; C es un dominio constante; X1 es un conector, X2 es una región Fc; n es 0 o 1 (Párr. [0012] - [0018]). Además, da a conocer métodos para su producción (Párr. [0235-0246]), métodos de diagnóstico a partir de detección de proteínas en muestras de pacientes (Párr. [0351-0409]), y composiciones farmacéuticas (Párr. [0348]).

El mismo documento D1 da a conocer las cadenas variables ligeras y pesadas de cada uno de los anticuerpos empleados en la construcción de proteínas de unión dual. Las figuras 2 y 3 presentan el alineamiento de la secuencias de cadenas variable pesada del anticuerpo NGF e IGF1 de la presente invención con las cadenas pesadas del anticuerpo divulgado en el documento D1, respectivamente.

CLUSTAL 2.1 multiple sequence alignment

```
60      QVQLQESGPGLVKPSSETLSLTCTVSGFSLIGYDLNWIRQPPGKGLEWIGIIWGDGTTDYN 60
D1_36  QVQLQESGPGLVKPSSETLSLTCTVSGFSLIGYDLNWIRQPPGKGLEWIGIIWGDGTTDYN 60
      *****

60      SAVKSRVTISKDTSKNQFSKLSSVTAADTAVYYCARGGYWYATSYFYDYWGQGLTVTVS 120
D1_36  SAVKSRVTISKDTSKNQFSKLSSVTAADTAVYYCARGGYWYATSYFYDYWGQGLTVTVS 120
      *****
```

60 S 121
D1_36 S 121
*

Figura 2. Porcentaje de identidad del 100% entre la secuencia 60 (cadena variable pesada de anticuerpo NGF) de la presente solicitud y la secuencia 36 del documento D1 . Alineamiento realizado con ClustalX (EMBL-EBI).

CLUSTAL 2.1 multiple sequence alignment

```
46 QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMNPNSGNTGY 60
D1_32 QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMNPNSGNTGY 60
*****
46 AQKFQGRVTMTRNTSISTAYMELSSLRSED TAVYYCARDPYYYYYGMDVWGQGT TTVTVSS 120
D1_32 AQKFQGRVTMTRNTSISTAYMELSSLRSED TAVYYCARDPYYYYYGMDVWGQGT TTVTVSS 120
*****
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Figura 3. Porcentaje de identidad del 100% entre la secuencia 46 (cadena pesada de anticuerpo IGF1,2) de la presente solicitud y la secuencia 32 del documento D1 . Alineamiento realizado con ClustalX (EMBL-EBI).

La diferencia entre la invención, definida en la reivindicación 1 y la proteína de unión divulgada en el documento D1 consiste en que el primer sitio blanco de unión funcional es para NGF y el que el segundo sitio blanco de unión funcional es para IGF1,2.

El efecto que dicha diferencia concede a la invención es que la proteína se une a NGF e IGF1,2.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar la proteína de unión dual conocida en el documento D1 para que se una a NGF e IGF1,2.

Sin embargo, el mismo documento D1 establece que las proteínas de unión de la invención son capaces de unirse a dos blancos seleccionados de Abeta, TNF-α, (IL-1β), IGF1, IGF2, IL-18, IL-6, RAGE, NGF, EGFRCD-20 y RGMA, de modo que resulta una tarea rutinaria la selección de IGF1,2 y NGF como un par de antígenos blanco a los que la proteína de unión dual de la presente solicitud tuviera afinidad, más aún cuando dicho documento revela las secuencias los dominios variables. Además, la utilización de anticuerpos anti-NGF y anti-IGF1 ya estaba sugerida en el documento D2, el cual revela el efecto de anticuerpos neutralizantes en un modelo de lesión del nervio facial donde la ramificación colateral del axón se ve notoriamente disminuida con anticuerpos Anti-NGF, anti-BDGF y anti-IGF. Debido al efecto de demostrado de anticuerpos anti-NGF y anti-IGF1 en el documento D2 para mejorar la calidad de inervación del axón, para una persona medianamente versada en la materia habría resultado previsible la selección de dichos dos blancos de la lista de antígenos del documento D1. En consecuencia, la solución se considera obvia y sin altura inventiva.

La reivindicación 1 no cumple con el requisito de nivel inventivo (Art. 18).

Las reivindicaciones 2 – 18 no incluyen características adicionales a la luz de los documentos D1 y D2, por lo cual tampoco cumplen con el requisito de nivel inventivo.

10. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 20090304693 A1	1 - 18	Nivel inventivo
D2	Streppel et al. 2002	1 - 18	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-08-26

Elaboró: Edna Carolina Bonilla Leon
Revisó: Sandra Carolina Crespo

Focal application of neutralizing antibodies to soluble neurotrophic factors reduces collateral axonal branching after peripheral nerve lesion

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Keywords: axotomy, facial nerve, motoneuron, nerve conduit, rat, retrograde labelling

Abstract

A major reason for the insufficient recovery of function after motor nerve injury are the numerous axonal branches which often re-innervate muscles with completely different functions. We hypothesized that a neutralization of diffusable neurotrophic factors at the lesion site in rats could reduce the branching of transected axons. Following analysis of local protein expression by immunocytochemistry and by *in situ* hybridization, we transected the facial nerve trunk of adult rats and inserted both ends into a silicon tube containing (i) collagen gel with neutralizing concentrations of antibodies to NGF, BDNF, bFGF, IGF-I, CNTF and GDNF; (ii) five-fold higher concentrations of the antibodies and (iii) combination of antibodies. Two months later, retrograde labelling was used to estimate the portion of motoneurons the axons of which had branched and projected into three major branches of the facial trunk. After control entubulation in collagen gel containing non-immune mouse IgG 85% of all motoneurons projecting along the zygomatic branch sprouted and sent at least one twin axon to the buccal and/or marginal-mandibular branches of the facial nerve. Neutralizing concentrations of anti-NGF, anti-BDNF and anti-IGF-I significantly reduced sprouting. The most pronounced effect was achieved after application of anti-BDNF, which reduced the portion of branched neurons to 18%. All effects after a single application of antibodies were concentration-dependent and superior to those observed after combined treatment. This first report on improved quality of reinnervation by antibody-therapy implies that, in rats, the post-transectional collateral axonal branching can be reduced without obvious harmful effects on neuronal survival and axonal elongation.

Introduction

Peripheral nerve injury initiates a complex series of changes. Approximately 24 h after disconnection, the axons in the distal fragment begin to lyse. When resorption of debris is complete, the Schwann cells re-arrange in the chains of Büngner (Büngner, 1891) which bridge the interfragmentary gap and form guiding channels for the regenerating axons to their target(s). This so-called Wallerian degeneration creates an environment that is highly supportive for axonal re-growth and ensures that the vast majority of axons will enter the distal stump (Bisby, 1995). Nevertheless, complete recovery of function is only rarely achieved. Despite the use of modern microsurgical techniques for nerve repair, the aberrant reinnervation of motor targets, e.g. facial muscles, causes abnormally associated movements and altered reflexes (Kimura *et al.*, 1975; Anonsen *et al.*, 1986; Baker *et al.*, 1994).

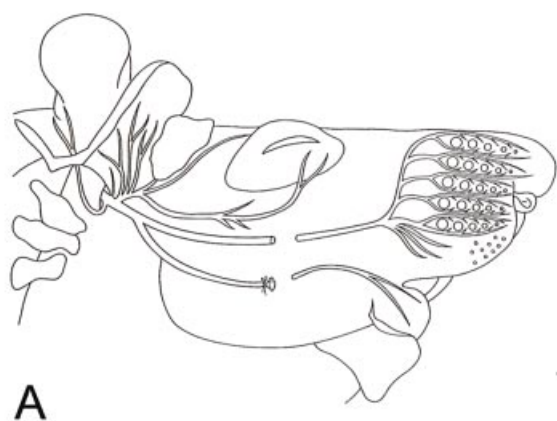
At the site of injury, this aberrant reinnervation has two components. First, due to malfunctioning axonal guidance, a muscle

gets re-innervated by a foreign axon which has been simply mis-routed along the wrong nerve fascicle (Esslen, 1960; Aldskogius & Thomander, 1986; Brushart & Seiler, 1987). Second, due to the presence of supernumerary branches from all transected axons (Shawe, 1954; Morris *et al.*, 1972; Mackinnon *et al.*, 1991), this given muscle can be re-innervated by branches stemming from several motoneurons (Ito & Kudo, 1994), a state known as polyneuronal innervation (Brown *et al.*, 1981; Rich & Lichtman, 1989) or hyperinnervation (Angelov *et al.*, 1996). Though claimed to be transient (Hennig & Dietrichs, 1994), this aberrant innervation may persist for extended periods (Mackinnon *et al.*, 1991; Madison *et al.*, 1999) with deleterious effects on synchronized function. Whereas numerous aspects of the post-transectional aberrant reinnervation of muscles have been extensively documented (Brushart, 1993), little is known how this phenomenon could be prevented. Attempts to act on the first component of the aberrant reinnervation, achieving a fascicular or topographic specificity (Mackinnon *et al.*, 1986; Evans *et al.*, 1991), have failed; so far, it is technically impossible to guide correctly the growth cones of several thousands of axons (and their branches) originating from the proximal stump of a transected nerve.

