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RGD-dependent mechanisms in the endoneurial phagocyte response and axonal regeneration in the nervous system of the snail *Lymnaea stagnalis*

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SUMMARY

Activation of phagocytic cells in the injury zone is a crucial step in the regeneration of peripheral axons. Many aspects of the mechanisms underlying the recruitment of active phagocytes remain, however, unclear. Notably, our understanding of the interactions between injury, extracellular matrix (ECM) degradation and phagocyte activation is limited. Most animal cell types, phagocytes included, interact with proteins of the ECM through one or more members of the integrin family, transmembrane cell adhesion receptors that typically bind their ligands through short linear amino acid sequences. This study focused on the role of one of the most common of such integrin recognition sequences, the Arg-Gly-Asp (RGD) motif in the recruitment and activation of endoneurial phagocytes in the injury response of the nervous system of the pond snail *Lymnaea stagnalis*. Like the mammalian nervous system, the *Lymnaea* nervous system responds to injury with recruitment and activation of endoneurial phagocytes (i.e. phagocytes residing in *Lymnaea*'s nerves), a process involving substantial changes in the morphology, motility and adhesion status of these cells. Using synthetic water-soluble RGD-peptides, we investigated the relevance of RGD-dependent mechanisms in the activation of endoneurial phagocytes and injury response of the organ-cultured nervous system of *Lymnaea.* Our results show that RGD-peptides modulate various aspects of phagocyte activation (i.e. spreading response, particle engulfment, oxidative burst) *in vitro* and *in situ* and significantly affect nerve regeneration in this model system. Surprisingly, while linear RGD-analogues suppressed both phagocyte activation and axonal regeneration, a circularized RGD-peptide analogue modulated these parameters in a concentration-dependent, biphasic manner. Collectively, these results emphasize the significance of RGD-dependent mechanisms in the regenerative response of the *Lymnaea* nervous system and implicate regulation of the cellular immune response as one of the factors in this context.

Key words: integrins, phagocytosis, spreading response, oxidative burst, axonal regeneration.

INTRODUCTION

The ability to guard against the harmful effects of invading pathogens, foreign objects and degenerating cellular or acellular constituents of the body appeared very early in the evolution of multizoa. Phagocytes, a variety of cell types capable of recognizing and internalizing foreign particulate objects, are a key component of this innate host-defence system. This study focused on the role of phagocytes in nerve injury repair. In addition to their role in host defence, phagocytes are indispensable in tissue repair processes, including the regeneration of the nervous system of vertebrates and invertebrates (Bale et al., 2001; Battisti et al., 1995; Brown et al., 1997; Clatworthy and Grose, 1999; Frostick et al., 1998; Fu and Gordon, 1997; Moffett, 1995; Moffett, 1996; Shen et al., 2000; Zochodne, 2000). In mammals, peripheral nervous system regeneration is preceded by a characteristic degenerative process during which distal elements of the injured neuronal processes are absorbed and extensive remodelling of the extracellular environment in the injured nerve occurs. Recruitment and activation of various populations of phagocytes, including Schwann cells assuming a phagocytic phenotype and resident and haematogenous macrophages, are essential to this process, which is known as Wallerian degeneration (Mueller et al., 2001; Mueller et al., 2003; Shen et al., 2000; Stoll et al., 1989). Although less exhaustively investigated, several studies indicate that repair of the invertebrate nervous system also critically depends on the recruitment of active phagocytes to the injury zone (Bale et al., 2001; Hermann et al., 2005).

In the absence of injury or inflammatory challenges, phagocytes typically maintain a dormant state. Although numerous factors have been identified that contribute to phagocyte activation and recruitment to the injury site, many unanswered questions remain (Bruck, 1997; Rothshenker, 2003; Zeev-Brann et al., 1998). In particular, our understanding of the role of components of the extracellular matrix (ECM) in the regulation of phagocyte responses to injury is limited. The ECM is a complex three-dimensional structure containing a variety of glycoproteins, proteoglycans and other components. Cells, including phagocytic cell types, interact with many of these ECM components through a variety of transmembrane cell adhesion receptors. One of the main classes of these receptors is the integrins, a family of evolutionarily conserved, obligate heterodimeric transmembrane receptors (Burke, 1999; Davids et al., 1999; Giancotti and Ruoslathi, 1999; Hughes, 2001; Hynes, 1992; Plows et al., 2006; Wildering et al., 1998). Integrins typically interact with their ligands, which include ECM glycoproteins such as fibronectins, laminins and collagens, through short linear amino acid motifs. One

of the most common of these so-called integrin recognition motifs is the three amino acid sequence Arg-Gly-Asp, also known as the RGD-motif. The RGD-motif acts as a binding site for a sub-class of the integrin family, including the most common fibronectin receptors (Hynes, 1992; Ruoslahti, 1996; Ruoslahti and Pierschbacher, 1987). The current study focused on the significance of the RGD-motif in axonal regeneration in the snail *Lymnaea stagnalis*, with particular emphasis on the regulation of the injury response of phagocytes residing in the animal's nerves (i.e. endoneurial phagocytes). Although RGD-dependent mechanisms have been implicated in the regulation of the activity of various circulating vertebrate and invertebrate phagocytic cell types outside the nervous system (Ballarin and Burighel, 2006; Ballarin et al., 2002; Berton and Lowell, 1999; Davids and Yoshino, 1998; Gresham et al., 1989; Hanayama et al., 2002; Pech and Strand, 1995; Plows et al., 2006), comparatively little is known about their role in the regulation of immune effector cells residing in the nervous system. Yet, recent studies suggest that they may serve a similar function in the control of microglial activity in inflammatory conditions of the rodent nervous system (Milner and Campbell, 2003; Milner et al., 2007).

The current study aimed to examine the significance of RGD-motifs in the regenerative response of the nervous system of *Lymnaea* using a model system we recently developed (Hermann et al., 2000; Hermann et al., 2005; Wildering et al., 2001). Because of their comparatively superior repair capacity, invertebrates like *Lymnaea* have enjoyed wide usage as model systems in the study of (functional) axonal repair (Hermann et al., 2000; Hermann et al., 2005; Koert et al., 2001; Moffett, 1995; Moffett, 1996; Wildering et al., 2001). Yet, compared with the regenerative process in mammals, we know very little about the non-neuronal factors contributing to successful regeneration in these model systems. Recently, we identified a class of phagocytic cells residing in *Lymnaea*'s nerves as a key player in the regenerative response of this model system (Hermann et al., 2005). Here, we took a pharmacological approach based on the use of synthetic water-soluble RGD-peptides to investigate the relevance of RGD-dependent mechanisms in the activation of endoneurial phagocytes and the injury response of the nervous system of *Lymnaea*. In a previous study, we demonstrated this strategy to be effective in selectively antagonizing adhesive interactions of *Lymnaea* cells with substrates known to display the RGD-motif (Wildering et al., 1998).

