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published in

The Journal of Neuroscience

2000

document version

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

citation for published version (APA)

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Neurotrophic Actions of a Novel Molluscan Epidermal Growth Factor

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The mammalian epidermal growth factor (EGF) is expressed in the developing and adult CNS, and it has been implicated in the control of cell proliferation, differentiation, and neurotrophic events. Despite extensive evolutionary conservation of the EGF motif in a range of different types of proteins, secreted EGF homologs with neurotrophic actions have not been reported in invertebrates. In this study, we present a novel member of the family of EGF-like growth factors, an EGF homolog from the mollusc *Lymnaea stagnalis* (L-EGF), and we demonstrate that this protein has neurotrophic activity. Purified L-EGF is a 43-residue peptide and retains the typical structural characteristics of the EGF motif. The *L-EGF* cDNA reveals a unique precursor organization. In contrast to the multidomain mammalian EGFs, it consists of only two domains, a signal peptide and a single EGF

motif. Conspicuously, the *L-EGF* precursor lacks a transmembrane domain, setting it apart from all other members of the EGF-family. *L-EGF* mRNA is expressed throughout embryonic development, in the juvenile CNS, but not in the normal adult CNS. However, expression in the adult CNS is upregulated after injury, suggesting a role of L-EGF in repair functions. This notion is supported by the observation that L-EGF evokes neurite outgrowth in specific adult *Lymnaea* neurons *in vitro*, which could be inhibited by an EGF receptor tyrosine kinase inhibitor. In conclusion, our findings further substantiate the notion that the EGF family has an early phylogenetic origin, and our data support a neurotrophic role for L-EGF during development and injury repair.

Key words: epidermal growth factor; neurotrophic factors; neurite outgrowth; mollusc; development; regeneration.

Epidermal growth factor (EGF), a member of the EGF growth factor family, is expressed in the developing and adult CNS (Rall et al., 1985; Schaudies et al., 1989; Lazar and Blum, 1992). Mammalian EGF is a polypeptide consisting of 53 amino acid residues characterized by six cysteine residues arranged in a conserved pattern of three disulfide bonds and three characteristic C-terminal amino acids (the EGF motif) (Carpenter and Cohen, 1990). This secreted polypeptide is the product of the proteolytic processing of a large, multi-domain transmembrane EGF precursor protein (Carpenter and Cohen, 1990; Plata-Salamán, 1991; Yamada et al., 1997). The EGF motif is present in many other multi-domain proteins throughout the animal kingdom, including a number of EGF family members, such as transforming growth factor α (TGF α) and amphiregulin (Engel, 1989; Carpenter and Cohen, 1990; Davis, 1990; Greenwald, 1990; Muskavitch and Hoffmann, 1990; Yamada et al., 1997). Although TGF α homologs have been

found in *Drosophila* (e.g., spitz and gurken; Rutledge et al., 1992; Neuman-Silberberg and Schüpbach, 1993), secreted forms of EGF itself have so far only been reported in mammals.

The present study was motivated by the observation that human EGF (hEGF) induces neurite outgrowth in motoneurons of the pond snail *Lymnaea stagnalis*. This result and the apparent conservation of the EGF motif prompted us to investigate the existence of an EGF homolog in *Lymnaea*. Although *Lymnaea* has been successfully used over the last decade to identify growth factor receptors (Roovers et al., 1995; Van Kesteren et al., 1998) and to characterize novel neuromodulatory actions of neurotrophic factors (Wildering et al., 1995; Fainzilber et al., 1996), attempts to identify endogenous growth factors have so far remained primarily unsuccessful. The single exception to this rule is cysteine-rich neurotrophic factor (Fainzilber et al., 1996). In this regard, it is of particular interest to investigate the existence of *Lymnaea* EGF homologs and their physiological activity.

In the present study, we purified, cloned, and characterized an EGF homolog, *Lymnaea* EGF (L-EGF) from the *Lymnaea* albumen gland, an organ that secretes fluid surrounding fertilized oocytes (Joosse and Geraerts, 1983). We show that L-EGF is a secreted growth factor with a surprisingly simple precursor organization. L-EGF selectively induces neurite outgrowth in adult *Lymnaea* neurons *in vitro*. An endogenous role for L-EGF in neuronal development and regeneration is suggested by the fact that, in addition to expression in the albumen gland, mRNA expression for this factor is highest during embryogenesis, in juvenile brains, and is upregulated in adult CNS after axotomy. This study shows for the first time that a secreted homolog of the peptide EGF is present in an invertebrate species and likely plays a neurotrophic role during development and axonal regeneration of adult neurons.

Received Feb. 15, 2000; revised May 22, 2000; accepted June 22, 2000.

This work was supported in part by National Science Foundation Grant IBN-9511773 (G.T.N.), grants from the Medical Research Council of Canada, and Human Frontiers Science Program Organization Grant RG0045/1997B. W.C.W. was supported by the Alberta Heritage Foundation for Medical Research (AHFMR) A.G.M.B. is an AHFMR Scientist. The L-EGF sequence has been deposited in the Protein Information Resource-International Protein Sequence Database under accession number A58998. The GenBank accession number for the *L-EGF* cDNA is AF187041. This work is dedicated to Dr. Michael J. Greenberg, codiscoverer of the first molluscan neuropeptide (FRMFamide) and former mentor (of G.T.N. and S.D.P.). We thank Drs. Marijke de Jong-Brink, Pamela de Boer, Nico de With, and Andries ter Maat for kindly providing initial supplies of *Lymnaea* albumen glands and Eric Schepens for technical assistance. We acknowledge the University of Texas Medical Branch (UTMB) Protein Chemistry Laboratory, which is supported by the UTMB Educational Cancer Center, for amino acid compositional and microsequence analysis.

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MATERIALS AND METHODS

Peptide extraction and purification of L-EGF. L-EGF was purified from extracts of ~500 albumen glands in batches of 10–20 glands. Albumen glands were removed from the adult gastropod mollusc *L. stagnalis*, lyophilized, extracted at 4°C in 0.1% heptafluorobutyric acid (HFBA) (Pierce, Rockford, IL) using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY), and sonicated. The extract was centrifuged for 20 min at 48,000 × g (4°C), and the supernatant was purified on four C18 Sep-Pak cartridges (Waters Associates, Milford, MA) connected in series; Sep-Paks were pre-treated with 5 ml of 100% acetonitrile (ACN) containing 0.1% HFBA and rinsed with 10 ml of 0.1% HFBA. The peptides were eluted with 5 ml of 50% ACN containing 0.1% HFBA and lyophilized. The lyophilizate was resuspended in 2.5 ml of 0.1% HFBA and applied to an analytical C18 reversed-phase (RP)-HPLC column (4.6 × 250 mm; Vydac, Hesperia, CA). The column was eluted with a two-step linear gradient of 0.1% HFBA and ACN containing 0.1% HFBA. The first step was 0–10% ACN containing 0.1% HFBA in 5 min, and the second step was 10–34% ACN containing 0.1% HFBA in 85 min. The column eluate was monitored at 214 nm, and 1 min (1 ml) fractions were collected. For chemical characterization studies, fractions containing L-EGF were pooled, lyophilized, reduced with 2-mercaptoethanol, alkylated with 4-vinylpyridine (Pierce) (Hawke and Yuan, 1987; Coligan et al., 1996), and purified by Vydac C18 RP-HPLC. The same gradient conditions were used as described above, except that 0.1% trifluoroacetic acid (TFA) (Pierce) was the counterion. The peak of interest was characterized by amino acid compositional and microsequence analysis. For bioassays studies, fractions containing L-EGF were not reduced and alkylated before Vydac C18 RP-HPLC. Approximately 175 pmol of L-EGF were obtained per gland.

Amino acid compositional analysis of L-EGF. Reagents for these analyses were purchased from PE/Applied Biosystems (Foster City, CA). Compositional analyses were performed using a PE/Applied Biosystems 420H Amino Acid Analyzer (Smith et al., 1991).

Edman microsequence analysis of L-EGF. For Edman degradations of L-EGF, formic acid-treated L-EGF, and Glu-C peptides, samples were applied to Biobrene Plus-treated glass fiber filters and subjected to automated N-terminal sequence analysis using a PE/Applied Biosystems Procise 494/HT Protein Sequencer. Pulsed liquid cycles were used for each analysis. Edman degradations of tryptic and pyroglutamate aminopeptidase (PGAP) peptides were performed using a PE/Applied Biosystems 477A Protein Sequencer connected to an on-line 120A Analyzer using sequencing protocols specified by, and reagents from, the instrument manufacturer. Data analyses were conducted by direct inspection of on-line analog chart recordings and compared with phenylthiohydantion-amino acid standards. Amino acid sequence analysis demonstrated that the peptide was blocked at the N terminus, and initial attempts to unblock the peptide were unsuccessful. Therefore, the glass fiber filter containing the reduced and alkylated peptide was placed back into a sequencer reaction cartridge, 15 μl of 1 M cyanogen bromide in 70% formic acid was added, and the filter containing the peptide was incubated in the sequencer for 2 hr at 70°C under argon. The filter was dried with argon and then sequenced using normal pulsed liquid chemistry. Microsequence analysis revealed a partial 34-residue sequence corresponding to residues 7–40. The partial sequence indicated a Pro residue at position 1 of the 34-residue sequence, suggesting that an Asp-Pro bond was cleaved by the formic acid treatment. This was confirmed by incubation with 70% formic acid alone at 21°C, followed by microsequence analysis, which generated an identical sequence for the first 10 cycles. Formic acid cleavage of the Asp⁶-Pro⁷ bond (and Asp⁴¹-Pro⁴² bond; see below) presumably generated a 35-residue peptide (residue 7–41). To extend these data, fraction 1 was digested with endoproteinase Glu-C, and the resulting peptides were purified by RP-HPLC. Microsequence analysis of one peptide (residues 30–43) extended the C-terminal sequence by an additional three residues. Two tryptic peptides representing the N terminus (residues 1–12) and middle region (residues 13–38) of reduced and alkylated L-EGF were also characterized. The N-terminal tryptic peptide (residues 1–12) was treated with PGAP, and Edman degradation determined the sequence of residues 2–11. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), electrospray mass spectrometry (ES-MS), and ES collisionally induced dissociation (ES-CID) determined the identity of residue 1 of the N-terminal peptide (residues 1–12). Edman degradation confirmed the sequence of the second tryptic peptide (residues 13–38).