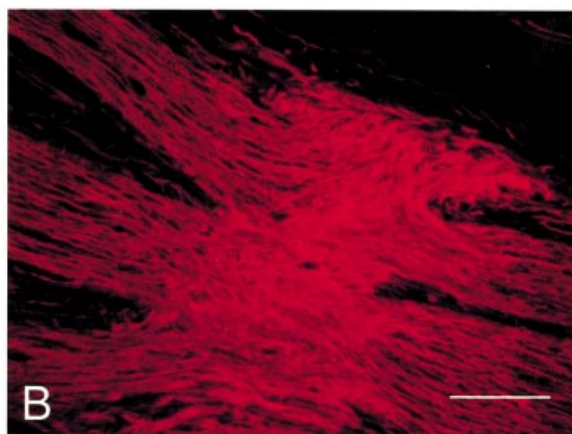
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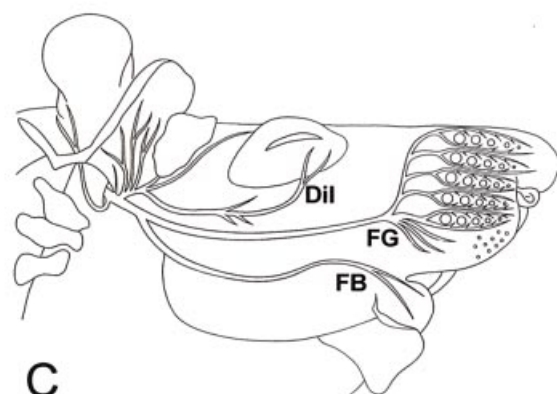
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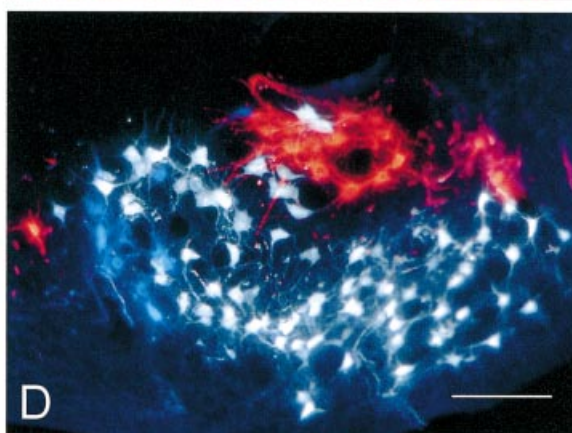
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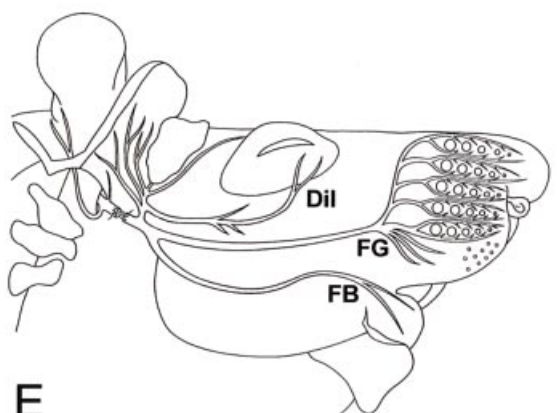
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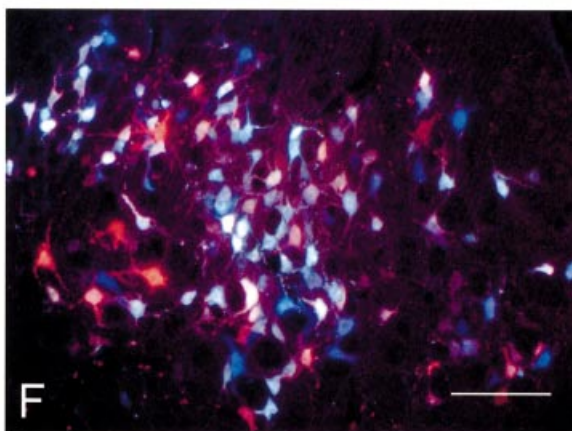
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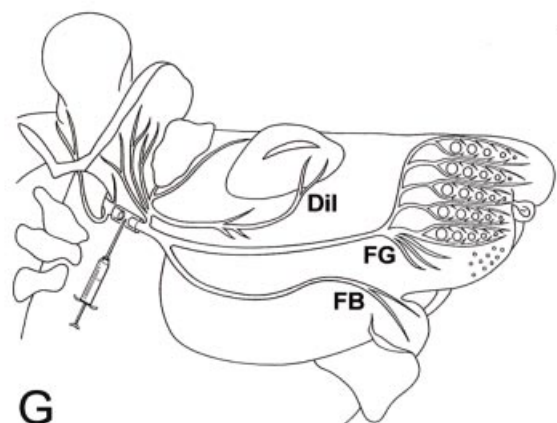
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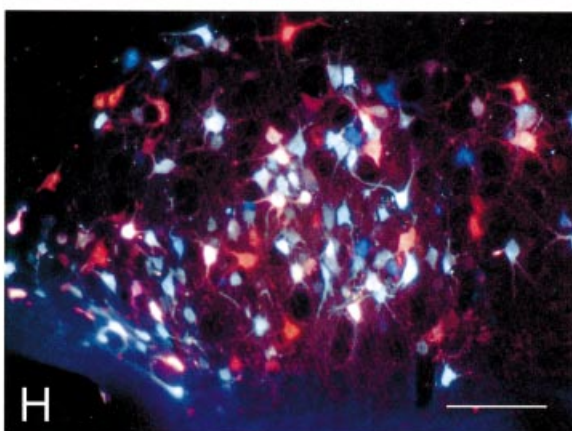
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Recently we tested whether application of extracellular matrix (ECM) proteins and cultured olfactory ensheathing cells at the lesion site would improve the accuracy of reinnervation. Our results clearly showed that, in spite of their known effect to support neurite elongation, both approaches failed to suppress the redundant axonal branching (Dohm *et al.*, 2000; Guntinas-Lichius *et al.*, 2001).

After studying the expression of neurotrophic molecules in the lesioned facial nerve, we hypothesized, that the aberrant axonal branching could, at least in part, be due to the increased expression of trophic molecules at the lesion site. Accordingly, an inhibition or blockade of these factors should reduce branching and improve the accuracy of reinnervation. In the present report we applied neutralizing antibodies to several neurotrophic agents and checked whether some of them would reduce the portion of branched axons.

Materials and methods

Adult female Wistar rats (175–200 g; strain HsdCpb : WU; Harlan Winkelmann, Borcheln, Germany) were used. Before and after experiments, all animals were kept on standard laboratory food (Ssniff, Soest, Germany) and tap water *ad libitum* with an artificial light-dark cycle of 12 h light on, 12 h off. All experiments were conducted in accordance with the German Law on the Protection of Animals, and the procedures used were approved by the local animal care committee (Bezirksregierung Koeln, Az. 23.203.2 K 35, 31/97).

The animals were divided in three major groups (A, B, and C) corresponding to the three major experimental sets of this study. The rats of group A (68 animals) were distributed in subgroups A₁–A₁₇ (each of four rats) and served to establish the time course of expression of: (i) nerve growth factor (NGF), (ii) brain-derived neurotrophic factor (BDNF) (iii) basic fibroblast growth factor (bFGF, FGF-2) (iv) ciliary neurotrophic factor (CNTF) (v) insulin-like growth factor I (IGF-I), and (vi) glial-cell-line derived neurotrophic factor (GDNF) in the proximal and distal fragments of a transected facial nerve as well as in its target (vibrissal) musculature. The rats of group B (12 animals) were used to establish the sources of secretion of trophic factors at the lesion site. Longitudinal sections of lesioned facial-nerve branches were processed for the detection of NGF mRNA, BDNF mRNA, and bFGF mRNA by *in situ* hybridization. The rats of group C (141 animals) were distributed in subgroups C₁–C₂₀ and served to establish the portion of motoneurons whose axons branched after entubulation of the facial nerve into a silicone tube filled with antibodies to neurotrophic factors.

Time course of trophic factor expression at the lesion site

Surgery

All procedures were carried out under an operating microscope (O.G.-L. and M.S.). After an intraperitoneal injection of Ketamin/

Xylazin (100 mg Ketanest® (Parke-Davis, Berlin, Germany) plus 5 mg Rompun® (Bayer, Leverkusen, Germany) per kg body weight), the buccal and marginal mandibular branches of the facial nerve were exposed. Thereafter the buccal facial branch (BFB) was transected and 1 mm of its nerve length removed. The marginal mandibular branch was also transected and its regeneration prevented by a proximal ligature (Fig. 1A). The latter procedure was necessary for eliminating the additional nerve supply of the whisker pad musculature by the marginal mandibular branch (Semba & Egger, 1986). The postoperative survival time was 6, 12, 18, 24, 48, 72, and 96 h, 5, 6, 7, 8, 10, 14, 18, 22, 26, and 30 days. Four animals were studied at each of these 17 time points.

Tissue processing

Rats were transcardially perfused with 0.9% NaCl in distilled water for 60 s followed by a fixation with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 under deep diethylether anaesthesia. To gain information about the expression of neurotrophic factors at the lesion site and in the target musculature, we employed the nerve-muscle-plate approach. The masseter muscle (with the nerves on it) and the levator labii superioris muscle (together with the whisker pad) were removed, postfixed, cryoprotected (in 30% sucrose in 0.1 M phosphate buffer, pH 7.4) and cut tangentially in 20 µm thick sections, which were mounted on 0.01% poly L-lysine coated slides.

Immunocytochemistry

(i) 5.0% (w/v) bovine serum albumin (BSA, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) in Tris-buffered saline (TBS) pH 7.4 for 60 min; (ii) incubation with the primary antibody in TBS plus 0.8% (w/v) BSA (see below) overnight at room temperature; (iii) 5.0% (v/v) normal goat serum (NGS, Alexis Deutschland GmbH, Grünberg, Germany) plus 0.8% BSA in TBS for 15 min; (iv) 1 : 400 matching biotinylated secondary antibody (see below) in TBS plus 0.8% NGS for 1 h; (v) FluoroLink™ Cy3™ – Streptavidin (1 : 100; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Steps (ii), and (v) were followed by four, and step (iv) by two 10 min washes in TBS. Finally, sections were dehydrated with ethanol and HistoClear (National Diagnostics, Atlanta, Georgia, USA) and coverslipped with Fluoromount (BDH Laboratory Supplies, Poole, England).

Primary antibodies. (i) Mouse monoclonal anti-NGF (1 : 50; Roche, Mannheim, Germany); (ii) mouse monoclonal anti-BDNF (1 : 1000; R & D Systems, Wiesbaden, Germany); (iii) mouse monoclonal anti-bFGF (1 : 50; UBI/Biomol, Hamburg, Germany); (iv) polyclonal goat anti-rat CNTF (1 : 100; R & D Systems); (v) mouse monoclonal anti-IGF-I (1 : 50; UBI/Biomol); (vi) mouse monoclonal anti-GDNF (1 : 500; R & D Systems); (vii) rabbit polyclonal anti-S-100 protein (1 : 1000; DAKO Diagnostika, Hamburg, Germany).