MATERIALS AND METHODS

Animals, CNS isolation and nerve crush procedure

Adult specimens (4–6 months of age) of laboratory-reared *Lymnaea stagnalis* L. were used in all experiments. The snails were fed *ad libitum* with lettuce and Trout Chow (Developer Trout Ration 5D06, Purina, St Louis, MO, USA). All snails were kept under constant environmental conditions (12 h:12 h light:dark, water temperature 20–21°C) in 100 l tanks containing aerated artificial pond water (0.26 g l^{-1} Instant Ocean salts, Aquarium Systems, Mentor, OH, USA, in reverse osmosis/deionized water). Anaesthesia and aseptic dissection of the CNS, including the buccal ganglia, were performed as described previously (Hermann et al., 2000; Hermann et al., 2005; Wildering et al., 2001). All nerves were cut as far distally as possible without inflicting damage to the proximal nerves and the rest of the nervous system. The lengths of the peripheral nerves that remained attached to the ganglia varied depending on the location of their target. The lengths of the visceral, right internal and external parietal nerves that were used for the various experiments described below were between 8 and 12 mm. After dissection, CNS were washed two times for 5 min in antibiotic Hepes-buffered saline (ABS) composed of (mmol l^{-1}): 51.3 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$ and 5 Hepes (pH 7.9), with 150 μg ml^{-1} gentamycin (Sigma Chemical Co., St Louis, MO, USA). Both right internal parietal (RIP) and right external parietal nerves were crushed as described previously (Hermann et al., 2000; Hermann et al., 2005; Wildering et al., 2001). This technique severs the axon fibres in the nerve, but preserves the nerve's perineurium and gross anatomy. Isolated CNS were pinned on small silicone rubber pads (~36 mm^2; RTV no. 616, General Electric, Waterford, NY, USA) with 0.1 mm insect pins and cultured at room temperature in a darkened chamber in ABS (2 CNS ml^{-1}) with or without the addition of the synthetic RGD-peptides Gly-Arg-Gly-Asp-Ser (G**RGD**S) or cyclic Gly-Arg-Gly-Asp-Ser-Pro-Ala (cG**RGD**SPA) or the 'control' peptide Ser-Asp-Gly-Arg-Gly (SDGRG). Note that the control peptide SDGRG contains the same amino acids as G**RGD**S but in reversed sequence. After 48 h, an incubation period shown previously to yield optimal results (Wildering et al., 2001), axonal regeneration was examined by retrograde nickel-lysine staining of the RIP nerve as described before (Hermann et al., 2000; Hermann et al., 2005; Wildering et al., 2001). The nickel-lysine was applied at the cut end of the nerve trunk, i.e. distal to the crush site (see Hermann et al., 2000). The extent of regeneration (i.e. re-growth of axons across the crush site) was determined by counting the number of retrograde-labelled cell bodies of right parietal A (RPA) group of motor neurons, known to project into the RIP nerve (see also Wildering et al., 2001). Analysis of the back-filled preparations was done in duplicate, in a blinded fashion by two individuals who were unaware of the treatment status of the samples.

Endoneurial phagocyte isolation

Endoneurial phagocytes were isolated from *Lymnaea*'s visceral, right internal and external parietal nerves using a procedure described previously (Hermann et al., 2005). Nerves were obtained from at least 15 CNS per experiment. Care was taken not to isolate haemocytes present in the blood vessel. Four samples of 100 μl each of the resulting cell suspension were added to 125 μl each of ABS only, ABS + SDGRG, ABS + G**RGD**S or ABS + cG**RGD**SPA. These suspensions were subsequently plated on poly-L-lysine (Sigma)-coated coverslips placed in 35 mm dishes containing 3 ml of ABS with compositions identical to the media in which the cells were suspended (i.e. ABS only, SDGRG, G**RGD**S or cG**RGD**SPA). The cells were cultured in the dark at room temperature for 48 h in the presence of a 1:7500 diluted suspension of monodispersed uncoated- or human plasma fibronectin coated-polystyrene carboxylated Fluoresbrite YG microspheres (0.75 μm; Polysciences, Warrington, PA, USA; see 'Covalent coupling of protein' below).

Morphology and phagocytosis assays

The morphology of the cells and phagocytic activity were examined 48 h after cell isolation. In order to examine their morphology, the cells were washed in 70% and 95% ethanol for 5 min each, stained for 1 min with fast green FCF (0.03% in 95% ethanol; Sigma), washed in 95% ethanol for 10 s and kept in absolute ethanol. Morphology and phagocytic activity were assessed by taking 10–14 differential interference contrast (DIC) and fluorescence images (excitation 480 nm/30 nm, emission 535 nm/40 nm) of 10–14 randomly selected areas in each culture dish at ×100 magnification. At ×100, individual microspheres were readily recognizable and

microsphere uptake was quantified at this magnification. Images were acquired with an intensified CCD camera (Stanford Photonics XR-GENIII+ Ultra-blue; Solamere Technology Group, Salt Lake City, UT, USA) coupled to an inverted microscope (Axiovert 100 TV; Zeiss, Oberkochen, Germany) and interfaced with a computer through an 8-bit frame grabber board (DT3155; Data Translation, Marlboro, MA, USA). Overlay pictures were used to confirm whether a cell had engulfed microspheres or if a particle aggregation lay outside a cell.

Covalent coupling of protein

Human plasma fibronectin was covalently coupled to the carboxylated polystyrene Fluoresbrite YG microspheres using the carbodiimide method as described by the supplier (Polysciences Inc., technical data sheet 238C). That is, after washing 0.5 ml of 2.5% microspheres twice in 0.1 mol l^{-1} carbonate buffer (pH 9.6) and three times in 0.2 mol l^{-1} phosphate buffer (pH 4.5), the microspheres were resuspended in a 50/50 phosphate buffer (pH 4.5)/2% carbodiimide solution (in phosphate buffer) and gently mixed for 4 h at room temperature. Subsequently, the microspheres were rinsed three times with the phosphate buffer. The microspheres were then resuspended in 0.2 mol l^{-1} borate buffer (pH 8.5), and human plasma fibronectin (Boehringer Mannheim, Indianapolis, IN, USA) was added to the microspheres to a final concentration of ~0.4 mg ml^{-1}. The microsphere suspension was then gently agitated overnight at room temperature. After centrifugation and removal of the supernatant, the microspheres were resuspended in 1.2 ml 0.2 mol l^{-1} borate buffer (pH 8.5) with the addition of 50 μl of 0.25 mol l^{-1} ethanolamine (2-aminoethanol; Sigma) and mixed gently for 30 min. Subsequently, non-specific binding sites were blocked by suspending the microspheres twice in 10 mg ml^{-1} BSA in borate buffer for 30 min at room temperature. The microspheres were resuspended and stored at 4°C in 0.5 ml storage buffer (1×PBS pH 7.4, 10 mg ml^{-1} BSA, 5% glycerol and 0.1% NaN_3). Spectrophotometric analysis of the supernatant remaining after termination of the coupling reaction revealed no detectable traces of protein, indicating that most fibronectin had bound to the microspheres.