Reduction and alkylation of L-EGF. L-EGF peptide was denatured, reduced, and cysteines alkylated with 4-vinylpyridine (Hawke and Yuan, 1987; Coligan et al., 1996). One microgram of peptide was dissolved in 17.5 μl of denaturing buffer (0.25 M Tris-HCl, 1.0 mM EDTA, and 6.0 M guanidine-HCl, pH 8.5), and 2.5 μl of 10% 2-mercaptoethanol (Aldrich, Milwaukee, WI) was added. After 2.5 hr at room temperature, 2 μl of neat 4-vinylpyridine (Aldrich) was added. After an additional 2 hr at room temperature, the solution was taken to dryness under vacuum with a SpeedVac concentrator (Savant Instruments, Inc., Hicksville, NY). The residue was dissolved in 25 μl of 1% TFA and desalted on a Hewlett Packard HP 1090 Liquid Chromatographic System using a 2.1 × 30 mm, 5 μm C18 column. The gradient solvents were aqueous 0.1% TFA (solvent A) and ACN/water/TFA, 80:20:0.01 (solvent B). After injection of the 25 μl sample and washing with 100% A, the column was developed with a linear gradient of 100% A to 25% A/75% B over 30 min at 0.175 ml/min.

The fraction containing the salt-free alkylated peptide was taken to dryness in the vacuum concentrator and stored dry at 4°C. The peptide was characterized by MALDI-TOF MS and ES-MS, and the reduced and alkylated peptide was found to have gained 630 Da, indicating that six cysteines had been alkylated (each pyridylethyl substitution adds 105 Da).

Reduction of L-EGF without alkylation. L-EGF peptide was denatured, reduced, and purified following the above procedure but without alkylation. The reduced peptide was characterized by MALDI-TOF MS and found to have gained 6 Da, indicating that three disulfide bonds had been reduced.

Endoproteinase Glu-C digestion of alkylated L-EGF peptide. An aliquot of reduced and alkylated L-EGF was digested with sequencing grade Glu-C (Boehringer Mannheim, Indianapolis, IN) by established procedures (Coligan et al., 1996). The resulting peptides were purified by C18 RP-HPLC (4.6 × 250 mm; Vydac) using a gradient of 0.1% TFA and 100% ACN containing 0.1% TFA and lyophilized, and the C-terminal Glu-C peptide (residues 30–43) was subjected to Edman microsequence analysis.

Trypsin digestion of the alkylated L-EGF peptide. One microgram of alkylated peptide was dissolved in 50 μl of 0.1 M ammonium bicarbonate, 0.025 μg of *N*-tosyl-phenylalanine chloromethyl ketone-treated trypsin (Worthington, Freehold, NJ) was added, and the digestion reaction was incubated at 37°C. The reaction was followed by MALDI-TOF MS and was complete after 2 hr. After the addition of 1 μl neat TFA, the reaction was fractionated by C18 RP-HPLC (2.1 × 30 mm column; PE/Applied Biosystems) using a 15 min linear gradient of 0–75% ACN containing 0.1% TFA at 0.3 ml/min and monitored at 214 nm. Alternatively, the reaction was fractionated by C18 RP-HPLC (2.1 × 150 mm column, 5 μm; Vydac) using a linear gradient of 0–60% ACN containing 0.1% TFA. Fractions were assayed for the desired alkylated peptide by MALDI-TOF MS. Two peptides were obtained representing the N terminus (residues 1–12) and middle (residues 13–38) portions of the L-EGF peptide. Appropriate fractions were pooled, taken to dryness in the vacuum concentrator, dissolved in 50% methanol/water containing 5% acetic acid, and further analyzed by ES-MS, ES-CID (residues 1–12), MALDI-TOF MS, and Edman degradation.

Pyroglutamate aminopeptidase digestion of alkylated N-terminal tryptic peptide (residues 1–12). PGAP (Boehringer Mannheim) digestion of the N-terminal tryptic peptide (residues 1–12) was performed in 100 mM potassium phosphate, 10 mM EDTA, 5 mM dithioerythritol, and 5% glycerol, pH 8.0. A ratio of 1 U of enzyme/mg of peptide was used, and the mixture was incubated at 37°C overnight. The resulting peptide from the above digests was desalted using a C18 ZipTip pipette tip (Millipore, Danvers, MA). Aliquots of the desalted sample were taken for MALDI-TOF MS and Edman degradation.

Mass spectrometry. ES-MS was performed in a Perkin-Elmer Sciex API III triple-quadrupole mass spectrometer (PE/Sciex, Thornhill, Ontario, Canada). When desalting was required, a liquid chromatograph (PE/Applied Biosystems model 140A) fitted with two 10 ml syringe cylinders was used in series with the API III mass spectrometer. Desalting was performed on an Aquapore Butyl C4 column (2.1 × 30 mm, 7 μm; PE/Applied Biosystems) with a gradient of 2–70% ACN containing 0.05% TFA in 20 min. MALDI-TOF MS was performed in a PE/PerSeptive Voyager-DE (PE/PerSeptive BioSystems, Framingham, MA) using α -cyano-4-hydroxycinnamic acid (Aldrich) for a matrix. The matrix-to-analyte ratio was ~1000:1 and the “dried drop technique” was used in spotting the sample plate.

Cloning of L-EGF. Based on the L-EGF amino acid sequence, two degenerate sense oligonucleotides, L-EGF-S1 (5'-agaagcttgngcnaa(t/c)tg(t/c)at(t/c/a)gcnta(t/c)ggg-3') and L-EGF-S2 (5'-agaagcttgcnaa(t/c/a)tg(t/c)ga(a/g)tg(t/c)ccntt(t/c)ggg-3'), were designed and used to PCR-amplify a L-EGF cDNA fragment from an albumen gland-specific λ ZAPII cDNA library. These primers were used in a nested PCR strategy in combination with primers directed against either the right λ arm (EV2, 5'-cgccagggtttccagtcacagc-3'; and T77, 5'-gcgtaaacgactactatagggcga-3') or the left λ arm (EV3, 5'-agcgatacaatttcacacagga-3'; and T33, 5'-ggcaattaaccctcaaaagg-3'). The L-EGF primers contained at the 5' end a recognition sequence for the restriction endonuclease *Hind*III. PCR was performed in a 50 μl solution containing ~10⁸ pfu of phenol-extracted and ethanol-precipitated library DNA, 200 μM each of the four deoxynucleotides, 50 pmol of L-EGF-s1, 12.5 pmol of EV2 or EV3, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM MgCl₂, and 0.5 U of *Taq* DNA polymerase (Goldstar). The PCR mixture was incubated in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk CT) for 42 cycles of 15 sec at 94°C, 30 sec at 46°C, and 3 min at 72°C. Of this PCR mixture, 1 μl was reamplified for 35 cycles under the same conditions, now using primers L-EGF-s2 in combination with T77 or T33. Amplified cDNA fragments were digested with *Eco*RI and *Hind*III, separated on agarose gel, cloned into pBluescript KS(+), and sequenced using an ABI 310 automated sequencer and the ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer Cetus). The cloned 300 bp L-EGF cDNA fragment was labeled with [α -³²P]dATP by random priming and used as a probe to screen ~100,000 recombinant clones of the albumen gland cDNA library. The clones were plated, absorbed to replica nylon filters (Boehringer Mannheim), prehybridized at 65°C for 16 hr, and hybridized at 65°C for 60 hr. The prehybridization and hybridization solutions contained 6× SSC (1× SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.4), 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA. The filters were washed two times in 1×

SSC/0.1% SDS at 65°C for 15 min, and hybridization signals were visualized using a GS-525 Molecular Imager System and Multi-Analyst 1.0 imaging software (both from Bio-Rad, Hercules, CA). Positive clones were isolated, and the insert-containing pBluescript phagemid (pBS-L-EGF) was rescued by *in vivo* excision and sequenced on both strands using vector-based primers and internal primers.

CNS organ culture. Adult *Lymnaea* CNS were dissected, washed 30 min in antibiotic saline (in mM: 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, and 5 HEPES, pH 7.9) containing 150 μg/ml gentamicin (Sigma, St. Louis, MO), and cultured for 24 hr in a modified Leibovitz L-15 defined medium (DM) (special order; Life Technologies, Rockville, MD) (Ridgway et al., 1991).

Northern blot analysis. Total RNA was isolated from albumen glands, acutely dissected adult CNS, and cultured adult CNS, using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987), and 15–20 μg of RNA was glyoxylated, fractionated on a 1.2% agarose gel, and transferred to a charged nylon membrane (Boehringer Mannheim). After prehybridization for 4 hr, the filter was hybridized at 65°C for 16 hr with a full-length [α -³²P]dATP-labeled *L-EGF* cDNA probe (specific activity, >10⁸ dpm/μg) and washed in 0.2× SSC, 0.1% SDS at 65°C for 30 min, and hybridization signals were visualized using a GS-525 Molecular Imager System and Multi-Analyst 1.0 imaging software.