FIG. 1. Schematic drawings of the infratemporal portion of the rat facial nerve indicating the site of transection and removal of 1 mm length from the buccal branch and the site of transection and ligation of marginal mandibular branch (A), the tracer application sites in the distal parts of the zygomatic, buccal and mandibular nerves are indicated by the 3 different labels applied, i.e. DiI, FG, and FB, respectively (C), the site of transection and end-to-end suture of the facial nerve trunk, i.e. facial-facial anastomosis (FFA) in E, and the site of entubulation of the facial nerve trunk after transection (G). (B) Longitudinal section through the transected buccal nerve showing S100-immunoreactivity in the Schwann cells (red fluorescence) at the lesion site 4 days after the lesion. 20 µm thick frozen section. Scale bar, 300 µm. (D) Facial nucleus after application of crystalline DiI to the zygomatic, FG to the buccal, and FB to the marginal mandibular branches of the facial nerve of an unoperated control rat. Note the myotopic organization of the nucleus; the DiI-labelled motoneurons (red) are localized in the dorsal, FG-labelled (white) in the lateral, and FB-labelled (blue) motoneurons in the intermediate facial subnucleus. 50 µm vibratome section. Scale bar, 100 µm. (F) Facial nucleus 8 weeks after FFA and 10 days after triple retrograde labelling identical to that in D. Note the complete lack of myotopic organization and the presence of double-labelled neurons. (H) Facial nucleus 8 weeks after NGF-neutralization and 10 days after triple retrograde labelling. 50 µm vibratome section. Scale bar, 100 µm.

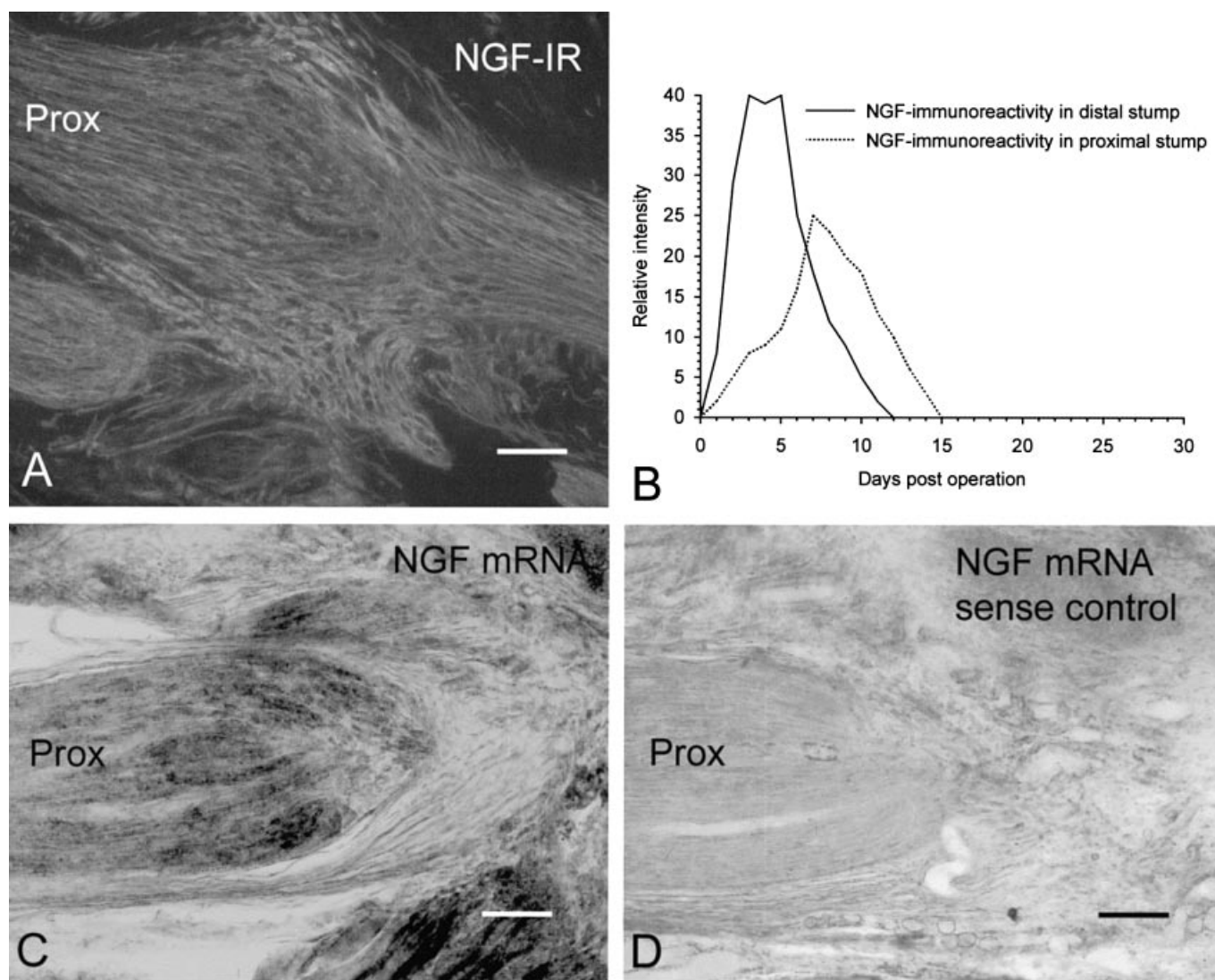


FIG. 2. Pattern of expression and sources of secretion of NGF. (A) Longitudinal section through the transected buccal nerve showing NGF-immunofluorescence in the periphery of small cells with long and slender cell processes running parallel to the long axis of the nerve. Immunopositive structures are observed in both proximal (Prox) and distal nerve segments 5 days after lesion. Scale bar, 200 µm. (B) Time course of the expression of NGF-immunoreactivity in the proximal and distal stumps of the transected buccal branch. Both, the presence as well as the intensity of the fluorescent immunostaining were estimated by two independent observers, who were blind to the previous treatment of the rat. A scale with a minimum of 0 and maximum of 40 was used. (C) NGF mRNA expression 5 days after transection of the buccal branch. *In situ* hybridization histochemistry with digoxigenin-labelled cRNA probes revealing reaction product in longitudinally arranged swollen Schwann cells which fill the proximal stump. Scale bar, 100 µm. (D) Sense control of the same animal as shown in C. Scale bar, 100 µm.

Secondary antibodies (all diluted 1 : 400). (i) Biotinylated goat anti-mouse IgG (Fab-specific; Sigma, Deisenhofen, Germany); (ii) biotinylated goat anti-mouse IgG (Fc specific, Sigma); (iii) biotinylated rabbit anti-goat IgG (DAKO Diagnostika, Hamburg, Germany); (iv) biotinylated goat anti-rabbit IgG (DAKO Diagnostika, No. E0432).

Controls. Omission of the primary or secondary antibody yielded blank sections. Incubation of sections with nonrelevant biotinylated secondary antibodies (e.g. goat anti-rabbit IgG for recognition of mouse primary antibodies) also yielded blank sections.

Fluorescence microscopy

The immunopositive (CY3-positive) structures were observed through Zeiss filter set 15 (excitation BP546/12, beamsplitter FT580, emission LP590).

Estimation. Both, localization of reaction product and intensity of fluorescent immunostaining were estimated separately and independently by two observers (N. Azzolin and D.N. Angelov). A scale with a minimum of 0 and maximum of 40 was used for graphical representation of the results (Figs 2B, 3E and 4E). According to this scale sections with no fluorescence received zero points and those with very strong fluorescence, 40 points. The levels between these extremes were evaluated between 5 and 10 points for very faint fluorescence, 15–20 points for faint fluorescence, and 25–30 points for fluorescence.

Sources of secretion of trophic factors at the lesion site

In situ hybridization was performed on free-floating 40 µm thick cryostat sections from the nerve-muscle plate (24 h to 5 days postsurgery) as previously described (Haas *et al.*, 1999). In brief, sections containing the proximal and distal stump of transected

TABLE 1. Number of retrogradely labelled motoneurons whose axons project through the zygomatic, buccal, or marginal mandibular branches of the facial nerve in intact rats, in rats after unilateral FFA, and after entubulation of a transected facial nerve into a silicone tube containing varying control substances

Surgery	Neurons sending axons through the zygomatic nerve only (DiI-only)	Neurons with axon sprouts in the zygomatic and buccal nerves (DiI + FG)	Neurons with axon sprouts in the zygomatic and marginal mandibular nerves (DiI + FB)	Total neurons, branched and unbranched projecting through the zygomatic nerve (DiI, DiI + FG, DiI + FB)	Neurons sending axons through the buccal nerve only (FG-only)	Neurons sending axons through the marginal mandibular nerve only (FB-only)
Group C1. Intact rats	306 ± 81* ^{2-5,13,15,16,18,19} 100%	0* ²⁻²⁰	0* ²⁻²⁰	306 ± 81* ^{12,15-17} 100%	1422 ± 39* ^{2,4,5,12-20}	288 ± 42* ²⁻²⁰
Group C2. FFA	132 ± 33* ^{1,6,7,9,10} 45%	99 ± 12* ^{1,5,12-20} 34%	63 ± 9* ^{1,5,12-18} 21%	294 ± 33* ^{12,14-18} 100%	1833 ± 120* ^{1,3,5-9,12-20}	402 ± 36* ^{1,4,5,10,12-20}
Group C3. Tube only	183 ± 33* ^{1,6,10} 53%	78 ± 24* ^{1,5,12-20} 23%	84 ± 9* ^{1,5,12-18} 24%	345 ± 66* ^{12,15-17} 100%	1 521 ± 111* ^{2,5,12-20}	462 ± 99* ^{1,5,10,12-20}
Group C4. Collagen only	192 ± 24* ^{1,5,6,10,15} 62%	93 ± 24* ^{1,5,12-20} 30%	24 ± 7* ^{1,5,12-18} 8%	309 ± 36* ^{12,15-17} 100%	1890 ± 141* ^{1,3,5,6-9,12-20}	501 ± 30* ^{1,2,5,10,12-20}
Group C5. Mouse IgG	80 ± 66* ^{1,4,6,7,9,10} 14%	305 ± 100* ^{1-4,6-11} 52%	196 ± 80* ^{1-4,6-11} 34%	581 ± 203 100%	2470 ± 292* ^{1-4,6-11}	2332 ± 236* ^{1-4,6-11}

Each value is a mean ± SD of nine rats. Postoperative survival time was 56 days post surgery plus 10 days post triple retrograde labelling. Small numbers indicate the groups with significantly different values. **P* < 0.05. The portions of motoneurons projecting through the zygomatic nerve with branched axons (DiI + FG or DiI + FB) and unbranched axons (DiI-only) are indicated as percentages below the absolute numbers.