Reactive oxygen species measurements

The production of reactive oxygen species (ROS) in the injured nerve was assessed by means of labelling with the redox-sensitive fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Burlington, ON, Canada). This chloromethyl derivative allows for covalent binding to intracellular components to enhance intracellular retention of the probe (see Molecular Probes manual 'Reactive oxygen species (ROS) detection reagents'; www.probes.invitrogen.com/media/pis/mp36103.pdf). To this end, CNS were isolated and RPA nerves were crushed as described above and cultured for 1 h (acute) or 48 h in ABS alone or ABS + cG**RGD**SPA. Subsequently, the CNS were incubated for 45 min in a solution of CM-H_2DCFDA (2 μmol l^{-1}) plus Pluronic F127 (0.008%; Molecular Probes) and washed with ABS only for 15 min. The CNS were placed on glass slides and the presence of ROS was immediately assessed by taking phase contrast and fluorescence images (excitation 480 nm/30 nm, emission 535 nm/40 nm) of the injured nerves using a Retiga Exi Fast cooled CCD camera attached to a Zeiss Axiovert 200M inverted microscope (Oberkochen, Germany). Illumination control, data acquisition and data analysis were done with Northern Eclipse version 7.0 (Mississauga, ON, Canada) and were identical for all preparations. The preparations were protected from prolonged exposure to light at all times. CM-H_2DCFDA is colourless and non-fluorescent until the acetate groups are hydrolysed by intracellular esterases and oxidation occurs within the cell, resulting in the green fluorescent chloromethyl-2′,7′-dichlorofluorescein (CM-H_2DCF). CM-H_2DCF fluorescence intensity was measured in and immediately distal and proximal to the crush zone.

Reagents, peptide reconstitution and application

The circularized RGD-peptide cG**RGD**SPA (Bachem, Torrance, CA, USA) was dissolved in H_2O to a stock concentration of 5 mmol l^{-1} and stored at –20°C. The linear RGD-peptide G**RGD**S (Bachem) and control peptide SDGRG (Bachem) were dissolved in H_2O to a stock concentration of 1 mmol l^{-1}, aliquoted, lyophilized and stored at –20°C. On the day of use, the peptides were further diluted to the appropriate concentration with ABS. CM-H_2DCFDA was dissolved in DMSO (anhydrous, 99.9+%; Sigma) to a stock concentration of 1 mmol l^{-1}, aliquoted and stored at –20°C. Aliquots were used within 7 days of preparation. The working concentration of DMSO was 0.0005% in the *in vitro* assays and 0.002% in the organ-culture studies.

Data analysis and statistics

All experiments were performed concurrently on matched experimental and control groups. All animals were randomly sampled from one and the same tank. The experiments were performed in triplicate. The numbers (*N* values) given in the text and figure legends reflect the total number of brains or total number of cells included under a particular condition. The effect of RGD-peptides on axonal regeneration of RPA neurons was tested by means of a one-way analysis of variance (ANOVA) or Student's unpaired *t*-test. When required (i.e. as indicated by Kolmogorov–Smirnov one-sample tests for normality), the data were logarithmically transformed prior to ANOVA. The effects of RGD-peptides on phagocytic activity were tested by means of Chi-squared tests. Associations between the spreading response and CM-H_2DCF fluorescence and the effect of different treatment conditions on this association were evaluated using a stratified analysis of 2×2 R×C tables with the Breslow–Day test for the interaction of risk ratio over the three strata (Breslow and Day, 1980). CM-H_2DCF fluorescence intensity distributions in RIP nerves were constructed with Northern Eclipse version 7.0. Averages and dispersion are given as arithmetic means and standard error of the mean (s.e.m.) throughout the text. Percentages of activated phagocytes are presented with their 95% confidence intervals ($CI_{95\%}$) of ratios as calculated from the *F* distribution according to a modified Wald method (Agresti and Coull, 1998). A two-sided critical value of statistical significance of $P<0.05$ was adopted throughout the paper. Image acquisition conditions were exactly the same for all preparations within an experiment.

RESULTS

The present study focused on the role of RGD-dependent mechanisms in the regulation of endoneurial phagocytic function during axonal regeneration in a *Lymnaea* nerve injury model. To test this general idea, RGD-mediated ligand–receptor interactions were antagonized with the following synthetic water-soluble RGD-peptides: the linear peptide Gly-Arg-Gly-Asp-Ser (G**RGD**S) and the circularized RGD analogue cyclic-Gly-Arg-Gly-Asp-Ser-Pro-Ala (cG**RGD**SPA). The latter peptide was included in this study because of its larger effective dose range (Wildering et al., 1998; Wildering et al., 2001), which allowed for examination of

concentration-dependent effects of RGD-peptides on axonal regeneration and phagocyte activation. To control for non-specific effects of these peptides we used Ser-Asp-Gly-Arg-Gly (SDGRG), a reverse sequence equivalent of G**RGD**S. The effectiveness of SDGRG as a negative control was previously confirmed in adhesion studies (Wildering et al., 1998).

RGD-dependent processes in axonal regeneration

To assess the general hypothesis that RGD-dependent processes are a key factor in axonal regeneration in *Lymnaea*, we tested the effect of G**RGD**S, SDGRG and cG**RGD**SPA on the regeneration of RPA motoneuron axons in the organ-cultured *Lymnaea* CNS. In the first set of experiments, a total of 82 isolated brains with crush-injured RIP nerves were cultured under one of the following three conditions: (1) ABS only (N=25); (2) ABS + SDGRG (100 μmol l^{-1}; N=27); and (3) ABS + G**RGD**S (100 μmol l^{-1}; N=30). As per our previous studies (Hermann et al., 2000; Hermann et al., 2005; Wildering et al., 2001), the extent of axonal regeneration of RPA group neurons was determined in each preparation by means of backfilling regenerated axons with the retrograde tracer nickel-lysine (Fig. 1Ai,Aii).

Fig. 1B shows that axonal regeneration was significantly reduced by treatment with G**RGD**S, while the two control groups (i.e. ABS only and ABS + SDGRG) were equally successful in the regeneration of RPA axons ($F_{2,79}$=13.13; P<0.001). Similar inhibitory effects of G**RGD**S were observed for the identified neurons right pedal dorsal 1 and visceral dorsal 2/3, all of which project axons through the RIP nerve (data not shown). Thus, RGD-dependent processes appear to be a vital factor in axonal regeneration in the organ-cultured *Lymnaea* CNS.