Reverse transcription-PCR. Total RNA was isolated from cultured adult CNS, acutely dissected adult CNS (from animals 10 weeks of age; shell length of 20–25 mm), acutely dissected juvenile CNS (from animals 3–4 weeks of age; shell length of 8–10 mm), albumen glands as above, or egg masses at various developmental stages. In each case, 1–2 μg of RNA was reverse transcribed into oligo-dT₁₇-primed cDNA using 200 U of M-MLV reverse transcriptase (SuperScript II; Life Technologies). A 256 bp *L-EGF* cDNA fragment was amplified using primers L-EGF-S3 (5'-tggtgcgaattgtattcctatgg-3') and L-EGF-AS1 (5'-ctttacaagttcataacctatagtc-3'). As positive controls for the cDNA synthesis, *Lymnaea* fructose 1,6-biphosphate aldolase cDNA was amplified using primers Lald-6 (5'-gctgctgaagatgcccc-3'; sense) and Lald-4 (5'-tagctttagagctcgccat-3'; antisense) (Van Kesteren et al., 1998), or *Lymnaea* tubulin cDNA was amplified using primers Ltub-s1 (5'-agggcgaatccaacatgaac-3') and Ltub-as1 (5'-ccctcagctttctctcatc-3'). PCR reactions were performed as described above. Thirty cycles (egg mass cDNA) or forty cycles (all other templates) were performed for 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C. Twenty microliters of the PCR mixtures were separated on a 2% agarose gel and photographed.

Neuron isolation and outgrowth assays. Identified neurons were isolated from the CNS of adult *Lymnaea* (10–14 weeks of age; shell length of 20–25 mm). Individual neurons were isolated according to the procedure described by Wildering et al. (1998). Neurons were cultured in DM, DM plus L-EGF, DM plus hEGF, or *Lymnaea* brain conditioned medium (CM) (Ridgway et al., 1991). Purified and lyophilized L-EGF or recombinant hEGF (PreproTech EC, Rocky Hill, NJ; gift of Dr. S. Weiss, University of Calgary, Calgary, Canada) were dissolved in DM to a final concentration in the plate of 100 nM (unless otherwise indicated). The protein kinase inhibitors K-252a (Kamiya, Seattle, WA) and PD153035 (gift of Dr. M. Hollenberg, University of Calgary; or supplied by Calbiochem-Novabiochem, San Diego, CA) were dissolved in DMSO before addition to the culture medium. The final DMSO concentrations in the plate were 0.02 and 0.1% v/v for K-252a and PD153035, respectively. Control plates contained the same concentration of DMSO, and all reagents were added before the isolation procedure. Neurons were plated in specially prepared polystyrene culture dishes (Falcon #3001; Becton Dickinson Labware, Franklin Lakes, NJ) containing a poly-L-lysine-coated small-volume (150 μl) center well (Wildering et al., 1998). To minimize evaporation from the center well, the outer part of the dish was also filled with DM without allowing contact between the inner and outer compartment. A variety of neurons were isolated and cultured for the outgrowth assays: right Parietal A cluster (RPA) and Pedal A cluster (PeA) motoneurons, the peptidergic neuron right Parietal Dorsal 2 (RPD2) and peptidergic neurons from the right Parietal B cluster (RPB), the dopaminergic interneuron right Pedal Dorsal 1 (RPED1), and the identified neurons Visceral Dorsal 2 and 3 (VD2/3). Neurons were considered to have extended neurites if they showed at least one primary neurite of more than one soma diameter in length with an active growth cone. Neurite outgrowth was scored ~48 hr after isolation, and all sessions included a control. Every outgrowth result of each experiment was derived from multiple isolation sessions. The identity of the dishes was unknown to the individuals involved in the plating as well as the scoring of outgrowth. The dose–response data regarding L-EGF-induced neurite outgrowth in RPA neurons were fitted with a nonlinear regression routine (Leverberg–Marquardt method) to a four-parameter logistic equation (i.e., sigmoidal dose–response curve with variable slope). The dose–response data regarding the inhibition of L-EGF-induced neurite outgrowth by the protein kinase inhibitor K-252a was analyzed by means of a χ^2 test for trend. Effects of single doses of K-252a or PD153035 on CM-, L-EGF- and hEGF-induced neurite outgrowth were analyzed by a Fisher's exact test. The number of neurons (*n*) given in the text or figure legends reflects the total, pooled number of neurons plated under that condition (i.e., the sum of multiple isolation sessions). The percentages of neurite outgrowth are presented with their 95% confidence intervals (CI_{95%}) of ratios as calculated from the F

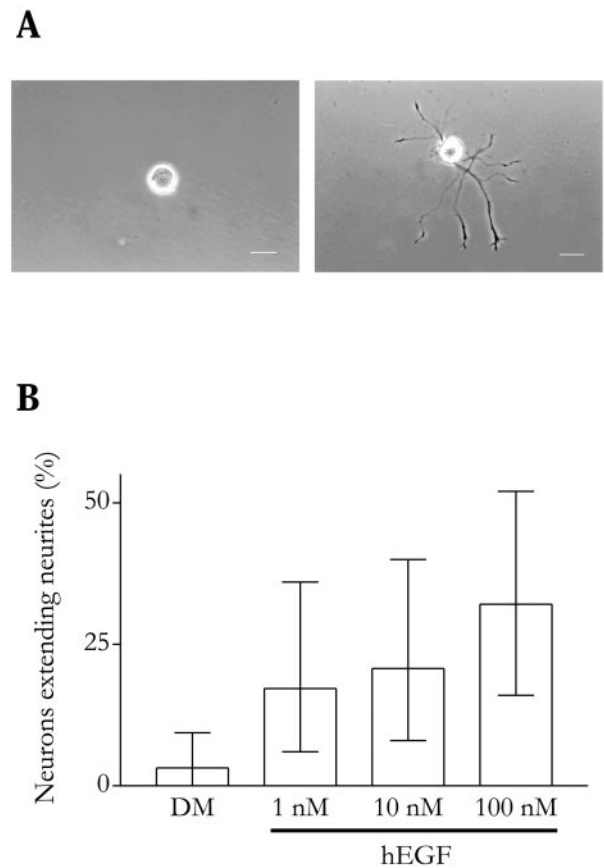


Figure 1. Human EGF induced neurite outgrowth in RPA motoneurons. *A*, Photomicrographs of RPA motoneurons cultured for 48 hr in either DM (left panel) or DM plus 100 nM hEGF (right panel). The addition of hEGF to the culture medium induced neurite outgrowth in this type of neuron. Scale bar, 50 μm. *B*, Different concentrations of hEGF induced neurite extension in RPA motoneurons after 48 hr in culture. The percentages are presented with their 95% confidence intervals.

distribution according to Sachs (1982). The two-sided critical value of statistical significance was $p < 0.05$.

RESULTS

Human EGF promotes neurite outgrowth from molluscan neurons

We tested the hypothesis that EGF can exert neurotrophic actions in adult *Lymnaea* neurons. To address this issue, we first assayed recombinant hEGF in our *in vitro* system. Previous studies showed that *Lymnaea* CM, murine 2.5 S nerve growth factor (NGF), and several extracellular matrix factors induce neurite outgrowth from RPA motoneurons in culture (Ridgway et al., 1991; Wildering et al., 1998). These neurons are readily isolated in large numbers and were used in our initial neurite outgrowth assays. RPA neurons were cultured in DM with the addition of 1 ($n = 29$), 10 ($n = 29$), or 100 ($n = 28$) nM hEGF (Fig. 1). In general, RPA neurons did not show neurite extension when cultured in DM ($n = 31$) (Fig. 1*A*, left; *B*; also see Fig. 5). Addition of hEGF to the culture medium induced neurite outgrowth in a percentage of the RPA motoneurons (Fig. 1*A*, right). Concentrations of 1, 10, and 100 nM hEGF induced outgrowth in 17, 21, and 32% of RPA neurons, respectively (Fig. 1*B*). These data show that hEGF can act as a neurotrophic factor on adult *Lymnaea* motoneurons, suggesting the existence of an endogenous *Lymnaea* EGF homolog.

Purification and characterization of *Lymnaea* EGF

Mammalian EGF is known to be involved in the control of cell proliferation and differentiation during development (Plata-Salamán, 1991; Gage et al., 1995; Weiss et al., 1996; Yamada et al.,

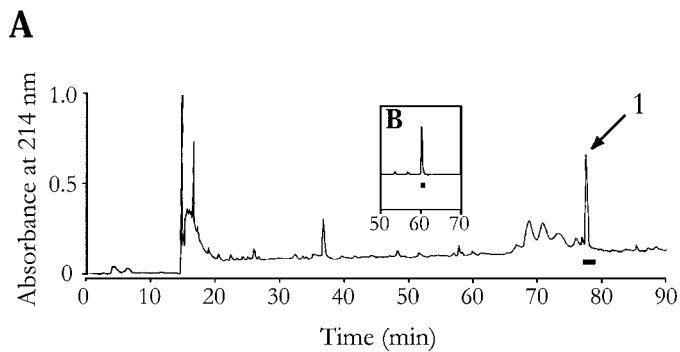


Figure 2. Purification of *Lymnaea* EGF. *A*, C18 RP-HPLC profile of an extract of 10 *Lymnaea* albumen glands that was fractionated using a gradient of 0.1% HFBA and ACN containing 0.1% HFBA. *B*, Fraction 1 in *A* was reduced, alkylated, and purified using a gradient of 0.1% TFA and ACN containing 0.1% TFA. The absorbance profile suggested that the peptide had been purified to homogeneity, which was confirmed by subsequent microsequence and mass spectrometric analyses (Table 2). Fractions containing L-EGF are indicated by solid bars.