buccal facial branch were proceeded through the following steps: (a) collection of sections in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0); (b) pre-treatment of sections in a 1 : 2 mixture of 2 × SSC-hybridization buffer (50% formamide, 4 × SSC, 50 mM NaH₂PO₄, 250 µg/mL heat-denatured salmon sperm DNA, 100 µg/mL tRNA, 5% dextran sulphate, and 1% Denhardt's solution) for 15 min; (c) pre-hybridization of sections in hybridization buffer for 60 min at 45 °C (for NGF mRNA and BDNF mRNA) and for 120 min at 55 °C (for bFGF mRNA); (d) all digoxigenin-labelled cRNA probes were generated by *in vitro* transcription using either plasmids or a polymerase chain reaction (PCR)-generated templates. A mouse NGF full-length cDNA inserted in pBluescript SK and a rat BDNF cDNA inserted in pBluescript SK (kind gift of R. Heumann, Bochum, Germany) were used to generate digoxigenin-labelled cRNAs. The following probe concentrations and hybridization temperatures were used: NGF, 100 ng/mL at 45 °C overnight (Haas *et al.*, 1999); BDNF, 100 ng/mL at 45 °C overnight (Haas *et al.*, 1999); bFGF, 12.5 µg/mL at 55 °C overnight (Grothe *et al.*, 2001); (e) washing of sections in 2 × SSC at room temperature; (f) washing in 2 × SSC plus 50% formamide; (g) washing in 0.1 × SSC and 50% formamide; (h) washing in 0.1 × SSC. Steps (f) to (h) were performed at a temperature 10 °C above the respective hybridization temperatures; (i) immunological detection of DIG-labelled hybrids with anti-DIG-AP was performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany); (j) colorimetric detection was performed using Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP); (k) development of the colour reaction was performed in the dark at room temperature and stopped after 30 min by transfer into 10 mM Tris/HCl, pH 8.0, 1 mM EDTA; (l) Sections were embedded in Moviol (Hoechst, Frankfurt/Main, Germany). Both antisense and sense (control) sections were used in all hybridizations. Because it has previously been shown that the rat central nervous tissue also expresses antisense transcript (Grothe & Meisinger, 1995; Li & Murphy, 2000) a tubulin sense probe of similar length (324 bases) was used instead of the bFGF sense probe.

Estimation of axonal branching

Triple retrograde labelling

After an intraperitoneal injection of Ketamin/Xylazin the zygomatic, buccal, and marginal mandibular ramus of intact rats were transected and instilled with crystals of DiI (1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Leiden, The Netherlands), Fluoro-Gold (FG; Fluorochrome Inc., Denver, Colorado, USA), and Fast Blue (FB; EMS-Chemie GmbH, Groß-Umstadt, Germany) respectively as shown in Fig. 1C. Ten days later, these animals (subgroup C₁) were fixed by perfusion, their brains cut in 50 µm thick vibratome sections, and all retrogradely labelled motoneurons in the brainstem counted (see below; Table 1).

Facial-facial anastomosis (FFA)

Transection and immediate end-to-end suture of the right facial nerve (facial-facial anastomosis, FFA) was performed in nine animals (subgroup C₂). Following an intraperitoneal injection of Ketamin/Xylazin, the main trunk of the facial nerve was exposed and transected close to its emergence out of the foramen stylomastoideum but distal to the posterior auricular branch (Fig. 1E). The proximal stump was then microsurgically reconnected to the distal stump with two 11–0 atraumatic sutures (Ethicon, Norderstedt, Germany). Finally the wound was closed by three 4–0 skin sutures (Ethicon). Triple retrograde tracing followed 8 weeks later.

Entubulation of the facial nerve trunk

Under anaesthesia, the main trunk of the facial nerve was transected and both stumps inserted into an empty silicone precision tube (subgroup C₃). The tube had an inner diameter of 1.47 mm and outer diameter of 1.96 mm (Aromando Medizintechnik, Düsseldorf; Germany; Fig. 1G). To prevent tension, both nerve ends were attached to the tube through epineural sutures with an interstump distance of 5 mm – literature data have shown that rat axons are able to bridge gaps of up to 10 mm through an artificial nerve conduit (Lundborg *et al.*, 1982; Labrador *et al.*, 1998).

In the following experimental subgroups C₄–C₂₀ the space between the proximal and distal nerve stumps with a volume of approximately 8 µL (5 mm × 0.735 mm × 0.735 mm × π^2) was filled with collagen type I (for subgroup C₄) or with collagen gel containing antibodies to trophic factors (see previous chapter for commercial sources) in neutralizing concentrations: C₆, 40 µg/mL anti-NGF (Bedi *et al.*, 1992; Diamond *et al.*, 1992; Ro *et al.*, 1996); C₇, 160 µg/mL anti-BDNF (Tonra *et al.*, 1998); C₈, 100 µg/mL anti-bFGF (Tuttle *et al.*, 1994; Murai *et al.*, 1996); C₉, 30 µg/mL anti-IGF-I (Zheng *et al.*, 1997); C₁₀, 3 µg/mL anti-GDNF (Vega *et al.*, 1996); C₁₁, 100 µg/mL anti-CNTF (Ding *et al.*, 1994; Tokiwa *et al.*, 1994).

The facial nerves of the animals in the following five subgroups (C₁₂–C₁₇) were transected and inserted into tubes with antibodies in a concentration 5-fold higher than the neutralizing one (Table 3). In groups C₁₈–C₂₀ the silicon tube contained combinations of the same antibodies to NGF, BDNF, and bFGF in neutralizing concentrations (Table 4).

We consider the entubulation of the facial nerve in a gel with 160 µg/mL mouse non-immune IgG (Sigma, Deisenhofen, Germany) as the most important control (group C₅) for the effect(s) of the antibodies; with the exception of anti-CNTF all of them were mouse monoclonals (see above).

The collagen gel was obtained from a collagen stock solution (Serva, Heidelberg, Germany) which was mixed with ×10 PBS and 0.1 M NaOH till the pH reached 7.4. To polymerize in a 3-dimensional gel the collagen/antibody mixture was left at 37 °C for 2 h (Guidry & Grinnell, 1987; Mauch *et al.*, 1988).

Fluorescence microscopy

Sections were observed with a Zeiss Axioskop 50 epifluorescence microscope through a custom-made Bandpass-filter set for Fast Blue (AHF Analysentechnik, Tübingen, Germany), which allows recognition only of FB-labelled motoneurons (appearing blue). Observations through a custom-made HQ-Schmalband-filter set for Fluoro-Gold (AHF Analysentechnik) visualized all motoneurons containing FG. Observations through a filter set 15 of Carl Zeiss (Excitation BP 546/12, Emission LP 590) revealed the red fluorescence of those motoneurons retrogradely labelled by DiI. The fluorescence cross-talk between the tracers was restricted with this filter combination *ad maximum*: (i) no DiI-labelled cells were visible through the Bandpass and HQ-Schmalband filter sets; (ii) no FG-labelled cells could be observed through filter set 15 or the Bandpass filter sets and (iii) no FB-labelled motoneurons could be observed using filter set 15.

Image analysis

Employing a CCD Video Camera System (Optronics Engineering Model DEI-470, Goleta CA USA) combined with the image analysing software Optimas 6.5. (Optimas Corporation, Bothell, Washington, USA), separate colour images of retrogradely labelled facial motoneurons were created through the different filter sets. All images of DiI-labelled motoneurons were used to create 'DiI-masks'

by the Optimas: frames were binarized, dilated, and the outlines of each DiI-labelled cell depicted. Using the arithmetic options dialogue from the image menu, the generated masks were superimposed over the FB- or FG-picture. In this way, all cells stained by DiI_{only}, FG_{only}, FB_{only}, as well as all those double stained by DiI + FG or DiI + FB could be readily identified and were manually counted on the computer screen (Dohm *et al.*, 2000).

Counting

Employing the fractionator principle (Gundersen, 1986) all retrogradely labelled motoneurons with visible cell nucleus in the 50 µm thick sections were counted in every third section through the facial nucleus on the operated and on the unoperated side. Details for this sampling technique were described previously (Guntinas-Lichius & Neiss, 1996). All counts were performed by two observers (S. Dohm and D. Felder) who had no information about the respective treatment of the rats.

Statistical evaluation

For an overall evaluation separate one-way ANOVA tests were used to compare the numbers of single- (DiI_{only}) and double- (DiI + FG and DiI + FB) labelled neurons in the facial nucleus. For a more detailed analysis we checked with the posthoc Bonferroni test differences between the numbers of labelled motoneurons in the different groups. A level of significance of $P < 0.05$ was used for all analyses. All computations were performed using SPSS®-software.

Results

NGF, BDNF, bFGF, IGF-I, and GDNF are differentially expressed in the proximal and distal stump of the transected buccal branch of the facial nerve

Current knowledge indicates that, with the exception of CNTF, which is synthesized by myelinating Schwann cells (Sendtner *et al.*, 1994), most other factors are target-derived. They are taken up at the synaptic terminals and reach the neuronal somata via retrograde axonal transport (Taniuchi *et al.*, 1988). Accordingly, it has been shown that sections through the intact facial nucleus of rats do not contain mRNA for BDNF, CNTF, and GDNF (Stöckli *et al.*, 1991; Baumgartner & Shine, 1997). In spite of that, we had to analyse the expression of neurotrophic factors under different conditions, i.e. using the nerve-muscle-plate approach and the selected neutralizing antibodies, which were also our therapeutic agents.