The affinity and activity of synthetic RGD-analogues can be modified substantially by substituting and/or adding amino acid residues surrounding the RGD core, or by altering the secondary structure of the peptides. One such modification, circularization, has been shown to be particularly effective in boosting the activity of RGD-analogues (Aumailley et al., 1991; Tung et al., 1993; Pfaff et al., 1993; Schense and Hubbell, 2000; Kato and Mrksich, 2004). Circularized RGD-analogues typically have an approximately 100-fold higher potency than the linear equivalents (Aumailley et al., 1991; Pfaff et al., 1993; Wildering et al., 1998; Wildering et al., 2002; Schense and Hubbell, 2000; Kato and Mrksich, 2004). In a previous study, we showed that circularized RGD-analogues are about 100-fold more potent than their linear equivalents in antagonizing adhesive interactions of *Lymnaea* cells with a fibronectin matrix (Wildering et al., 1998). Prompted by this observation, we tested the dose dependency of cG**RGD**SPA on axonal regeneration. A total of 180 preparations that had received a crush injury to their RIP nerves were divided into six groups and cultured in ABS only (N=30) or in ABS plus cG**RGD**SPA at five different concentrations ranging from 10 nmol l^{-1} (N=33) to 100 μmol l^{-1} (N=30 for each group; Fig. 1C). Treatment with cG**RGD**SPA affected axonal regeneration of RPA neurons in a non-linear, concentration-dependent manner ($F_{5,177}$=2.839; P<0.05; Fig. 1C). That is, over the range from 10 nmol l^{-1} to 1 μmol l^{-1}, the number of back-labelled RPA axons declined progressively. Above 1 μmol l^{-1}, however, the inhibitory effect of cG**RGD**SPA treatment reversed and at 100 μmol l^{-1} it culminated in a slight increase in the number of regenerated RPA axons. Note that the same concentration of the linear RGD-peptide GRGDS strongly suppressed axonal regeneration of RPA neurons (Fig. 1B).

In conclusion, the data presented above confirm that RGD-dependent processes are a crucial factor in axonal regeneration in *Lymnaea* nerves. In addition, the results show that the conformation in which the RGD-motif is presented has dramatic consequences for both the potency and nature of the effect of RGD-peptides on axonal regeneration in this model system.

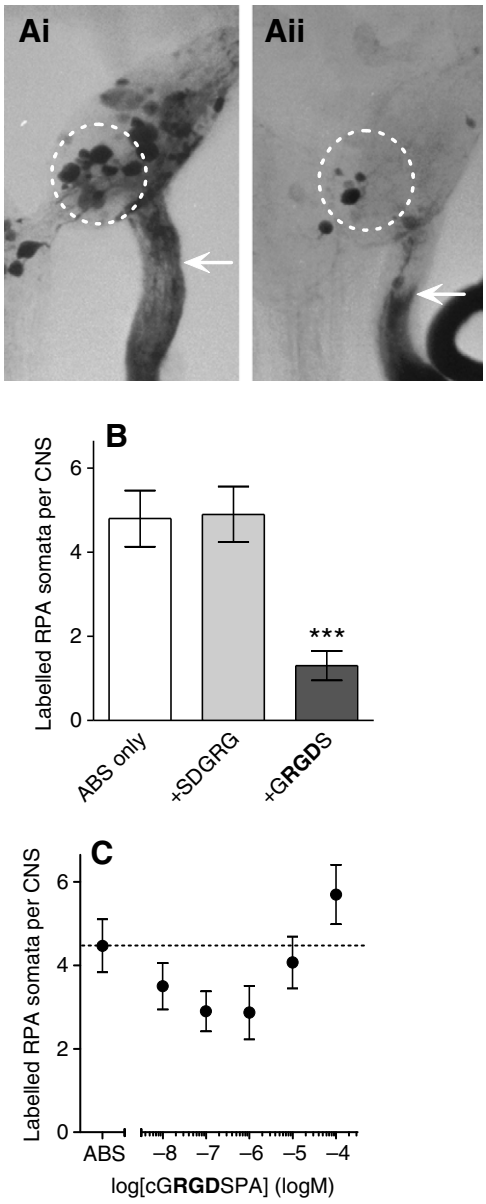

Fig. 1. Treatment with RGD-peptides modulates axonal regeneration in organ-cultured CNS. (Ai and Aii) Microphotographs of isolated CNS (dorsal view) that were cultured after receiving a crush to the right internal parietal (RIP) nerve (arrow) in ABS only (i) and ABS + 100 μmol l^{-1} G**RGD**S (ii). Comparison of the two photographs illustrates that the number of labelled right parietal A (RPA) somata was substantially reduced in the presence of GRGDS. (B) Average number of retrograde nickel-lysine labelled RPA somata in preparations that were cultured in ABS only, ABS + 100 μmol l^{-1} control peptide SDGRG and ABS + 100 μmol l^{-1} G**RGD**S. (C) Average number of retrograde nickel-lysine labelled RPA somata in preparations that were cultured in ABS only or in the presence of different concentrations of cG**RGD**SPA (10 nmol l^{-1} to 100 μmol l^{-1}). Treatment with cG**RGD**SPA had a concentration-dependent biphasic effect on the regeneration of RPA neurons projecting into the RIP nerve. ***P<0.001.

RGD-peptides modulate endoneurial phagocyte activity

Axonal regeneration in its native tissue context involves a complex interplay between many cellular and acellular factors, including the recruitment of activated phagocytes to the injury zone. Previously, we identified a class of phagocytes residing in the nerve as a key factor in *in vivo* axonal regeneration in *Lymnaea* (Hermann et al., 2005). Other studies in closely related gastropod species (*Biomphalaria glabrata*) show that RGD-dependent mechanisms are critical in the activation of blood-borne phagocytes (i.e. haemocytes) (Davids and Yoshino, 1998; Davids et al., 1999). Therefore, in view of the above results, we first tested whether RGD-peptides influence the activation and activity of *Lymnaea* endoneurial phagocytes *in vitro*.

To this end, we examined the effect of G**RGD**S and SDGRG using two assays of phagocyte activation and activity, i.e. the spreading response and microsphere engulfment of these cells. These experiments were performed under one of the following three conditions: (1) ABS only, (2) ABS + SDGRG (100 μmol l^{-1}) and (3) ABS + G**RGD**S (100 μmol l^{-1}) in the presence of either uncoated fluorescent latex microspheres or microspheres coated with human plasma fibronectin. The percentage of cells displaying a spreading response did not differ significantly under the two control conditions, independent of whether they were cultured in the presence of uncoated or fibronectin-coated microspheres (Fig. 2B,C). Treatment with G**RGD**S, however, significantly reduced the percentage of cells exhibiting a spreading response in the presence of uncoated ($\chi^2_{(2)}$=58.29, P<0.001, N=769; Fig. 2B) as well as fibronectin-coated microspheres ($\chi^2_{(2)}$=36.67, P<0.001, N=840; Fig. 2C).

Analysis of microsphere uptake showed that less than 2% of the non-spreading cells had internalized microspheres. In contrast, consistent with the notion that the spreading response is a hallmark of phagocyte activation (see also Hermann et al., 2005), more than 50% of the cells that displayed a spread phenotype under control conditions had internalized one or more microspheres (see columns labelled ABS only and +SDGRG in Fig. 3Bi and Ci). Treatment with G**RGD**S had no significant effect on the percentage of spreading cells that engulfed uncoated microspheres ($\chi^2_{(2)}$=0.298, P=0.86, N=410; Fig. 3Bi) or on the distribution of the number of uncoated microspheres taken up by individual cells (Fig. 3Bii). In contrast, the results were very different for fibronectin-coated microspheres. In comparison with both control conditions, treatment with G**RGD**S significantly reduced the percentage of spreading cells that had engulfed one or more fibronectin-coated microspheres ($\chi^2_{(2)}$=15.16, P<0.001, N=477; Fig. 3Ci). In addition, coating with fibronectin significantly enhanced the number of beads taken up by individual cells when cultured under control conditions (see ABS only and +SDGRG in Fig. 3Cii; compare with Fig. 3Bii). For example, the percentage of cells that engulfed more than 30 coated microspheres significantly increased from around 10% to more than 40% in both control groups (Z=4.79 and 5.83 for ABS only and SDGRG-treated cells, respectively; P<0.001). However, note that no such shift occurred in the presence of G**RGD**S (Z=1.12, P=0.26; Fig. 3Cii).