1997; Temple and Alvarez-Buylla, 1999). Thus, a potential source of *Lymnaea* EGF are the glands that secrete fluids surrounding the fertilized oocytes before they are packaged into egg masses (e.g., albumen gland, oothecal gland; Joosse and Geraerts, 1983). We prepared protein extracts from albumen glands and separated these using RP-HPLC. A representative RP-HPLC elution is shown in Figure 2*A*. A major absorbance peak corresponding to fraction 1 was reduced and alkylated and then further purified by RP-HPLC using a different counterion (Fig. 2*B*). The amino acid composition analysis indicated that purified fraction 1 (Fig. 2*B*) contained a peptide that was 40–43 residues in length (Table 1). Microsequence analysis and mass spectrometry confirmed a length of 43 residues and demonstrated that this peptide is 35% identical (47% with conserved substitutions) with mouse EGF, including the EGF-like six cysteine residues and three C-terminal residues (Table 2, Fig. 3*A*). Therefore, we named this peptide L-EGF.

The observed mass of native L-EGF agrees with that calculated for the determined sequence of the native structure containing three disulfide bonds (Table 2), thus precluding any additional residues or additional post-translational modifications. A sequence comparison of L-EGF with other members of the EGF family shows a strict conservation of all six cysteine residues (positions 8, 13, 19, 28, 30, and 39), as well as the three characteristic EGF-like residues in the C-terminal region (Tyr³⁴, Gly³⁶, and Arg³⁸) (Fig. 3*A*) (Carpenter and Cohen, 1990). The spacing between the first-second and third-fourth cysteine residues differs from the vertebrate EGF family members but is identical to the EGF-like motifs that are found in molecules such as Notch and Delta (Fig. 3*A*).

Cloning and structural characteristics of L-EGF

PCR on independent fractions of an albumen gland-specific cDNA library with degenerate oligonucleotide primers in combination with vector-based primers resulted in the identification of a single 300 bp PCR product in each fraction. Nucleotide sequencing of this product revealed that it encoded the C-terminal region of L-EGF, as predicted by the amino acid sequence data, followed by a stop codon, a short 3' untranslated region of 216 bp, and a poly(A⁺) tail. Using this cDNA fragment as a probe, the full-length L-EGF cDNA was isolated from one of the library fractions and completely sequenced on both strands. The full-length L-EGF cDNA is 451 bp in length and contains a single open reading frame encoding a 64 amino acid protein, preceded by several in-frame stop codons, indicating that the open reading frame is complete at the 5' end (Fig. 3*B*).

The predicted L-EGF precursor protein consists of a 21 amino acid signal peptide, which is predicted to be cleaved after Ala⁻¹ (Von Heijne, 1983). The remaining EGF domain is 43 amino acids

Table 1. Amino acid composition of *Lymnaea* EGF

Amino acid	Residues/mol peptide	
	Composition	Sequence ^a
Aspartic acid	5.1 (5)	5
Glutamic acid	4.1 (4)	5
Serine	3.0 (3)	3
Glycine	5.6 (5–6)	6
Arginine	2.0 (2)	2
Threonine	1.0 (1)	1
Alanine	3.3 (3)	3
Proline	3.1 (3)	3
Tyrosine	3.3 (3)	3
Valine	0.8 (1)	1
Cysteine	5.5 (5–6)	6
Isoleucine	4.5 (4–5)	4
Phenylalanine	1.1 (1)	1
Total	40–43	43

^aResidues quantitated by HPLC.

in length and is identical to the amino acid sequence of the purified L-EGF peptide (compare Fig. 3*B* with Fig. 3*A* and Table 2). Comparison of the L-EGF precursor with other vertebrate and invertebrate members of the EGF family of growth factor precursors (e.g., hEGF, TGF α , amphiregulin, and spitz) and precursors with EGF motifs not belonging to the EGF family, such as Notch 3 and Delta 1, shows that the L-EGF precursor is surprisingly simple (Fig. 3*C*). Whereas these other precursors are large and complex multiple-domain proteins, most of which have a transmembrane domain, the L-EGF precursor contains only a signal peptide and a single copy of L-EGF. Conspicuously, the L-EGF precursor lacks a transmembrane domain, setting it apart from all other members of the EGF family. The presence of a signal peptide together with the absence of a transmembrane domain strongly suggest that L-EGF is a secreted peptide.

L-EGF expression *in ovo*, albumen gland, juvenile, and organ-cultured adult CNS

Northern blot hybridization using the full-length L-EGF cDNA as a probe revealed a very abundant transcript of ~450 bp in the albumen gland, but no L-EGF transcript was detected in the adult CNS either when acutely dissected or after organ culture for 24 hr (Fig. 4*A*). The size of the transcript detected in albumen glands corresponds well with the size of the cloned L-EGF cDNA, confirming that this clone is indeed full-length.

Because the L-EGF mRNA might be expressed in the CNS at levels below the detection limit of a Northern blot hybridization, we extended our expression studies to reverse transcription (RT)-PCR analysis of cDNA derived from albumen gland, juvenile CNS, acutely dissected adult CNS, and organ-cultured adult CNS (Fig. 4*B*) (see Material and Methods). In agreement with the Northern blot hybridization, L-EGF cDNA could be amplified from albumen glands but not from acutely dissected adult CNS. RT-PCR revealed, however, abundant expression of L-EGF mRNA in juvenile CNS and an upregulation of L-EGF mRNA levels in the organ-cultured adult CNS (Fig. 4*B*, top panel). As a positive template control, *Lymnaea* fructose 1,6-bisphosphate aldolase was amplified with consistency from all cDNA preparations (Fig. 4*B*, bottom panel).

The expression of L-EGF in the albumen gland and the developing juvenile CNS suggests a role for L-EGF during embryonic development. Therefore, we next examined the expression of L-EGF mRNA in egg masses at different times from day 3 after deposition [embryonic day 3 (E3)] to the day of hatching (E13). Expression of L-EGF mRNA could be detected in all stages of *in*

Table 2. Chemical and mass spectrometric sequence analysis of *Lymnaea stagnalis* EGF

Treatment	Fragment	Sequence	Calculated observations	Mass Da (average)	Procedures
None	(1–43)		4541.0 ^a	4540.8	ES-MS
Reduced	(1–43)		4547.0	4546.9	MALDI
Red. & Alk.	(1–43)		5177.9	5177.5	MALDI
Red. & Alk./70% formic	(7–41)	PXQIVRXSYGANXIAYGDTAIXEPPFGYSGIRXQ			Edman
Tr. of Red. & Alk.	(1–12)		1404.5	1404.7	MALDI
	(1–12)	<QGDGEDPXQIVR		1403.8	ES-MS
	(13–38)	XSYGANXIAYGDTAIXEPPFGYSGI	3155.7	3155.4	Edman, MALDI
PGAP of (1–12)	(2–12)	GDGEDPXQIV	1293.4	1293.6	Edman, MALDI
Red. & Alk./Glu-C	(30–43)	XPPFGYSGIRXQDPS			Edman
Sequence	(1–43)	<QGDGEDPCQIVRCSYGANCIAYGDTAICECPFGYSGIRXQDPS			
		1 10 20 30 40			

(Q, Pyrrolidone carboxylic acid; Red. & Alk., reduction and alkylation; X, pyridylethyl cysteine; Tr, trypsin; Glu-C, endoproteinase Glu-C.

^aCalculated as oxidized peptide; therefore, three disulfide bonds.

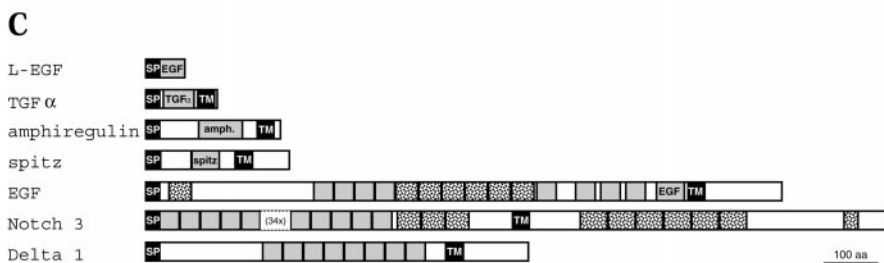
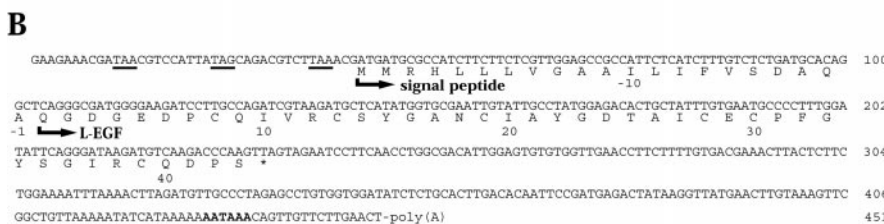
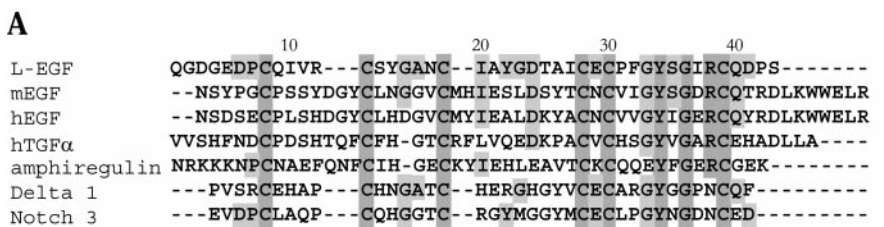