NGF-immunoreactivity

The use of the nerve-muscle-plate showed the presence of NGF-protein in both proximal and distal stumps of the transected BFB. In the proximal nerve stump, NGF was first detected at 3 days post axotomy (DPA), reached a maximum at 5 DPA and declined at 6 DPA. In the distal nerve stump, NGF-immunoreactivity appeared at 1 DPA, reached a maximum at 4–5 DPA, and was no more evident at 12 DPA (Fig. 2A and B). A parallel immunostaining for S-100 protein (Fig. 1B), considered as a general marker of Schwann cells, showed that the majority of the cells which expressed NGF were Schwann cells. NGF-protein was also found in the target musculature of the whisker pad at 4–5 DPA.

BDNF-immunoreactivity

BDNF-immunoreactivity was detected in the Schwann cells of the proximal nerve stump at 3–4 DPA, reached a maximum at 5 DPA and steeply declined after 6 DPA. In the Schwann cells of the distal

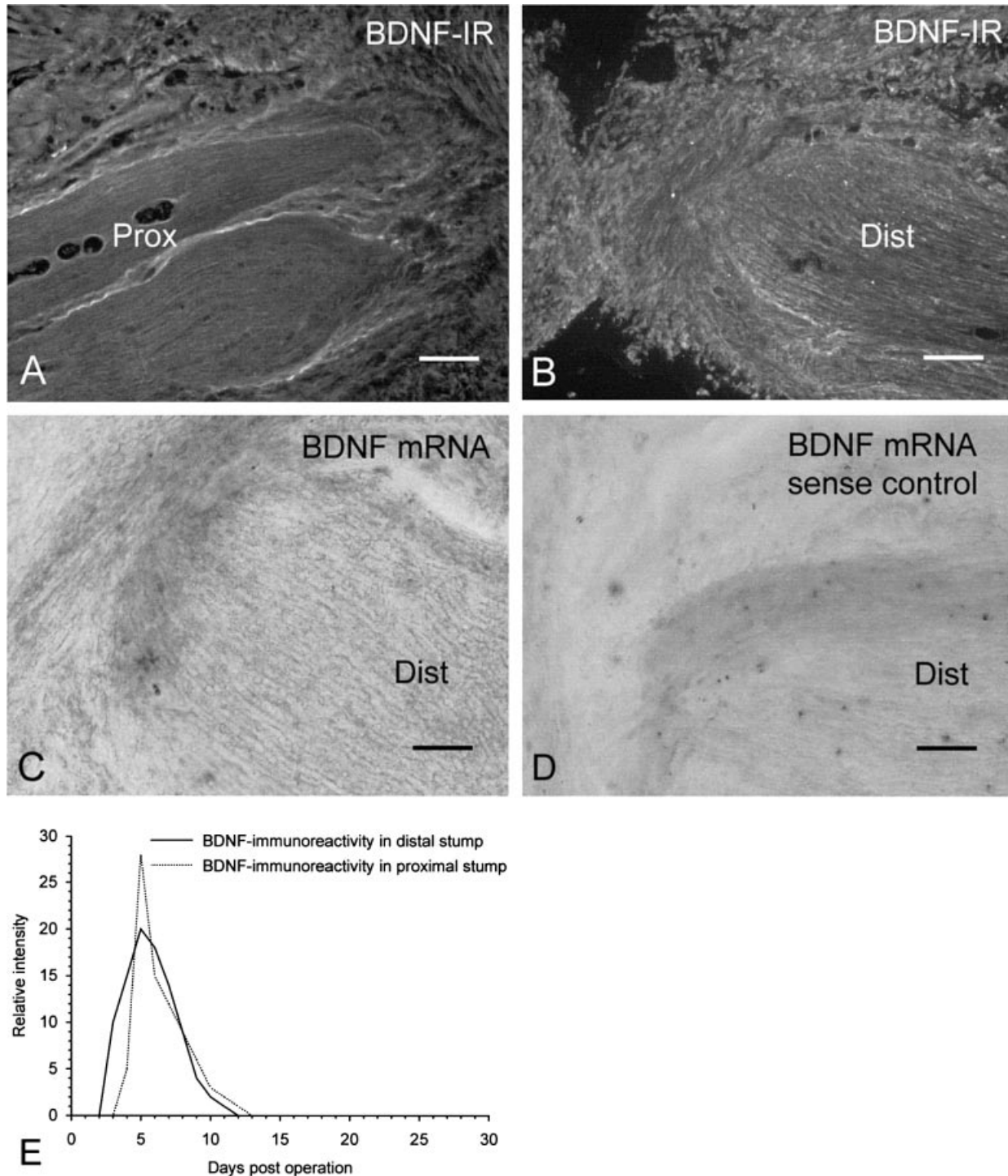


FIG. 3. Pattern of expression and sources of secretion of BDNF. Longitudinal sections through the transected buccal nerve showing BDNF-immunofluorescence in the Schwann cells of the proximal (A) and distal (B) nerve stump 4 days after the lesion. 20 μ m thick frozen section. Scale bar, 200 μ m. (C) BDNF mRNA expression 5 days after transection of the buccal branch. *In situ* hybridization histochemistry with digoxigenin-labelled BDNF cRNA probes reveals reaction product only in the peripheral cytoplasm of multivacuolated longitudinally arranged Schwann cells of the distal stump. Scale bar, 100 μ m. (D) Sense control of the same animal as shown in C. Scale bar, 100 μ m. (E) Time course of expression of BDNF-immunoreactivity in proximal and distal nerve stumps of the transected buccal branch estimated in a way identical to that described for Fig. 2B.

nerve stump, BDNF-immunoreactivity became evident at 4 DPA, reached its maximum at 5 DPA and was no more evident at 12 DPA (Fig. 3A, B and E). These findings support, though indirectly, earlier observations that the retrogradely transported BDNF prevents injury

induced death of facial motor neurons in neonatal rats (Hughes *et al.*, 1993; Koliatsos *et al.*, 1993; Yan *et al.*, 1993). No BDNF-immunoreactivity was detected in the target musculature of the whisker pad.

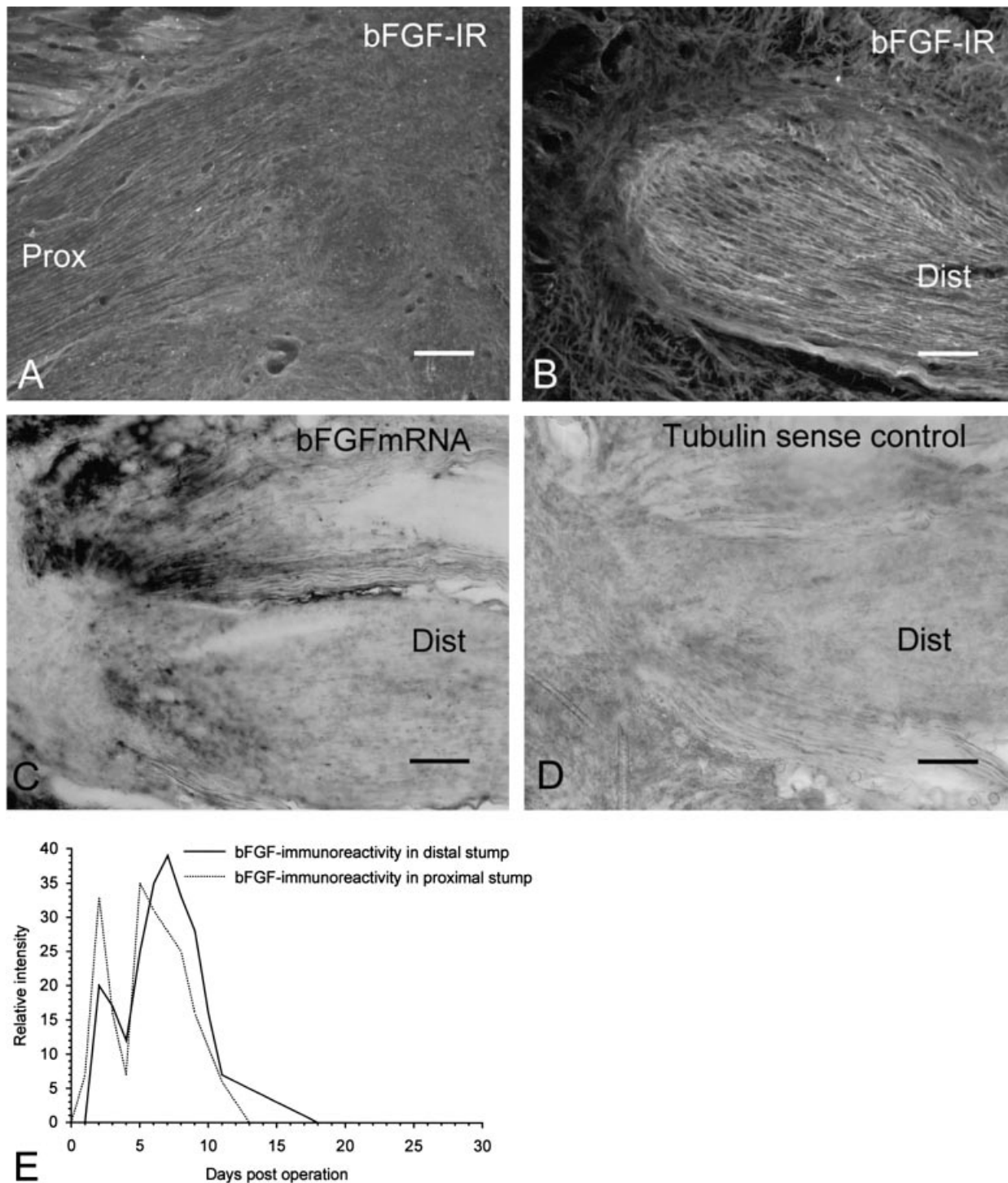


FIG. 4. Pattern of expression and sources of secretion of bFGF (FGF-2). Longitudinal sections through the transected buccal nerve showing bFGF-immunofluorescence in the Schwann cells of the proximal nerve segment (A) and around the neurofilamentous swelling of the distal nerve stump (B) 5 days after the lesion. Scale bar, 200 μ m. (C) bFGF mRNA expression 4 days after transection of the buccal branch. *In situ* hybridization histochemistry showing abundant and intensively dark reaction product in the cytoplasm of swollen Schwann cells from the distal stump. Scale bar, 100 μ m. (D) Tubulin sense control of the same animal as shown in C. Scale bar, 100 μ m. (E) Time course of expression of bFGF-immunoreactivity in the proximal and distal nerve stumps of the transected buccal branch depicting a characteristic double peak.