Considering the remarkable biphasic concentration-dependent effect of cG**RGD**SPA on axonal regeneration, we next tested the dose dependency of cG**RGD**SPA on phagocytosis by endoneurial phagocytes at concentrations of 100 μmol l^{-1} and lower doses. Endoneurial phagocytes, isolated as before, were cultured in the presence of fibronectin-coated fluorescent microspheres in ABS only (N=288), ABS + 10 nmol l^{-1} cG**RGD**SPA (N=347), ABS + 1 μmol l^{-1} cG**RGD**SPA (N=408) or ABS + 100 μmol l^{-1} cG**RGD**SPA (N=307). Phagocyte activation under these conditions was quantified as before by monitoring cells for the occurrence of a spreading response and counting the number of fluorescent microspheres absorbed by individual cells. As shown in Fig. 4A, cG**RGD**SPA affected phagocyte spreading cells in a non-linear fashion. Like the linear G**RGD**S, cG**RGD**SPA significantly repressed the spreading response at doses of 1 μmol l^{-1} or lower ($\chi^2_{(3)}$=51.25; P<0.001, N=1350). However, increasing cG**RGD**SPA dosage above 1 μmol l^{-1} did not further enhance the peptide's inhibitory actions on phagocyte spreading. Rather, the percentage of spreading cells increased to a value slightly below the non-peptide control level. As shown in Fig. 4B, which shows the percentage of spread (i.e. activated) phagocytes that internalized fibronectin-coated microspheres under each of the four aforementioned conditions, a similar hyperbolic concentration-dependent trend was observed in the effect of cG**RGD**SPA on microsphere uptake ($\chi^2_{(3)}$=31.57; P<0.001). Again, the strongest inhibitory effect of cG**RGD**SPA was reached at an intermediate concentration of 1 μmol l^{-1} ($\chi^2_{(1)}$=28.19; P<0.001). At the highest concentration of cG**RGD**SPA tested, fibronectin-coated microsphere uptake was not significantly different from the value observed in the ABS-only controls ($\chi^2_{(1)}$=3.043; P>0.05). Fig. 4C

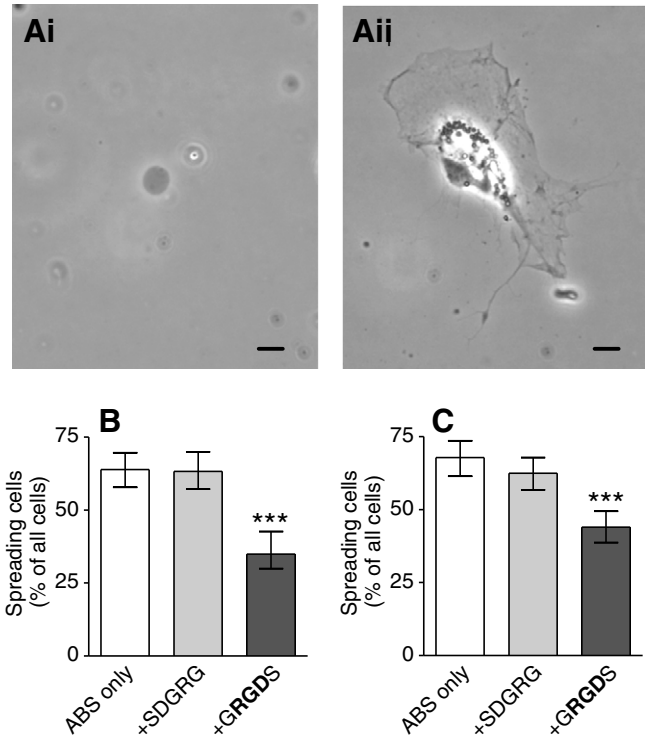

Fig. 2. Linear RGD-peptides reduce the percentage of spreading endoneurial phagocytes *in vitro*. (Ai and Aii) Photographs of non-spreading (i) and spreading (ii) endoneurial phagocytes cultured in ABS. (B) Percentage of endoneurial phagocytes showing a spreading response after culturing in the presence of uncoated monodispersed polystyrene carboxylated Fluoresbrite YG microspheres in ABS only, ABS + 100 μmol l^{-1} SDGRG and ABS + 100 μmol l^{-1} G**RGD**S. The addition of G**RGD**S significantly reduced the percentage of spreading cells. (C) Percentage of endoneurial phagocytes showing a spreading response after culturing in the presence of fibronectin-coated microspheres in ABS only, ABS + 100 μmol l^{-1} SDGRG and ABS + 100 μmol l^{-1} G**RGD**S. Again, the addition of G**RGD**S significantly reduced the percentage of spreading cells. Scale bar in A, 10 μm. ***P<0.001.