Figure 3. cDNA and deduced amino acid sequence of *L-EGF* and alignment of *L-EGF* with vertebrate and invertebrate EGF-related proteins and precursor molecules. *A*, Amino acid sequence alignment of *L-EGF* and EGF-like motifs from mouse EGF (*mEGF*) (GenBank accession number G1494001; Savage et al., 1972), hEGF (GenBank accession number NP 001954; Bell et al., 1986), human transforming growth factor α (*hTGF α*) (GenBank accession number AAA61158; Derynck et al., 1984), human amphiregulin (*amphiregulin*) (GenBank accession number NP 001648; Plowman et al., 1990), human Delta-1 (*Delta 1*) (GenBank accession number NP 005609; unpublished), and human Notch 3 (*Notch 3*) (GenBank accession number NP 000426; Lewis, 1996). Note the conservation of all six cysteine residues (positions 8, 13, 19, 28, 30, and 39), as well as the characteristic EGF-like residues in the C terminal, i.e., Tyr³⁴, Gly³⁶, and Arg³⁸. *B*, cDNA sequence and deduced amino acid sequence of *L-EGF*. Nucleotide positions are numbered at the right; amino acid positions are numbered below the amino acid sequence. The first amino acid of the signal sequence and of the *L-EGF* domain are indicated by arrows. In frame, stop codons in the 5' untranslated region are underlined; asterisk denotes the stop codon; a putative polyadenylation signal in the 3' untranslated region is in bold. *C*, Comparison of the *L-EGF* precursor molecule with other EGF-like precursor molecules, i.e., *TGF α* , amphiregulin, *Drosophila* spitz, hEGF, Notch 3, and Delta 1. Gray blocks indicate EGF-like motifs; dotted blocks indicate non-EGF-like repeat motifs. SP, Signal peptide; TM, transmembrane domain.

ovo development (Fig. 4C, top panel). As a positive template control, *Lymnaea* tubulin was amplified consistently from all cDNA preparations (Fig. 4C, bottom panel).

L-EGF induces neurite outgrowth

The upregulation of *L-EGF* mRNA in axotomized adult CNS suggests a role for L-EGF in injury repair. Thus, we examined whether L-EGF has neurotrophic activity in *Lymnaea*. To this end, RPA motoneurons were cultured in DM containing L-EGF at

concentrations ranging from 300 pM to 1 μ M ($n > 25$ for each concentration) (Fig. 5). As a control, RPA neurons were cultured in DM only ($n = 89$). Our experiments showed that L-EGF induced neurite outgrowth from RPA neurons in a dose-dependent manner. The trophic activity of the peptide extended over a three log range of concentrations (Fig. 5). The threshold dose was ~ 1 nM, whereas the response reached a plateau at ~ 1 μ M, with an EC₅₀ of 22 nM (CI_{95%} of 8.8–54 nM) (Fig. 5). In accord with previous studies, only a small percentage (<5%) of the neurons cultured under control

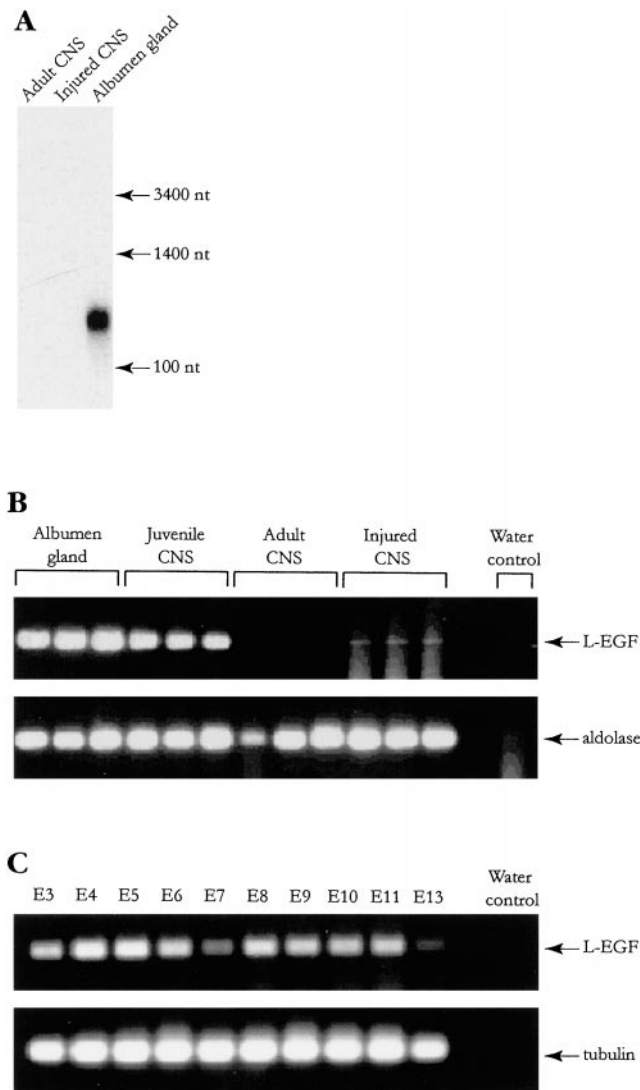


Figure 4. L-EGF expression in the developing and adult CNS. *A*, Northern blot hybridization of adult CNS, injured cultured adult CNS, and *Lymnaea* albumen gland show only transcript of a 450 nucleotides in the albumen gland. No L-EGF mRNA was found in the acutely dissected or injured cultured adult CNS. *B*, RT-PCR analysis of expression of L-EGF in the albumen gland, juvenile CNS, and injured cultured adult CNS. Acutely dissected adult CNS did not express L-EGF. Oligonucleotide primers against fructose 1,6-biphosphate aldolase generated a PCR product of the expected size in all tissues. PCR water controls are shown in the right lanes. *C*, RT-PCR analysis of expression of L-EGF in developing *Lymnaea* embryos. L-EGF mRNA expression was observed in egg masses from 3 d after egg mass deposition (E3) up to hatching (E13). Oligonucleotide primers against *Lymnaea* tubulin generated a PCR product of the expected size in all tissues. PCR water controls are shown in the right lanes.

conditions (i.e., DM alone) exhibited neurite outgrowth (Ridgway et al., 1991; Wildering et al., 1998) (Figs. 1*A,B*, 5).

Specificity of L-EGF-induced neurite outgrowth

We expanded our study to ask whether L-EGF could induce outgrowth from neurons other than RPA motoneurons. To this end, we isolated and cultured a number of different types of identified neurons: another group of motoneurons (PeA neurons), two types of neurosecretory cells (RPB neurons and the unique cell designated as RPD2), and an interneuron (RPeD1, also known as the giant dopamine cell). We also examined a pair of identical neurons, VD2/3, which are of unknown function but whose morphology suggests a visceral function (Magoski and Bulloch, 1997). As a positive control, neurons were cultured in CM, which is a potent stimulator of neurite outgrowth in all types of *Lymnaea*

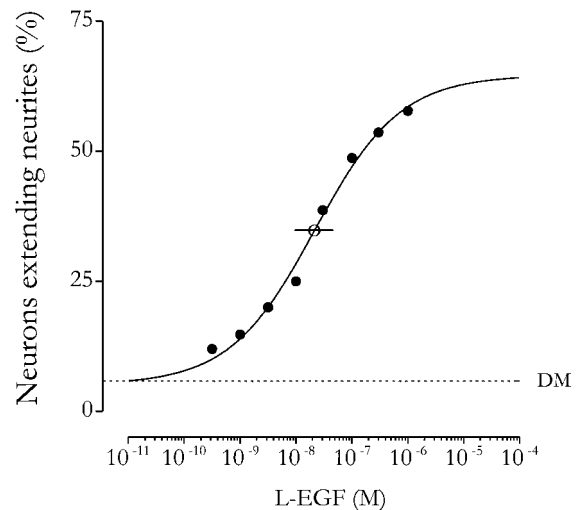


Figure 5. Dose–response curve of *Lymnaea* EGF. Neurite outgrowth in RPA neurons after 48 hr in culture. The lowest concentration of L-EGF that induced neurite outgrowth was ~1 nM, whereas maximal outgrowth was observed at 1 μ M. The EC₅₀ (open circle) as derived from the fitted model was 22 nM. The 95% confidence interval of the EC₅₀ was 8.8–54 nM (horizontal bar). The number of neurons cultured per concentration was >25. The total number of neurons cultured in L-EGF was >230.

neurons tested to date (Ridgway et al., 1991). As expected, all of the six types of neurons tested in CM exhibited robust outgrowth after 1–2 d of culture (Fig. 6). The morphology of cells that responded to CM (Fig. 6*A*) was similar to previous studies (Ridgway et al., 1991; Wildering et al., 1998). In contrast, L-EGF induced neurite outgrowth in only three of the six cell types tested, these being the RPA and PeA motoneurons and the visceral neurons VD2/3 (Fig. 6). The appearance of the sprouted cells was strikingly different in L-EGF compared with CM but was similar to that observed in hEGF; the neurites were relatively thick and short, i.e., the axons often extended only a few soma diameters in length, even after extended culture periods (Figs. 1*A*, 6*A*). Despite these different morphological phenotypes, the percentage of cells that responded to L-EGF was not different from CM (Fig. 6*B*). The three cell types that did not respond to L-EGF (i.e., RPB, RPD2, and RPeD1) always remained spherical without any sign of process outgrowth (Fig. 6*A*).

L-EGF signaling

Given that EGF is known to signal via a tyrosine kinase receptor in vertebrate cells (Yamada et al., 1997), we assayed the effects of kinase inhibitors on L-EGF- and hEGF-evoked responses (Fig. 7). RPA motoneurons were cultured in the presence of either L-EGF (100 nM) or hEGF (100 nM) with (experimental) or without (control) the addition of K-252a, a broad-spectrum protein kinase inhibitor (Fig. 7*A*) (Kase et al., 1986, 1987; Ruegg and Burgess, 1989; Knüsel and Hefti, 1992). A similar percentage of the neurons cultured in the presence of L-EGF (49%, $n = 47$) (Figs. 5, 6) or hEGF (32%, $n = 38$) (Fig. 1*B*) exhibited neurite outgrowth (Fig. 7*A*). In the presence of the inhibitor, L-EGF-induced neurite outgrowth was significantly reduced in a dose-dependent manner ($p < 0.0001$). The addition of 10 nM K-252a ($n = 39$) reduced the responsiveness of neurons to L-EGF. The addition of 100 nM K-252a completely blocked neurite outgrowth, and the neurons remained spherical in both L-EGF ($n = 38$) and hEGF ($n = 46$, $p < 0.0001$) (Fig. 7*A*).