bFGF-immunoreactivity

bFGF-immunoreactivity was detected in the proximal stump at 1 DPA, reached a first peak at 2 DPA and declined at 4 DPA in three out of four rats. At 5–6 DPA there occurred a second peak in the

bFGF-immunoreactivity (in four out of four rats), which was followed by a gradual decline to zero at 8 DPA. Identical changes in the bFGF-immunoreactivity were detected in the Schwann cells of the distal nerve stump (Fig. 4A, B and E). There was a faint bFGF-

immunoreactivity in the target muscles of the whisker pad. Similar findings have been reported by Chen *et al.* (1999).

IGF-I-immunoreactivity

IGF-I-immunoreactivity followed a pattern of expression identical to that of bFGF. However, the IGF-I-immunoreactivity in the target muscles (detected at 1 DPA) was stronger and persisted till 5 DPA (data not shown). This result is in line with earlier data about IGF-I immunoreactivity mainly in Schwann cells until 1 week after the lesion, when strongly IGF-1 positive macrophages invade the lesioned nerve (Glazner *et al.*, 1994; Ishii *et al.*, 1994; Cheng *et al.*, 1996).

GDNF-immunoreactivity

GDNF-immunoreactivity in the proximal nerve stump appeared at 1–2 DPA, reached a maximum at 5 DPA, and disappeared at 6 DPA. In the distal nerve stump, GDNF-immunoreactivity appeared at 3 DPA, reached a maximum at 5 DPA and was no more evident at 6 DPA (data not shown). These results are in accordance with those in an earlier report about retrograde transport of GDNF to facial motoneurons (Yan *et al.*, 1995). There was no GDNF-immunoreactivity in the target musculature of the whisker pad.

CNTF-immunoreactivity

There were no detectable amounts of CNTF in the whole nerve-muscle-plate after transection of the BFB (not shown). This finding is consistent with previous results showing that the expression of CNTF mRNA and protein in the peripheral nerve decreases following nerve lesion (Friedman *et al.*, 1992; Sendtner *et al.*, 1992; Smith *et al.*, 1993).

Blood-borne macrophages and Schwann cells are the major sources of NGF, BDNF, and bFGF at the lesion site

It is well known that the level of plasma NGF is very high in stressed animals (Alleva *et al.*, 1996). To consider the trauma-induced appearance of NGF after surgery, we needed to establish the exact sources of trophic factors secretion at the lesion site. This is why free-floating 40 μ m thick cryostat sections from the nerve-muscle plate were processed for detection of NGF mRNA, BDNF mRNA, and bFGF mRNA by *in situ* hybridization.

General features

(i) Twenty-four and 48 h after removal of 1 mm length from the buccal branch of the facial nerve, the only cells which expressed NGF-, BDNF-, and bFGF mRNA at the lesion site were blood-borne cells, presumably monocytes/macrophages. Confirming the results of a very recent work (Barouch *et al.*, 2001), this finding is also in accordance with earlier data stating that BDNF was synthesized neither by motoneurons nor by the Schwann cells during the early postlesion period (Ernfors *et al.*, 1990; Meyer *et al.*, 1992; Funakoshi *et al.*, 1993). (ii) The motor target (whiskerpad musculature) showed no mRNA expression for NGF, BDNF, and bFGF.

NGF mRNA. Three days after the lesion, the Schwann cells of both the proximal and the distal nerve stump expressed NGF mRNA. Five days after the lesion only rows of swollen Schwann cells of the proximal nerve stump contained NGF mRNA (Fig. 2C). The distal stump was negative. These results are in line with those in earlier reports about a compensatory increase in the expression of NGF by non-neuronal (Schwann) cells at the site of lesion (Heumann *et al.*, 1987a; Lindholm *et al.*, 1987; Raivich *et al.*, 1991; Meyer *et al.*, 1992).

BDNF mRNA. Starting from 3 days after the lesion, the only cells that contained BDNF mRNA were the Schwann cells of the distal nerve stump. Thus, the onset of gene expression is in agreement with the findings of Meyer *et al.* (1992) and Funakoshi *et al.* (1993). At present we have not established whether the expression of BDNF mRNA gradually increased over a 3–4-week period as previously described (Meyer *et al.*, 1992; Funakoshi *et al.*, 1993) – we were interested in the sources of trophic factors during a relatively short period (24 h to 5 days) after axotomy. The proximal stump remained negative (Fig. 3C and D).

bFGF mRNA. Three days after lesion, the Schwann cells of both, proximal and distal nerve stumps expressed bFGF mRNA, a result which is consistent with earlier observations (Grothe *et al.*, 1997, 2001; Huber *et al.*, 1997). At 4 days after lesion, the bFGF mRNA in the proximal nerve stump was down-regulated. Thus, the major source of cells which contained bFGF mRNA were the Schwann cells of the distal nerve stump (Fig. 4C and D).

Application of neutralizing antibodies to trophic agents reduces post-transectional axonal branching and improves the quality of reinnervation

Unoperated rats

In intact rats, the zygomatic branch of the facial nerve contained the axons of 306 ± 81 motoneurons located in the dorsal facial subnucleus, the buccal branch of 1422 ± 39 motoneurons in the lateral subnucleus, and the marginal mandibular branch of 288 ± 42 motoneurons in the intermediate subnucleus (group C₁ in Table 1). No double-labelled motoneurons were observed (Fig. 1C) and all motoneurons projecting through the zygomatic branch contained only DiI. No fluorescent perikarya were ever found in the medial or ventromedial facial subnucleus, the motoneurons of which project through the posterior auricular and cervical branch, respectively; these nerves were not transected or labelled.

General features in the facial nucleus after transection of the facial nerve

Eight weeks after unilateral FFA/entubulation and another 10 days after triple retrograde labelling, three general changes were detected in the lesioned facial nucleus. First, the myotopic organization into subnuclei was no longer observed, i.e. all retrogradely labelled motoneurons were scattered throughout the facial nucleus (Fig. 1F and H). Second, as a rule there were always more retrogradely labelled motoneuronal cell somata than in unoperated animals (Tables 1–4). The reason for this is the postoperative hyperinnervation of targets (Angelov *et al.*, 1996), i.e. labelling of motoneurons which, under normal conditions, do not send axons into the three facial fascicles under study. After transection of the facial nerve, however, these motoneurons develop axonal branches, which adjoin the wrong fascicles and thus reach the sites of tracer application. Third, numerous double-labelled motoneurons occurred after the lesion (appearing pink-orange or purple in Fig. 1F and H) which demonstrates that twin axons projected into more than one branch of the facial nerve. All double- and single-labelled motoneurons labelled were counted. The mean values were statistically analyzed (Tables 1–4).

Analysis of the total number of neurons projecting through the marginal mandibular branch (FB-only)

Controls. With the exception of C₅, the values for the surgically treated control groups (C₂–C₄ in Table 1) did not differ from those obtained after application of antibodies in neutralizing concentrations (Table 2). All numbers, however, were significantly lower than those

TABLE 2. Number of retrogradely labelled motoneurons whose axons project through the zygomatic, buccal, or marginal mandibular branches 56 days after entubulation of a transected facial nerve into a silicone tube containing antibodies to trophic factors in neutralizing concentrations

Surgery	Neurons sending axons through the zygomatic nerve only (DiI-only)	Neurons with axon sprouts in the zygomatic and buccal nerves (DiI + FG)	Neurons with axon sprouts in the zygomatic and marginal mandibular nerves (DiI + FB)	Total neurons, branched and unbranched projecting through the zygomatic nerve (DiI, DiI + FG, DiI + FB)	Neurons sending axons through the buccal nerve only (FG-only)	Neurons sending axons through the marginal mandibular nerve only (FB-only)
Group C6. Anti-NGF (40 µg/mL)	302 ± 66 ^{*2-5} 77%	65 ± 36 ^{*1.5.12-20} 16%	27 ± 16 ^{*1.5.12-20} 7%	394 ± 85 ^{*12.15-17} 100%	1168 ± 502 ^{*2.4.5.12-20}	572 ± 206 ^{*1.5.12-20}
Group C7. Anti-BDNF (160 µg/mL)	270 ± 82 ^{*2.5} 82%	47 ± 25 ^{*1.5.12-20} 14%	14 ± 10 ^{*1.5.12-20} 4%	331 ± 107 ^{*12.15-17} 100%	1152 ± 357 ^{*2.4.5.12-20}	434 ± 179 ^{*5.12-20}
Group C8. Anti-bFGF (100 µg/mL)	196 ± 101 ^{*10} 78%	35 ± 26 ^{*1.5.12-20} 14%	19 ± 9 ^{*1.5.12-20} 8%	250 ± 127 ^{*12.15-17} 100%	1040 ± 314 ^{*2.4.5.12-20}	675 ± 282 ^{*1.5.12-20}
Group C9. Anti-IGF-I (30 µg/mL)	282 ± 77 ^{*2.5} 73%	74 ± 45 ^{*1.5.12-20} 20%	28 ± 16 ^{*1.5.12-20} 7%	386 ± 127 ^{*12.15-17} 100%	1365 ± 270 ^{*2.4.5.12-20}	480 ± 162 ^{*5.12-20}
Group C10. Anti-GDNF (3 µg/mL)	392 ± 74 ^{*2-5.8} 70%	131 ± 69 ^{*1.5.12-20} 23%	40 ± 22 ^{*1.5.12-20} 7%	563 ± 128 ^{*12.15-17} 100%	1466 ± 444 ^{*5.12-20}	818 ± 281 ^{*1-5.12-20}
Group C11. Anti-CNTF (100 µg/mL)	252 ± 98 77%	55 ± 28 ^{*1.5.12-20} 17%	20 ± 8 ^{*1.5.12-20} 6%	327 ± 121 ^{*12.15-17} 100%	1501 ± 344 ^{*5.12-20}	542 ± 121 ^{*1.5.12-20}

Each value is a mean ±SD of nine rats. Small numbers indicate the groups with significantly different values.