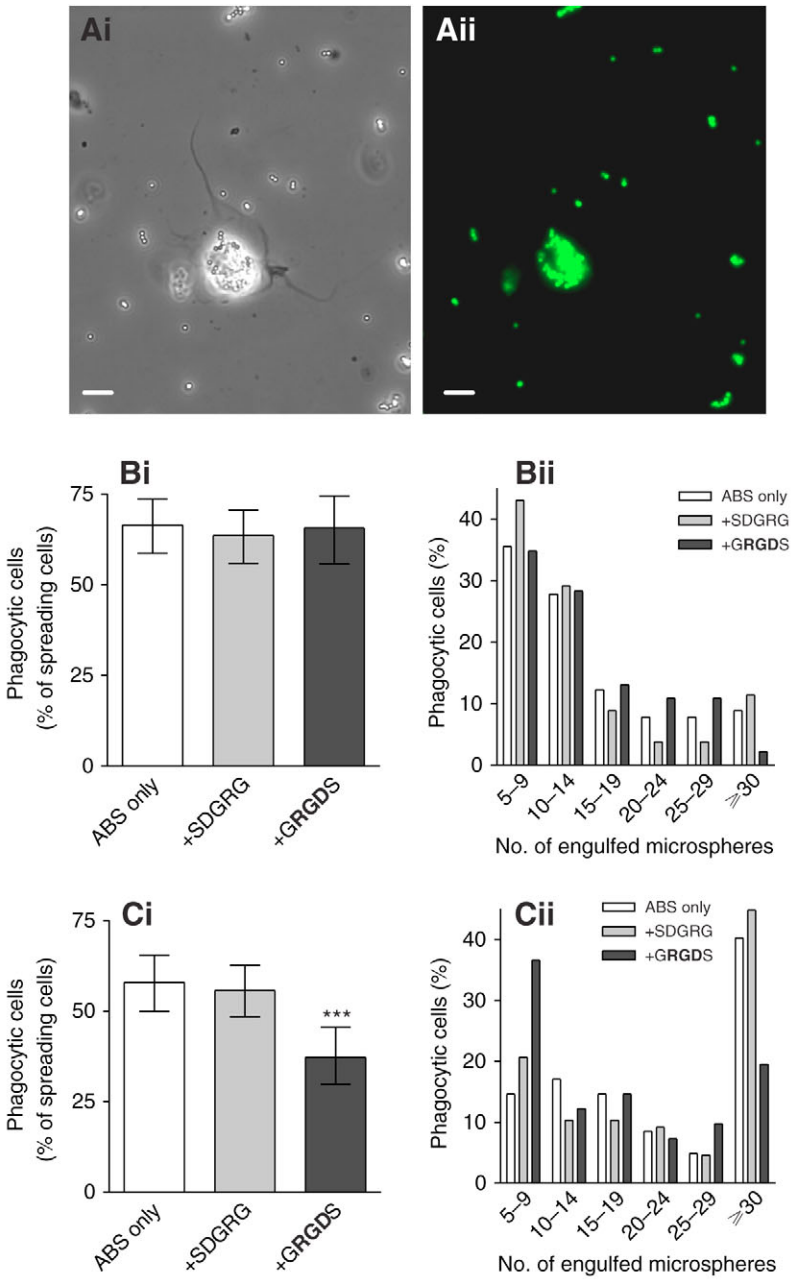

Fig. 3. Linear RGD-peptides reduce the percentage of phagocytic active cells *in vitro*. (Ai) Photograph of an isolated endoneurial phagocyte cultured in the presence of monodispersed polystyrene carboxylated Fluoresbrite YG microspheres. (Aii) Photomicrograph showing the large number of fluorescent microspheres engulfed by the phagocyte shown in Ai. (Bi) Percentage of spreading cells that internalized uncoated microspheres when cultured in ABS only, ABS + 100 μmol l^{-1} SDGRG and ABS + 100 μmol l^{-1} G**RGD**S. (Bii) The distribution of the number of uncoated microspheres engulfed by spreading cells under the three culture conditions. Note that the addition of G**RGD**S had no effect on the percentage of phagocytic cells engulfing uncoated microspheres nor on the distribution of the number of engulfed uncoated microspheres. (Ci) Percentage of spreading cells that phagocytized fibronectin-coated microspheres when cultured in ABS only, ABS + 100 μmol l^{-1} SDGRG and ABS + 100 μmol l^{-1} G**RGD**S. The addition of G**RGD**S significantly reduced the percentage of phagocytic cells. (Cii) Distribution of the number of engulfed fibronectin-coated microspheres by spreading cells is shifted to the right (i.e. more microspheres are engulfed) when the cells were cultured in ABS or ABS + 100 μmol l^{-1} SDGRG. In contrast, treatment with G**RGD**S did not result in a similar increase in internalization of fibronectin-coated microspheres. Scale bar in A, 10 μm. ***P<0.001.

summarizes the qualitative effects of cG**RGD**SPA in that it expresses the data in terms of the percentage of activated phagocytes that had successfully internalized a minimal number of fibronectin-coated microspheres. Note that 1 μmol l^{-1} cG**RGD**SPA also reduced the number of microspheres engulfed per phagocytic active cell (Fig. 4C). Thus, consistent with our observations above (Fig. 1C), endoneurial phagocyte activation is modulated by cG**RGD**SPA in a concentration-dependent, biphasic manner.

Taken together, these results lead to the following conclusions: (1) activation of *Lymnaea* endoneurial phagocytes *in vitro* is characterized by a spreading response; (2) the spreading response involves RGD-dependent mechanisms; and (3) RGD-peptides antagonize the uptake of fibronectin-coated (i.e. RGD-containing) but not uncoated latex microspheres, suggesting that *Lymnaea* endoneurial phagocytes utilize both RGD-dependent and RGD-independent internalization mechanisms.

RGD-peptides modulate the phagocytic response to nerve injury

The results described above indicate that RGD-peptides modulate various aspects of endoneurial phagocyte physiology. To evaluate the relevance of these *in vitro* observations in the much more complex context of whole nerve tissue, we examined whether RGD-peptides affect the injury response of endoneurial phagocytes in organ-cultured CNS preparations. Since neither phagocyte spreading nor microsphere uptake can be effectively monitored in the intact nerve, we approached this question by measuring the production of reactive oxygen species (ROS). Enhanced ROS production, also known as the 'oxidative burst', is a hallmark of phagocyte activity that can be monitored through the use of oxidation-sensitive fluorescent probes (Donko et al., 2005; Lehmann et al., 2000; Park, 2003; Sumimoto et al., 2005). In these experiments we used 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), a cell-permeant indicator for ROS with enhanced intracellular retention characteristics.

To validate CM-H_2DCFDA for use in our applications, we first tested this compound on endoneurial phagocytes *in vitro*. Cells were isolated and cultured for 48 h as described before under the following three conditions: ABS only (N=122), ABS + 1 μmol l^{-1} cG**RGD**SPA (N=134) and ABS + 100 μmol l^{-1} cG**RGD**SPA (N=134). At the end of the incubation period the cells were loaded with CM-H_2DCFDA. After allowing sufficient time for hydrolysis of the probe to its active form (i.e. chloromethyl-2′7′-dichlorodihydrofluorescein or CM-H_2DCF), the shape of the cell and the presence of fluorescent labels were examined qualitatively using a combination of differential interference contrast (DIC) and epifluorescence microscopy. As shown before (Fig. 4), cG**RGD**SPA suppressed phagocyte spreading at a concentration of