Second, we tested the effects of PD153035, a tyrosine kinase inhibitor reported to be specific for the EGF receptor (EGFR) (Fry et al., 1994), on outgrowth induced by CM, 100 nM L-EGF, or 100 nM hEGF (Fig. 7*B*). Without inhibitor, 56, 43, and 38% of the neurons showed neurite outgrowth in CM ($n = 50$), L-EGF ($n = 84$), or hEGF ($n = 39$), respectively (Fig. 7*B*). Importantly, the addition of 100 nM PD153035 did not affect the outgrowth of RPA

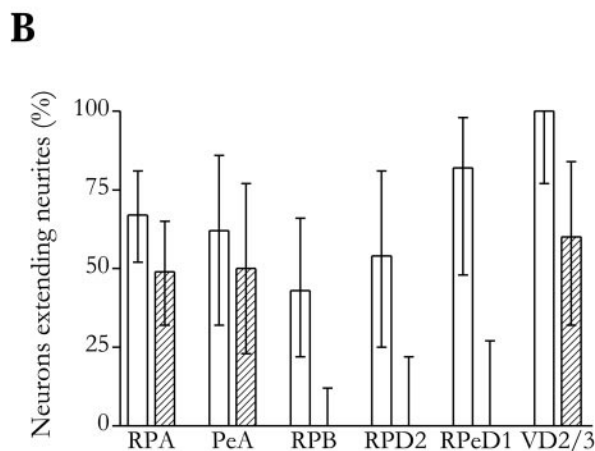
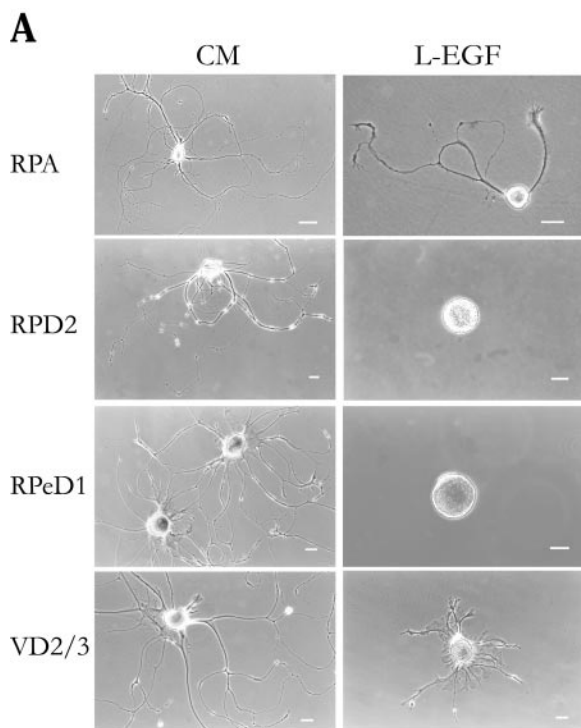


Figure 6. Specificity of L-EGF-induced neurite outgrowth. *A*, Photomicrographs of different types of neurons cultured for 48 hr in either CM (left panels) or DM with 100 nM L-EGF (right panels). RPA motoneurons, the neurosecretory neuron RPD2, the dopaminergic interneuron RPeD1, and the neurons VD2/3 developed multiple neurites when cultured in CM. In contrast, only RPA neurons and VD2/3 showed neurite outgrowth in the presence of 100 nM L-EGF. The neurons RPD2 and RPeD1 remained spherical. Scale bar, 50 μ m. *B*, The percentage of neurons developing neurites after culturing for 48 hr in either CM (open bars) or DM plus 100 nM L-EGF (hatched bars). The majority of RPA and PeA motoneurons ($n = 46$ and 13, respectively), the neurosecretory RPB neurons ($n = 21$) and RPD2 ($n = 13$), the interneuron RPeD1 ($n = 11$), and the identified VD2/3 ($n = 14$) showed neurite outgrowth in the presence of CM. When cultured in the presence of 100 nM L-EGF, only RPA ($n = 39$) and PeA ($n = 14$) motoneurons and VD2/3 ($n = 15$) extended neurites. The RPB neurons ($n = 30$) and the identified neurons RPD2 ($n = 15$) and RPeD1 ($n = 12$) always remained spherical in the presence of 100 nM L-EGF. The percentages are presented with their 95% confidence intervals.

neurons in CM ($n = 48$, $p = 0.69$) (Fig. 7*B*), indicating that the inhibitor had no nonspecific adverse effect on the cells. The presence of PD153035, however, significantly reduced the percentage of neurons extending neurites in L-EGF ($n = 90$, $p < 0.0005$) (Fig. 7*B*) and in hEGF ($n = 46$, $p < 0.025$) (Fig. 7*B*).

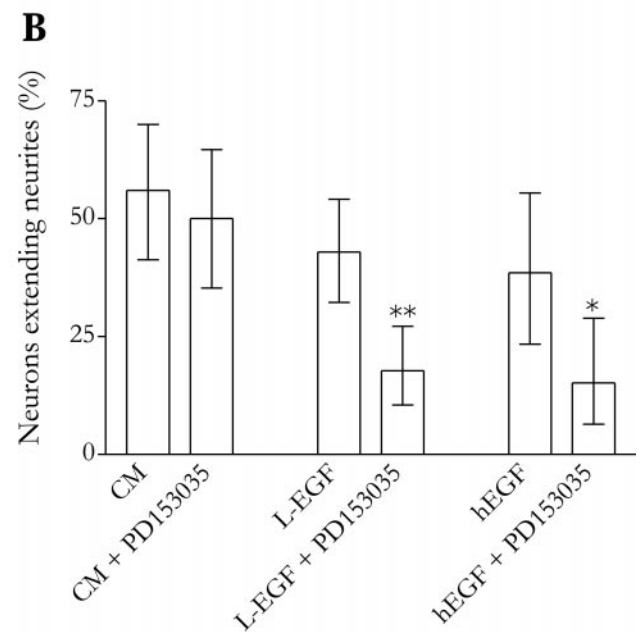
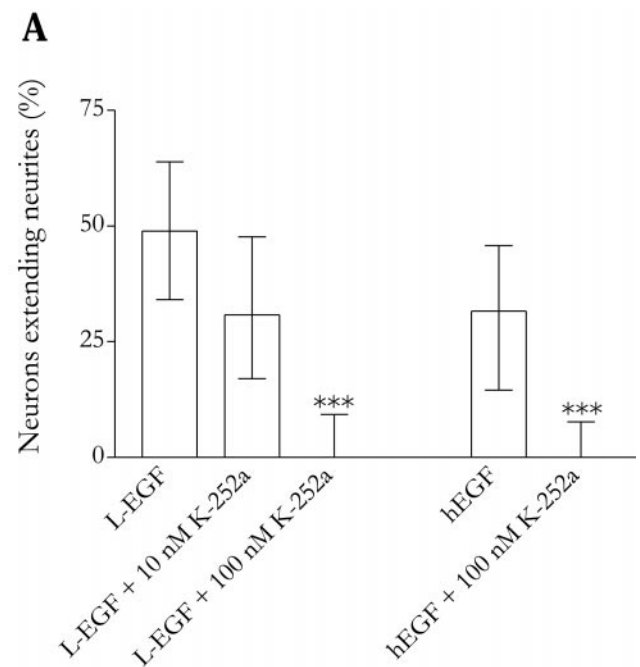


Figure 7. Receptor tyrosine kinase-mediated L-EGF signaling in RPA motoneurons. *A*, The protein kinase inhibitor K-252a significantly reduced L-EGF-induced neurite outgrowth in a dose-dependent manner (χ^2 test for trend) and hEGF-induced neurite outgrowth (Fisher's exact test). The percentages are presented with their 95% confidence intervals. *B*, The specific EGFR tyrosine kinase inhibitor PD153035 (100 nM) significantly reduced L-EGF- and hEGF-induced neurite outgrowth (Fisher's exact test). In contrast, CM-evoked neurite outgrowth was not affected by 100 nM PD153035 ($p = 0.69$; Fisher's exact test). The percentages are presented with their 95% confidence intervals. * $p < 0.025$; ** $p < 0.0005$; *** $p < 0.0001$.

DISCUSSION

In this study, we purified and cloned a *Lymnaea* epidermal growth factor homolog (L-EGF) and characterized its neurotrophic actions. Expression patterns suggest that L-EGF has a developmental role in embryos and in the juvenile CNS and is involved in injury response of the adult CNS. The latter notion is supported by our

finding that L-EGF evokes neurite outgrowth in some but not all identified adult neurons *in vitro*, suggesting differential neurotrophic actions.

Molecular structure of L-EGF

The L-EGF precursor is rather unique and surprisingly simple compared with the precursor of other EGF family members (e.g., EGF, TGF α , amphiregulin, spitz, and gurken) and other proteins containing an EGF motif (e.g., Notch and Delta). The L-EGF precursor molecule contains only a signal peptide and a single copy of L-EGF and lacks a transmembrane domain. Whereas mammalian EGF precursors require proteolytic processing to release EGF, we predict that L-EGF is synthesized as a nonmembrane-bound peptide and subsequently secreted.