* $P < 0.05$. The portions of motoneurons projecting through the zygomatic nerve with branched axons (DiI + FG or DiI + FB) and unbranched axons (DiI-only) are indicated as percentages below the absolute numbers.

TABLE 3. Number of retrogradely labelled motoneurons whose axons project through the zygomatic, buccal, or marginal mandibular branches 56 days after entubulation of a transected facial nerve into a silicone tube containing antibodies to trophic factors exceeding the neutralizing concentration 5 fold

Surgery	Neurons sending axons through the zygomatic nerve only (DiI-only)	Neurons with axon sprouts in the zygomatic and buccal nerves (DiI + FG)	Neurons with axon sprouts in the zygomatic and marginal mandibular nerves (DiI + FB)	Total neurons, branched and unbranched projecting through the zygomatic nerve (DiI, DiI + FG, DiI + FB)	Neurons sending axons through the buccal nerve only (FG-only)	Neurons sending axons through the marginal mandibular nerve only (FB-only)
Group C12. Anti-NGF (200 µg/mL)	198 ± 56 34%	214 ± 75 ^{*1-4.6-11} 37%	166 ± 73 ^{*1-4.6-11} 29%	578 ± 121 ^{*1-11} 100%	2524 ± 572 ^{*1-4.6-11}	2058 ± 252 ^{*1-4.6-11}
Group C13. Anti-BDNF (800 µg/mL)	152 ± 28 ^{*1.10} 32%	200 ± 50 ^{*1-4.6-11} 42%	125 ± 68 ^{*1-4.6-11} 26%	477 ± 118 100%	2680 ± 84 ^{*1-4.6-11}	2109 ± 98 ^{*1-4.6-11}
Group C14. Anti-bFGF (500 µg/mL)	136 ± 103 ^{*10} 24%	254 ± 156 ^{*1-4.6-11} 44%	190 ± 97 ^{*1-4.6-11} 32%	580 ± 340 ^{*2} 100%	2194 ± 125 ^{*1-4.6-11}	1710 ± 714 ^{*1-4.6-11}
Group C15. Anti-IGF-I (150 µg/mL)	85 ± 58 ^{*1.4.6.7.9.10} 5%	626 ± 364 ^{*1-4.6-11} 56%	412 ± 255 ^{*1-4.6-11} 39%	1123 ± 362 ^{*1-11.13.14.18-20} 100%	2643 ± 150 ^{*1-4.6-11}	1966 ± 274 ^{*1-4.6-11}
Group C16. Anti-GDNF (15 µg/mL)	143 ± 63 ^{*1.10} 25%	248 ± 76 ^{*1-4.6-11} 44%	172 ± 57 ^{*1-4.6-11} 31%	563 ± 88 ^{*1-4.6-11} 100%	3214 ± 857 ^{*1-4.6-11}	2339 ± 437 ^{*1-4.6-11}
Group C17. Anti-CNTF (500 µg/mL)	267 ± 105 43%	225 ± 46 ^{*1-4.6-11} 36%	133 ± 22 ^{*1-4.6-11} 20%	616 ± 128 ^{*1-4.6-11} 100%	2773 ± 171 ^{*1-4.6-11}	1972 ± 194 ^{*1-4.6-11}

Each value is a mean ±SD of four rats.

*The level of significance is $P < 0.05$. The portions of motoneurons projecting through the zygomatic nerve with branched axons (DiI + FG or DiI + FB) and unbranched axons (DiI-only) are indicated as percentages below the absolute numbers.

TABLE 4. Number of retrogradely labelled motoneurons whose axons project through the zygomatic, buccal, or marginal mandibular branches 56 days after entubulation of a transected facial nerve into a silicone tube containing combinations of antibodies to neurotrophic factors in neutralizing concentrations

Surgery	Neurons sending axons through the zygomatic nerve only (DiI-only)	Neurons with axon sprouts in the zygomatic and buccal nerves (DiI + FG)	Neurons with axon sprouts in the zygomatic and marginal mandibular nerves (DiI + FB)	Total neurons, branched and unbranched projecting through the zygomatic nerve (DiI, DiI + FG, DiI + FB)	Neurons sending axons through the buccal nerve only (FG-only)	Neurons sending axons through the marginal mandibular nerve only (FB-only)
Group C18. Anti-NGF and anti-BDNF	91 ± 56* ^{1,6,7,9,10} 17%	306 ± 195* ^{1-4,6-11} 56%	206 ± 127* ^{1-4,6-11} 38%	544 ± 232* ² 100%	2582 ± 352* ^{1-4,6-11}	2602 ± 424* ^{1-4,6-11}
Group C19. Anti-BDNF and anti-bFGF	175 ± 21* ^{1,10} 35%	202 ± 91* ^{1-4,6-11} 41%	121 ± 67 24%	496 ± 152 100%	2770 ± 314* ^{1-4,6-11}	1966 ± 229* ^{1-4,6-11}
Group C20. Anti-bFGF and anti-NGF	191 ± 88* ¹⁰ 42%	196 ± 107* ^{1-4,6-11} 43%	72 ± 39 15%	460 ± 216 100%	2690 ± 406* ^{1-4,6-11}	1791 ± 208* ^{1-4,6-11}

Each value is a mean ± SD of six rats.

*The level of significance is $P < 0.05$. The portions of motoneurons projecting through the zygomatic nerve with branched axons (DiI + FG or DiI + FB) and unbranched axons (DiI-only) are indicated as percentages below the absolute numbers.

obtained after treatment with 5-fold higher concentrations of neutralizing antibodies (Table 3) and after combined treatments (Table 4).

Neutralizing concentrations of the antibodies. All values were lower than those obtained after treatment with 5-fold higher concentrations and after combined treatments (compare the values in the right column of Table 1 to those in Table 3 and Table 4).

Analysis of the total number of neurons projecting through the buccal branch (FG-only)

Controls. With the exception of group C₃ each of the other operated controls (C₂, C₄, and C₅) showed a value that was significantly higher than that obtained for intact rats (Table 1). Compared to the number obtained after application of antibodies in neutralizing concentrations (Table 2), the value for C₂, C₄, and C₅ was significantly higher. Compared to those obtained after 5-fold higher concentrations (Table 3), and after combined treatments (Table 4), these numbers were significantly lower.

Neutralizing concentrations of the antibodies. All values were significantly lower than those after treatment with 5-fold higher concentrations (Table 3) and after combined treatments (Table 4).

Analysis of the total number of neurons projecting through the zygomatic branch (DiI, DiI + FG, DiI + FB)

We selected the zygomatic branch as a representative of the whole facial nerve plexus to evaluate the degree of axonal branching after surgery. The reason for this was the relatively small number of motoneurons projecting through the zygomatic branch before and after surgery. Thus, the distribution of the tracer DiI was of special interest and we carefully differentiated the portions of motoneuronal perikarya labelled by DiI_{only}, by DiI + FG, or by DiI + FB. Each percentage is indicated below the absolute mean number in the first four columns of all tables. A beneficial effect of a particular treatment was recognized only if a given value was close to the distribution pattern in intact rats (first row in Table 1).

Controls. There were no differences in the total number of neurons, whose axons projected through the zygomatic nerve among the surgically treated control groups (C₂–C₅) of the present study (Table 1). All control values were significantly lower than the numbers obtained after treatment with 5-fold higher concentrations of some neutralizing antibodies (Groups C₁₂, C₁₅–C₁₇; Table 3).

Neutralizing concentrations of the antibodies. Compared to the values in the control groups (C₂–C₅) and to the combinations of antibodies (C₁₈–C₂₀) the treatment with neutralizing concentrations of all antibodies caused no reduction in the total number of neurons, whose axons projected postoperatively through the zygomatic branch of the facial nerve (Tables 1, 2 and 4) These values were, however, significantly lower than the numbers obtained after treatment with 5-fold higher concentrations of some neutralizing antibodies (C₁₂, C₁₅–C₁₇; Table 3).

Analysis of the number of double-labelled motoneurons projecting through the zygomatic branch (DiI + FG and DiI + FB)

Controls. With the exception of the control group C₅ (where 86% of all motoneurons had branched) there were no significant differences in the number of DiI + FG and DiI + FB-labelled perikarya among

the groups C₂–C₄ (Table 1). In general, all control values were significantly lower than the numbers obtained after treatment with 5-fold higher concentrations of all neutralizing antibodies (Groups C₁₂–C₁₇; Table 3) and after combined treatment (groups C₁₉, C₂₀; Table 4).

Neutralizing concentrations. This treatment did not reduce the number of double-labelled (i.e. branched) neurons (DiI + FG and DiI + FB) when compared to the values in groups C₂–C₄. All values (Table 2), however, were significantly lower (i.e. showed reduced branching) than that in the control group C₅ (Table 1) and than those after treatment with 5-fold higher concentrations of all neutralizing antibodies (Groups C₁₂–C₁₇; Table 3) and after combined treatments (Groups C₁₈–C₂₀; Table 4).

Analysis of the number of single-labelled motoneurons projecting through the zygomatic branch (DiI-only)

There were no differences among the surgically treated control groups (C₂–C₅). In general, treatment with neutralizing concentrations of NGF, BDNF, IGF-I, and GDNF (Groups C₆, C₇, C₉, C₁₀; Table 2) significantly increased the number of motoneurons sending a single axon only through the zygomatic branch, i.e. this treatment reduced axonal branching.

Discussion

Our present data show for the first time that collateral axonal branching from the proximal stump of the lesioned peripheral nerve can be experimentally reduced. Focal application of neutralizing antibodies to neurotrophic factors in suitable dose diminished the number of axonal branches without any notable harmful affect on neuronal survival and axonal elongation.