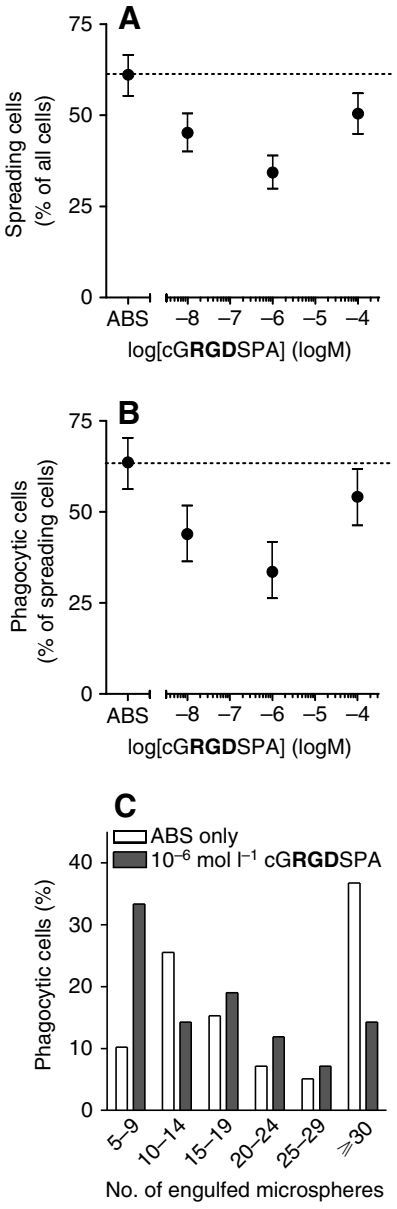

Fig. 4. Circularized RGD-peptides modulate activation of endoneurial phagocytes *in vitro*. (A) Percentage of endoneurial phagocytes showing a spreading response after culturing in the presence of fibronectin-coated microspheres in ABS only, and in the presence of ABS + 10 nmol l^{-1} cG**RGD**SPA, ABS + 1 μmol l^{-1} cG**RGD**SPA or ABS + 100 μmol l^{-1} cG**RGD**SPA. (B) Percentage of spreading endoneurial phagocytes that engulfed fibronectin-coated microspheres when cultured in ABS only, and in the presence of ABS + 10 nmol l^{-1} cG**RGD**SPA, ABS + 1 μmol l^{-1} cG**RGD**SPA or ABS + 100 μmol l^{-1} cG**RGD**SPA. Treatment with cG**RGD**SPA had a significant concentration-dependent biphasic effect on both the percentage of spreading cells and the percentage of phagocytic active cells. (C) Distribution of the number of engulfed fibronectin-coated microspheres by spreading cells is shifted to the right, i.e. more microspheres are engulfed, when the cells were cultured in ABS only compared with the cells cultured in the presence of 1 μmol l^{-1} cG**RGD**SPA.

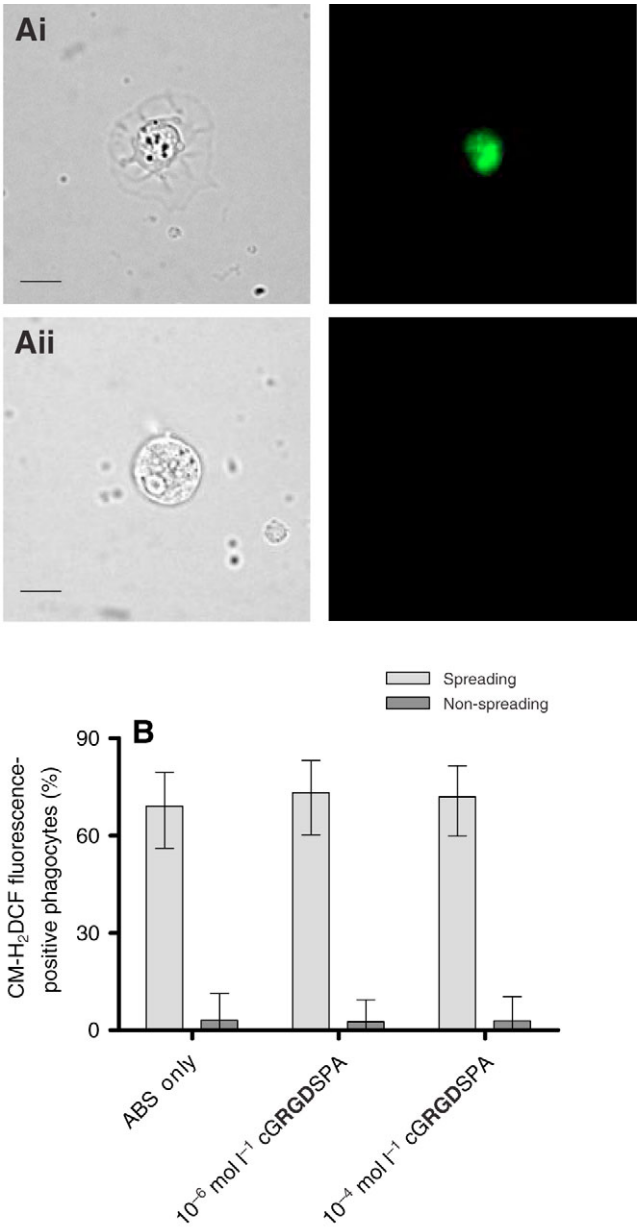

Fig. 5. Reactive oxygen species production depends on the activation status of endoneurial phagocytes. (Ai and Aii, left panels) DIC images of a spreading endoneurial phagocyte (i) and non-spreading endoneurial phagocyte (ii). (Ai and Aii, right panels) Epifluorescence images showing CM-H_2DCF fluorescence (i), or the absence thereof (ii), in the same cells as shown in the corresponding left panels. Note that the CM-H_2DCF fluorescence is contained inside the activated endoneurial phagocyte. (B) Percentage of CM-H_2DCF fluorescence-positive endoneurial phagocytes when cultured in ABS only, ABS + 1 μmol l^{-1} cG**RGD**SPA and ABS + 100 μmol l^{-1} cG**RGD**SPA. Note that independent of culture condition, the majority of the spreading cells are CM-H_2DCF fluorescence positive while only a very small percentage (<3%) of the non-spreading cells display CM-H_2DCF fluorescence. Image acquisition conditions were exactly the same for all cells. Scale bar in A, 10 μm.

1 μmol l^{-1} but had little effect at a concentration of 100 μmol l^{-1}. While the percentage of spreading cells differed substantially between the cultures treated with 1 μmol l^{-1} cG**RGD**SPA (31.7%) and those maintained in ABS only (67.6%) and ABS + 100 μmol l^{-1} cG**RGD**SPA (51.5%), we found a strong association between cell spreading and fluorescence labelling under all conditions (Fig. 5). That is, the percentage of spread phagocytes that were positively labelled was very similar under all conditions (Fig. 5B). Likewise, the percentage of fluorescently labelled non-spreading phagocytes was equally low under all three conditions