Importantly, the cDNA clone of *L-EGF* predicts the same amino acid sequence as the purified peptide. Together, the purification and cloning data show that L-EGF has the typical arrangement of six cysteine residues and three characteristic amino acid residues in the C-terminal region (Tyr³⁴, Gly³⁶, and Arg³⁸) that are found in most EGF-related proteins (Carpenter and Cohen, 1990). The cysteine residues form three intramolecular disulfide bonds that are necessary for maintaining the biological activity of EGF (Taylor et al., 1972; Savage et al., 1973). Although our data do not support any assignment, the canonical EGF disulfide pattern would predict cystinyl bridges between positions 8 and 19, 13 and 28, and 30 and 39 (Table 2).

Whereas EGF itself has only been reported in mammals, EGF-like domains have been found in genes from a wide variety of organisms ranging from the protist *Plasmodium falciparum* (Kaslow et al., 1988) to invertebrate and vertebrate multizoa (Greenwald, 1990; Muskavitch and Hoffmann, 1990; Yamada et al., 1997). This suggests that the EGF motif has an ancient origin and is conserved in eukaryotes. Although the six cysteine residues are invariably present in all EGF-like domains, the spacing between the first two pairs of cysteine residues varies (Greenwald, 1990; Muskavitch and Hoffmann, 1990; Rutledge et al., 1992; Yamada et al., 1997; Van de Poll et al., 1998). L-EGF in this regard shares a higher similarity with the EGF-like repeats in proteins such as Notch and Delta than with EGF itself (Fig. 3A). It is suggested that size and composition of the cysteine loops are an important determinant of receptor binding by EGF (Van de Poll et al., 1998). However, although hEGF and L-EGF differ in their cysteine residue spacing, the fact that both peptides induced neurite outgrowth from *Lymnaea* neurons *in vitro* suggests that receptor-binding properties are conserved. This notion is supported by the observation that the neurotrophic effect of both L-EGF and hEGF could be inhibited by PD153035, a specific inhibitor of the EGFR (see Results and below, L-EGF signaling).

Developmental significance of L-EGF

Mammalian EGF is expressed in the developing and adult CNS (Rall et al., 1985; Schaudies et al., 1989; Lazar and Blum, 1992). In this study, we showed that L-EGF is synthesized and that its mRNA is expressed at high levels in the albumen gland of adult *Lymnaea*. The albumen gland is part of the female reproductive tract, and it secretes the perivitelline fluid that surrounds the fertilized oocytes (for review, see Joosse and Geraerts, 1983). Moreover, *L-EGF* mRNA is expressed throughout embryonic development. With regard to neurogenesis it is interesting to note that, at stage E3, cell groups from the ectodermal epithelium begin to differentiate into ganglion cells and the first elements of the nervous system appear (Verdonk, 1973; Croll and Voronezhskaya, 1996). Thus, L-EGF is already expressed during the earliest stages of neurogenesis. This notion is further supported by preliminary RP-HPLC and mass spectrometry data indicating that intact L-EGF is present in the egg masses, from either embryonic or maternal origin (G. T. Nagle, unpublished observations). Together, this strongly suggests a role for L-EGF during embryonic development of *Lymnaea*.

In addition to the albumen gland, high levels of *L-EGF* mRNA

are expressed in the CNS of animals ~3–4 weeks after hatching. In *Lymnaea*, development of the CNS is incomplete at hatching, and neuronal proliferation, circuit formation, and other developmental phenomena continue for 6–8 weeks after hatching (Croll and Chiasson, 1989; Smit et al., 1992; Serfozo et al., 1998; Croll et al., 1999). Thus, the expression of L-EGF in the CNS during extraovular development of the nervous system strongly suggests a role of L-EGF in maturation of the juvenile CNS. Together, our data suggest neurotrophic and proliferative roles for L-EGF both during the development of the *Lymnaea* embryo and in the juvenile CNS. This view complements studies in mammals that point to proliferative and neurotrophic roles for vertebrate EGF during CNS development (for review, see Plata-Salamán, 1991; Yamada et al., 1997). For example, mammalian EGF is thought to be involved in the regulation of cell proliferation and differentiation (for review, see Gage et al., 1995; Weiss et al., 1996; Temple and Alvarez-Buylla, 1999), as well as in the survival and process outgrowth of embryonic neurons (Morrison et al., 1987; Abe et al., 1991; Casper et al., 1991; Maiese et al., 1993).

Neurotrophic actions of L-EGF

Whereas some neurotrophic actions of vertebrate EGF in the developing CNS are described (see above), the neurotrophic function of EGF in the adult CNS is less well defined. In our study, mRNA expression of *L-EGF* was not detectable in the acutely isolated adult CNS. However, L-EGF is transcribed in the adult CNS after it was isolated (a procedure that axotomizes most neurons) and maintained for 24 hr in organ culture. Thus, our data imply that L-EGF is involved in injury response in the adult CNS. This is supported by our observation that L-EGF supports neurite outgrowth from adult *Lymnaea* neurons and the report that L-EGF, in a concentration range of 400–800 nM, can support soma–soma synapse formation of adult *Lymnaea* neurons *in vitro* (Hamakawa et al., 1999). The only other suggestion for such a role of EGF is by Toma et al. (1992) who showed spatiotemporal upregulation of the EGFR in Schwann cells and fibroblasts after transection of the sciatic nerve in adult rats. This observation indicates that EGF or one of the other EGFR ligands (e.g., TGF α , amphiregulin, betacellulin, ephregulin, and heparin-binding epidermal growth factor-like factor) (for review, see Riese and Stern, 1998), might enhance axonal regeneration.

We showed that L-EGF is able to differentially induce neurite outgrowth in *Lymnaea* neurons. The specificity of L-EGF *in vitro* appears to differ from both murine NGF and rat CNTF. Whereas L-EGF induced neurite outgrowth primarily in *Lymnaea* motoneurons, both NGF and CNTF induced neurite outgrowth in motoneurons and interneurons (Ridgway et al., 1991; Syed et al., 1996). CNTF also induced neurite outgrowth in a small percentage of neurosecretory cells. Together, these studies suggest that, at least *in vitro*, growth factor receptors are differentially expressed in *Lymnaea* neurons. Furthermore, it suggests that some adult molluscan neurons express multiple growth factor receptors, a feature shared with many mammalian neurons (Korsching, 1993).

L-EGF signaling

Given that EGF acts via a tyrosine kinase receptor in vertebrate cells, we assayed the effects of protein kinase inhibitors on L-EGF-evoked neurite outgrowth. We showed that L-EGF-induced neurite outgrowth of RPA neurons can be inhibited by a broad-spectrum protein kinase inhibitor, K-252a (Kase et al., 1986, 1987; Ruegg and Burgess, 1989; Knüsel and Hefti, 1992). This is consistent with findings by others showing that EGF-dependent actions on primary cultures of hippocampal and cerebellar neurons can be inhibited by K-252a (Abe et al., 1992).

An even more convincing indication for the involvement of an EGFR in L-EGF-evoked neurite outgrowth is the demonstration that PD153035, reported to be a specific inhibitor of the EGFR (Fry et al., 1994; Tropepe et al., 1999), inhibits L-EGF-induced neurite outgrowth in RPA motoneurons. In view of the specificity of the inhibitor, our observation that hEGF-induced neurite out-

growth can be inhibited by PD153035 indicates that L-EGF and hEGF act via the same receptor. Intriguingly, PD153035 did not affect CM-induced neurite outgrowth in RPA motoneurons. This could indicate that either L-EGF is not present in CM and other growth factors with overlapping activities fully substitute for the absence of L-EGF, or L-EGF is present but its neurotrophic actions are masked by other growth factors present in CM. Alternatively, it may indicate that CM does not contain functional L-EGF because of its degradation during production of CM (Ridgway et al., 1991). The hypothesis that L-EGF is present in CM, whether in active or inactive form, corresponds more closely with the observation that *L-EGF* mRNA expression is upregulated in organ-cultured CNS, i.e., the source of CM-contained growth factors. Moreover, this idea is supported by our observation that L-EGF-induced neurite outgrowth is morphologically different from CM-induced outgrowth in the same types of cells, as well as by another study implicating other types of growth factors in the control of neurite outgrowth in *Lymnaea* (Ridgway et al., 1991).

In conclusion, our data introduce L-EGF as the first secreted form of EGF in invertebrates and suggest the involvement of L-EGF in injury response. Furthermore, high levels of L-EGF mRNA expression *in ovo* as well as in juvenile CNS suggests a significant role for this peptide in development of the *Lymnaea* nervous system. Moreover, our results indicate that the neurotrophic actions of EGF are likely to have an ancient origin. In view of the apparent absence of neurotrophin family homologs in invertebrates, this raises the question as to whether the neurotrophic actions of EGF evolved preceding those of other families of neurotrophic factors.