Neurobiological importance of post-transectional axonal branching

When an axon is severed, the proximal cut end rapidly closes with a membrane seal (Fishman *et al.*, 1990; Spira *et al.*, 1993) and soon afterwards forms a terminal swelling or end-bulb (Friede & Bischhausen, 1980; Fried *et al.*, 1991). Within the following 3 h numerous axonal branches begin to emerge from the end-bulb and start to elongate (Bisby & Pollock, 1983) at a rate of approximately 4 mm/day (Tetzlaff & Bisby, 1989; Fawcett & Keynes, 1990). Under ideal conditions only one of these branches should reach its original target.

The navigation of these immature axons is under the control of short-range acting guidance cues, most of them deriving from the distal nerve stump. Thus, postlesional axonal branching is considered to represent the search of axons for local guidance cues necessary for their navigation (Al-Majed *et al.*, 2000). In general, however, exactly these actively searching axons not only choose a wrong way, but also branch and project simultaneously along several different fascicles (subdivisions, rami) of a transected nerve trunk (Angelov *et al.*, 1999). Thus, the process of branching, which is originally aimed at both neuronal- and target-survival, turns out to be responsible for the failure of axons to re-innervate solely their original domain (Fu & Gordon, 1997).

In a recent set of experiments, we conceptually divided the overall process of axonal re-growth into axon elongation and axonal branching. We hypothesized that accelerated axonal elongation through extracellular matrix proteins fostering neurite outgrowth might reduce axonal branching and improve recovery of function.

The subsequent neuron counts showed that, despite their known effect to support neurite elongation, the tested extracellular matrix proteins failed to suppress axonal branching in the facial nerve plexus (Dohm *et al.*, 2000). In the present study we approached the problem in a completely other, actually in an exactly opposite way, i.e. blocking the entire process of neurite re-growth we hoped to achieve a reduction in axonal branching.

We succeeded to do this only using antibodies to neurotrophic factors in neutralizing concentrations (groups C₆–C₁₂) whereby the numbers of neurons projecting through each of the investigated branches was very close to those in intact rats. Three groups of substances, however, the mouse IgG (group C₅), the 5-fold higher concentrations (groups C₁₂–C₁₇) as well as the combinations of antibodies (groups C₁₈–C₂₀) were probably nonsupportive to neurite elongation. Massive axonal branching followed.

Possible role(s) of trophic factors in axonal re-growth and effect of their neutralization

Most of the neurotrophic agents are produced by the target tissue and reach the intact facial motoneurons via retrograde axonal transport. Accordingly, after axotomy trophic factors are taken up by regenerating axons to keep the neurons trophically satisfied until the axons regain their target-derived source (Di Stefano *et al.*, 1992; Unsicker *et al.*, 1992; Yan *et al.*, 1992; Friedman *et al.*, 1995; Sendtner *et al.*, 1997).

Apart from this central effect on neuronal cell bodies (promoting survival), however, numerous reports show another peripheral effect of the trophic factors. Accordingly, the synthesis of receptors for NGF, BDNF, bFGF, GDNF, CNTF, and IGF-I is up-regulated at the lesion site following axotomy (Meyer *et al.*, 1992; Raivich & Kreutzberg, 1993; McMahon & Priestley, 1995; MacLennan *et al.*, 1999). However, it should be reminded that the expression of p75^{NTR} in Schwann cells following facial nerve surgery was not found necessary for motoneuron survival or prompt regeneration (Ferri *et al.*, 1998). This, in turn, was a major reason for our attempt to neutralize the trophic agents themselves and not their receptors.

The quantitative changes in post-transectional axonal branching after neutralization of this peripheral effect were the main object of this study. In the following sections we will try to interpret the results and correlate them with other already established events at the lesion site.

The ciliary neurotrophic factor (CNTF) increases the rate of axonal elongation, axonal sprouting, number of re-innervated muscle end-plates and improves functional recovery (Gurney *et al.*, 1992; Curtis *et al.*, 1993; Sahenk *et al.*, 1994; Ulenkate *et al.*, 1994; Newman *et al.*, 1996; Oyesiku & Wigston, 1996). Treatment with 100 µg/mL anti-CNTF (group C₁₁) decreased the number of double-labelled neurons when compared to the mouse IgG control, but not vs. the other controls (Table 2). Based on these results we cannot ascribe any obvious effect of the anti-CNTF-treatment (100 µL/mL) on the branching of axons stemming from a transected rat facial nerve.

The glial-cell-line derived neurotrophic factor (GDNF) has a marked stimulatory effects on neurite outgrowth (Ebendal *et al.*, 1995; Rosenblad *et al.*, 1996; Sinclair *et al.*, 1996; Wang *et al.*, 1996; Wilby *et al.*, 1999). It has been shown that its neutralization inhibits branching and reduces sprouting (Batchelor *et al.*, 2000). Our present results, however, do not support these earlier data. Treatment with 3 µg/mL anti-GDNF (group C₁₀) caused a significant increase in the total number of motoneurons projecting through the zygomatic branch of the facial nerve when compared to the values obtained after FFA (group C₂) and after neutralization of bFGF (group C₈; Table 2). This suggests that the neutralization of GDNF potentiated, rather than

inhibited axonal branching from motoneurons. This in turn, diminishes the value of the otherwise very important large number of DiI_{only} labelled cells, which were significantly more than those after FFA (group C₂), mere entubulation (group C₃), entubulation in collagen (group C₄), and entubulation in mouse IgG (group C₅).

The insulin-like growth factors (IGFs) control Schwann cell viability (Meier *et al.*, 1999; Syroid *et al.*, 1999), promote neurite outgrowth of motoneurons (Caroni & Grandes, 1990; Lewis *et al.*, 1993; Vergara *et al.*, 1993) increase regeneration rate *in vivo* after lesions of the sciatic nerve (Sjoberg & Kanje, 1989; Pu *et al.*, 1999), and enhance axonal regeneration in chronically injured neurons (Houle *et al.*, 1996). It has been shown that their neutralization causes a sustained reduction in the regeneration rate (Glazner *et al.*, 1993) as well as inhibition of epithelial cell proliferation (Zheng *et al.*, 1997) and spontaneous regeneration (Kanje *et al.*, 1989; Near *et al.*, 1992). In our experimental set, treatment with 30 µg/mL anti-IGF-I (group C₉) decreased the number of double-labelled neurons when compared to the mouse IgG control (Table 2). Based on these data we can state that the neutralization of IGF-I with 30 µg/mL anti-IGF-I reduced the post-transectional axonal branching.

The basic fibroblast growth factor (FGF-2) stimulates *in vivo* neurite outgrowth from the proximal stump of transected peripheral nerves and contributes to the enlargement of axon calibre (Aebischer *et al.*, 1989; Unsicker *et al.*, 1993; Laquerriere *et al.*, 1994; Fujimoto *et al.*, 1997). Its neutralization causes a significant decrease in the number of regenerating axons (Chen *et al.*, 1999). Our results, however, fail to confirm these recent data. Treatment with 100 µg/mL anti-bFGF (group C₈) decreased the number of double-labelled neurons only when compared to the mouse IgG control. There was really a reduction in the number of DiI_{only} labelled cells, however, only when compared to the values obtained after treatment with 3 µg/mL anti-GDNF (group C₁₀). Thus, we may conclude that an inhibition of bFGF employing therapeutic neutralizing concentrations of antiserum had no significant effect on the post-transectional branching of motoneurons.

The brain-derived neurotrophic factor (BDNF) influences the dynamic branching of axonal arbours (Sawai *et al.*, 1996; Inoue & Sanes, 1997; Cohen-Cory, 1999; Lom & Cohen-Cory, 1999) and effectively promotes axonal outgrowth (Novikov *et al.*, 1997; Novikova *et al.*, 1997; Bamber *et al.*, 2001). Its neutralization reduces the arborizations of axons (Cohen-Cory & Fraser, 1995), retards the length of regenerated nerves (Zhang *et al.*, 2000) and decreases the average axon length (Ghosh *et al.*, 1994; Nawa *et al.*, 1994). Our present results are in line with these findings. Treatment with 160 µg/mL anti-BDNF (group C₇) decreased the number of double-labelled neurons when compared to the mouse IgG control. When compared to FFA there was a significant increase in the number of DiI_{only} labelled cells. These values were not accompanied by an increase in the total number of neurons projecting through the zygomatic nerve (Table 2).

The nerve growth factor (NGF) can preserve the Schwann cells, whereupon they facilitate regeneration from the proximal stump and modulate neurite outgrowth and axon branching (Aloe *et al.*, 1985; Heumann *et al.*, 1987b; Lindsay, 1988; Snider & Johnson, 1989; Gloster & Diamond, 1992; Kimpinski *et al.*, 1997; Galo & Letourneau, 1998; McAllister *et al.*, 1999; Mohiuddin *et al.*, 1999). When applied to silastic nerve bridges between the ends of transected sciatic and facial nerves NGF increases the regeneration rate (Chen *et al.*, 1989; Derby *et al.*, 1993; Spector *et al.*, 1993). Its neutralization by neutralizing antibodies or antisense oligonucleotides reduced the maladaptive axonal branching and the detrimental sprouting (Yip *et al.*, 1984; Mearow & Kril, 1995; Krenz *et al.*,

1999). The results of our work confirm these earlier data. Treatment with 40 µg/mL anti-NGF decreased the number of double-labelled neurons when compared to the mouse IgG control. When compared to FFA, mere entubulation, and entubulation with mouse IgG there was a significant increase in the number of DiI_{only} labelled cells. These values were not accompanied by an increase in the total number of neurons projecting through the zygomatic nerve (Table 2).

Taken together our present results show that treatment of a transected rat facial nerve with anti-NGF or anti-BDNF in neutralizing concentrations significantly reduces the post-transectional axonal branching. In this way focal intraoperative antibody-treatment, that should be carefully controlled in human patients, may open new perspectives in the surgical strategies to improve the quality of reinnervation.

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Abbreviations

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; FB, Fast Blue; FG, Fluoro-Gold; GDNF, glial-cell-line derived neurotrophic factor; IGF-I, insulin-like growth factor I; mRNA, messenger ribonucleic acid; NGF, nerve growth factor.

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