REFERENCES

- Abe K, Takayanagi M, Saito H (1991) Basic fibroblast growth factor and epidermal growth factor promote survival of primary cultured cerebellar neurons from neonatal rats. *Jpn J Pharmacol* 56:113–116.
- Abe K, Takayanagi M, Saito H (1992) Neurotrophic effects of epidermal growth factor on cultured brain neurons are blocked by protein kinase inhibitors. *Jpn J Pharmacol* 59:259–261.
- Bell GI, Fong NM, Stempien MM, Wormsted MA, Caput D, Ku LL, Urdea MS, Rall LB, Sanchez-Pescador R (1986) Human epidermal growth factor precursor: cDNA sequence, expression in vitro and organization. *Nucleic Acid Res* 14:8427–8446.
- Carpenter G, Cohen S (1990) Epidermal growth factor. *J Biol Chem* 265:7709–7712.
- Casper D, Mytilineou C, Blum M (1991) EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. *J Neurosci Res* 30:372–381.
- Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT (1996) Current protocols in protein science. New York: Wiley.
- Croll RP, Chiasson BJ (1989) Postembryonic development of serotonin-like immunoreactivity in the central nervous system of the snail, *Lymnaea stagnalis*. *J Comp Neurol* 280:122–142.
- Croll RP, Voronezhskaya EE (1996) Early elements in gastropod neurogenesis. *Dev Biol* 173:344–347.
- Croll RP, Voronezhskaya EE, Hiripi L, Elekes K (1999) Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis*. II. Postembryonic development of central and peripheral cells. *J Comp Neurol* 404:297–309.
- Davis CG (1990) The many faces of epidermal growth factor repeats. *New Biol* 2:410–419.
- Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV (1984) Human transforming growth factor- α : precursor structure and expression in *E. coli*. *Cell* 38:287–297.
- Engel J (1989) EGF-like domains in extracellular matrix proteins: localized signals for growth and differentiation? *FEBS Lett* 251:1–7.
- Fainzilber M, Smit AB, Syed NI, Wildering WC, Hermann PM, Van der Schors RC, Jiménez C, Li KW, Van Minnen J, Bulloch AGM, Ibáñez CF, Geraerts WPM (1996) CRNF, a molluscan neurotrophic factor that interacts with the p75 neurotrophin receptor. *Science* 274:1540–1543.
- Fry DW, Kraker AJ, McMichael A, Ambroso LA, Nelson JM, Leopold WR, Connors RW, Bridges AJ (1994) A specific inhibitor of the epidermal growth factor tyrosine kinase. *Science* 265:1093–1095.
- Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci USA* 95:11879–11883.
- Greenwald I (1990) Genetic and molecular analysis of EGF-related genes in *Caenorhabditis elegans*. *Mol Reprod Dev* 27:73–79.
- Hamakawa T, Woodin MA, Bjorgum MC, Painter SD, Takasaki M, Lukowiak K, Nagle GT, Syed NI (1999) Excitatory synaptogenesis between identified *Lymnaea* neurons requires extrinsic trophic factors and is mediated by receptor tyrosine kinases. *J Neurosci* 19:9306–9312.
- Hawke DH, Yuan PM (1987) S-pyridylethylation of cysteine residues. Applied Biosystems User Bulletin #28, Foster City, CA.
- Joosse J, Geraerts WPM (1983) Endocrinology. In: The mollusca, Vol 4, Physiology, Pt 1 (Saleuddin ASM, Wilbur KM, eds). New York: Academic.
- Kase H, Kazuyuki I, Matsuda Y (1986) K-252a, a potent inhibitor of protein kinase C from microbial origin. *J Antibiot (Tokyo)* 34:1059–1065.
- Kase H, Iwashi I, Nakanishi S, Matsuda Y, Yamada K, Takashi M, Murakata C, Sato A, Kaneko M (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent kinases. *Biochem Biophys Res Commun* 142:436–440.
- Kaslow DC, Quakyi IA, Syn C, Raum MG, Keister DB, Coligan JE, McCutchan TF, Miller LH (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. *Nature* 333:74–76.
- Knüsel B, Hefti F (1992) K-252 compounds: modulators of neurotrophin signal transduction. *J Neurochem* 59:1987–1996.
- Korsching S (1993) The neurotrophic factor concept: a reexamination. *J Neurosci* 13:2739–2748.
- Lazar L, Blum M (1992) Regional distribution and developmental expression of epidermal growth factor and transforming growth factor- α mRNA in mouse brain by a quantitative nuclease protection assay. *J Neurosci* 12:1688–1697.
- Lewis J (1996) Neurogenic genes and vertebrate neurogenesis. *Curr Opin Neurobiol* 6:3–10.
- Magoski NS, Bulloch AGM (1997) Localization, physiology, and modulation of a molluscan dopaminergic synapse. *J Neurobiol* 33:247–264.
- Maiese K, Boniece I, DeMeo D, Wagner JA (1993) Peptide growth factors protect against ischemia in culture by preventing nitric oxide toxicity. *J Neurosci* 13:3034–3040.
- Morrison RS, Kornblum HI, Leslie FM, Bradshaw RA (1987) Trophic stimulation of cultured neurons from neonatal rat brain by epidermal growth factor. *Science* 238:72–75.
- Muskavitch MAT, Hoffmann FM (1990) Homologs of vertebrate growth factors in *Drosophila melanogaster* and other invertebrates. *Curr Top Dev Biol* 24:289–328.
- Neuman-Silberberg FS, Schüpbach T (1993) The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGF α -like protein. *Cell* 75:165–174.
- Plata-Salamán CR (1991) Epidermal growth factor and the nervous system. *Peptides* 12:653–663.
- Plowman GD, Green JM, McDonald VL, Neubauer MG, Distecher CM, Todaro GJ, Shoyab M (1990) The *amphiregulin* gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. *Mol Cell Biol* 10:1969–1981.
- Rall LB, Scott J, Bell GI, Crawford RJ, Penschow JD, Niall HD, Coghlan JP (1985) Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues. *Nature* 313:228–231.
- Ridgway RL, Syed NI, Lukowiak K, Bulloch AGM (1991) Nerve growth factor (NGF) induces sprouting of specific neurons of the snail, *Lymnaea stagnalis*. *J Neurobiol* 22:377–390.
- Riesse II DJ, Stern DF (1998) Specificity within the EGF family/ErbB receptor family signaling network. *BioEssays* 20:41–48.
- Roovers E, Vincent ME, Van Kesteren E, Geraerts WP, Planta RJ, Vreugdenhil E, Van Heerikhuizen H (1995) Characterization of a putative molluscan insulin-related peptide receptor. *Gene* 162:181–188.
- Ruegg UT, Burgess GM (1989) Staurosporine, K-252a and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol Sci* 10:218–220.
- Rutledge BJ, Zhang K, Bier E, Jan YN, Perrimon N (1992) The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. *Genes Dev* 6:1503–1517.
- Sachs L (1982) Applied statistics. A handbook of techniques, Ed 2. New York: Springer.
- Savage Jr CR, Inagami T, Cohen S (1972) The primary structure of epidermal growth factor. *J Biol Chem* 247:7612–7621.
- Savage Jr CR, Hash JH, Cohen S (1973) Epidermal growth factor. Location of disulfide bonds. *J Biol Chem* 248:7669–7672.
- Schaudies RP, Christian EL, Savage Jr CR (1989) Epidermal growth factor immunoreactivity material in the rat brain. Localization and identification of multiple species. *J Biol Chem* 264:10447–10450.
- Serfozo Z, Elekes K, Varga V (1998) NADPH-diaphorase activity in the nervous system of the embryonic and juvenile pond snail, *Lymnaea stagnalis*. *Cell Tissue Res* 292:579–586.
- Smit AB, Jiménez CR, Dirks RW, Croll RP, Geraerts WPM (1992) Characterization of a cDNA clone encoding multiple copies of the neuropeptide APGWamide in the mollusc *Lymnaea stagnalis*. *J Neurosci* 12:1709–1715.
- Smith JS, Miller BT, Knock SL, Kurosky A (1991) Biotinylated peptides/proteins. I. Determination of stoichiometry of derivation. *Anal Biochem* 197:247–253.
- Syed N, Richardson P, Bulloch AGM (1996) Ciliary neurotrophic factor,

- unlike nerve growth factor, supports neurite outgrowth but not synapse formation by adult *Lymnaea* neurons. *J Neurobiol* 29:293–303.
- Taylor JM, Mitchell WM, Cohen S (1972) Epidermal growth factor. Physical and chemical properties. *J Biol Chem* 247:5928–5934.
- Temple S, Alvarez-Buylla A (1999) Stem cells in the adult mammalian central nervous system. *Curr Opin Neurobiol* 9:135–141.
- Toma JG, Pareek S, Barker P, Mathew TC, Murphy RA, Acheson A, Miller FD (1992) Spatiotemporal increases in epidermal growth factor receptors following peripheral nerve injury. *J Neurosci* 12:2504–2515.
- Tropepe V, Sibilio M, Ciruna BG, Rossant J, Wagner EF, Van der Kooy D (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol* 208:166–188.
- Van de Poll MLM, Van Vugt MJH, Lenferink AEG, Van Zoelen EJJ (1998) Identification of the minimal requirements for binding to the human epidermal growth factor (EGF) receptor using chimeras of human EGF and an EGF repeat of *Drosophila* Notch. *J Biol Chem* 273:16075–16081.
- Van Kesteren RE, Fainzilber M, Hauser G, Van Minnen J, Vreugdenhil E, Smit AB, Ibáñez CI, Geraerts WPM, Bulloch AGM (1998) Early evolutionary origin of the neurotrophin receptor family. *EMBO J* 17:2534–2542.
- Verdonk NH (1973) Gene expression in early development of *Lymnaea stagnalis*. *Dev Biol* 35:29–35.
- Von Heijne G (1983) A new method for predicting signal sequence cleavage sites. *Nucleic Acid Res* 11:4683–4690.
- Weiss S, Reynolds BA, Vescovi AL, Morshead C, Craig CG, Van der Kooy D (1996) Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci* 19:387–393.
- Wildering WC, Lodder JC, Kits KS, Bulloch AGM (1995) Nerve growth factor (NGF) acutely enhances high-voltage activated Ca^{2+} currents in adult molluscan neurons. *J Neurophysiol* 74:2778–2781.
- Wildering WC, Hermann PM, Bulloch AGM (1998) Neurite outgrowth, RGD-dependent, and RGD-independent adhesion of identified molluscan motoneurons on selected substrates. *J Neurobiol* 35:37–52.
- Yamada M, Ikeuchi T, Hatanaka H (1997) The neurotrophic action and signalling of epidermal growth factor. *Prog Neurobiol* 51:19–37.