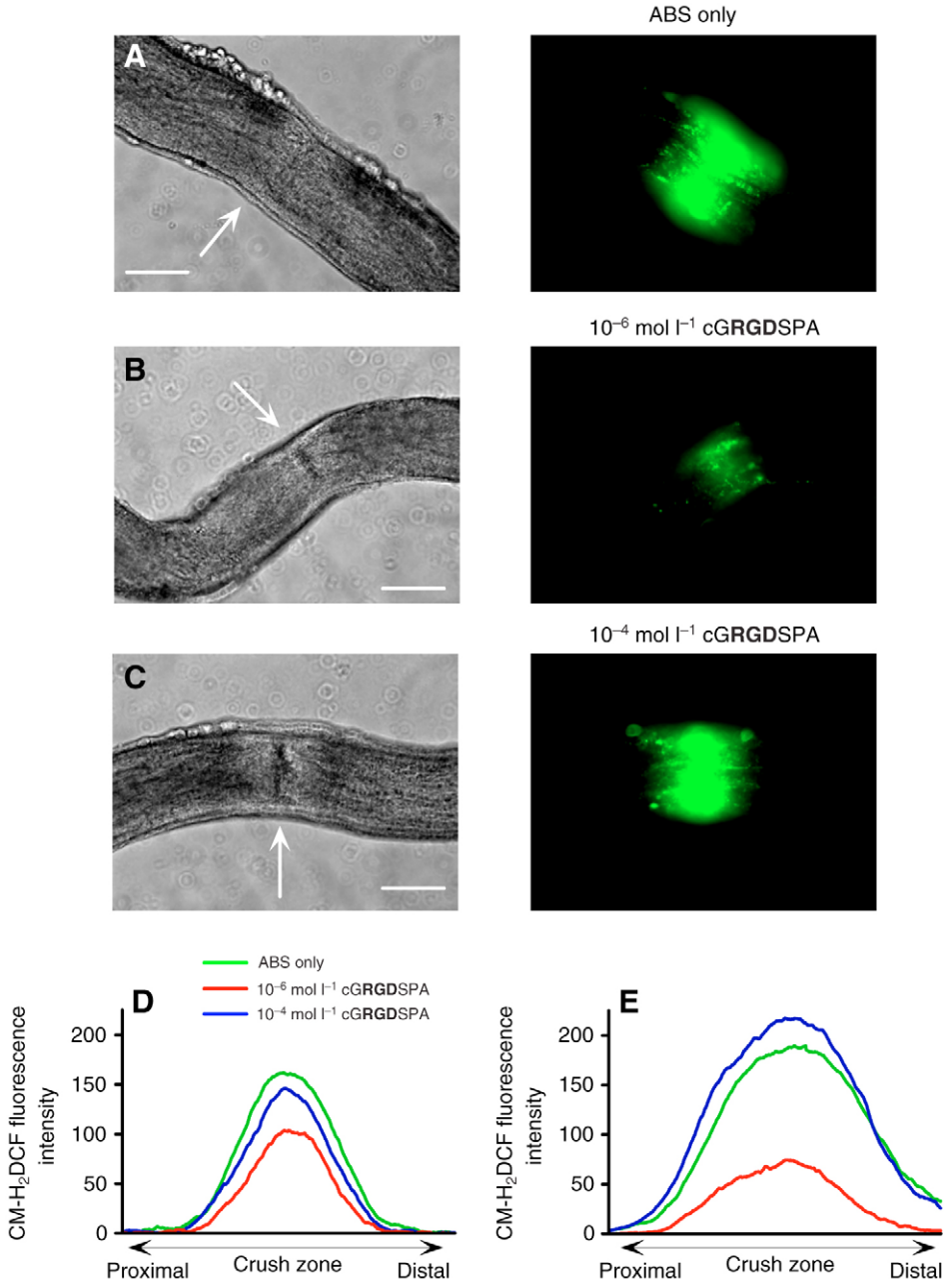

Fig. 6. Circularized RGD-peptides modulate oxidative burst in the injured nerve. (A–C, left panels) Phase contrast photomicrographs showing the injured RIP nerve cultured in ABS only, 1 μmol l^{-1} cG**RGD**SPA and 100 μmol l^{-1} cG**RGD**SPA, respectively. Arrows indicate crush zones. (A–C, right panels) Corresponding CM-H_2DCF fluorescence signal of the nerves shown in the left panels. (D) Average CM-H_2DCF fluorescence intensity of preparations cultured for 1 h in ABS only, ABS + 1 μmol l^{-1} cG**RGD**SPA or ABS + 100 μmol l^{-1} cG**RGD**SPA. (E) Average CM-H_2DCF fluorescence intensity of preparations cultured for 48 h in ABS only, ABS + 1 μmol l^{-1} cG**RGD**SPA or ABS + 100 μmol l^{-1} cG**RGD**SPA. Note the attenuated CM-H_2DCF fluorescence signal in the preparations cultured in the presence of 1 μmol l^{-1} cG**RGD**SPA. Image acquisition conditions were exactly the same for all preparations. Scale bar in A–C, 100 μm.

(Fig. 5Aii,B). Consequently, we found no significant difference in the association between spreading and CM-H_2DCF fluorescence labelling across the three experimental conditions (Breslow–Day test for interaction of risk ratio over strata: χ^2= 0.0673, P=0.967, d.f.=2). Overall, spreading cells were fluorescently labelled with a more than 25 times higher likelihood than non-spreading cells (Mantel–Haenszel adjusted risk ratio=25.2, $CI_{95\%}$=11.19–55.78). Hence, we conclude that CM-H_2DCFDA is a reliable indicator of *Lymnaea* endoneurial phagocyte activation and that, once cells are activated, ROS production in itself is insensitive to treatment with RGD-peptides. Importantly, the data also indicated that CM-H_2DCF fluorescence was contained inside activated cells (pilot studies with the parent molecule H_2DCFDA showed a substantial extracellular signal distribution, data not shown). Obviously, this observation is consistent with the purportedly enhanced intracellular retention of CM-H_2DCFDA. The fact that CM-H_2DCF is largely insensitive to the accumulation of extracellular ROS provides an advantage when monitoring the number of activated phagocytes in injured nerves. Taken together, these results demonstrate that CM-H_2DCFDA is a reliable and effective indicator of endoneurial phagocyte activity that is very well suited to quantify the phagocytic response in injured RIP nerves.

To determine whether RGD-peptides affected ROS generation in the injured nerve, isolated *Lymnaea* brains with a crush injury to their RIP nerve were cultured in ABS only, ABS + 1 μmol l^{-1} cG**RGD**SPA or ABS + 100 μmol l^{-1} cG**RGD**SPA. To assess both immediate and delayed effects of RGD-peptides on phagocyte activity, two separate sets of these experiments were performed. In the first set, measurement of CM-H_2DCFDA fluorescence in the injury zone commenced within 1 h post-injury (21 preparations per test group). The second set was evaluated 48 h post-injury (12 preparations per test group).

Both data sets showed that a nerve crush triggers a substantial oxidative burst in the injury zone and adjacent parts of the nerve (Fig. 6). In both data sets, CM-H_2DCF fluorescence intensity was distributed in a parabolic fashion centred on the site of the injury (Fig. 6D,E). However, while essentially similar CM-H_2DCF fluorescence distributions were found in all experimental groups, absolute values differed across the groups. Fig. 6D,E illustrates that, both immediately and 48 h after injury, CM-H_2DCF fluorescence in the injury zone was less intense in the preparations treated with 1 μmol l^{-1} cG**RGD**SPA as compared with the other two test groups. While this difference was statistically significant in both the preparations cultured for 1 h ($F_{2,2697}$=59.97; P<0.001) and those cultured for 48 h ($F_{2,2697}$=535.7; P<0.001), comparison of the data from the two experiments shows that it was most prominent in the latter group (cf. Fig. 6D,E). We conclude from these data that treatment with 1 μmol l^{-1} cG**RGD**SPA significantly reduces acute (i.e. within 2–3 h) as well as long-term (i.e. 48 h post-injury) injury-induced ROS production in *Lymnaea* nerves, whereas treatment with a higher dose of cG**RGD**SPA has little or no effect.

DISCUSSION

In this study we investigated the significance of the RGD-motif in the regenerative response of the organ-cultured *Lymnaea stagnalis* nervous system. Our results are summarized as follows: (1) RGD-peptides antagonize morphological changes associated with the activation of endoneurial phagocytes *in vitro* (i.e. the spreading response); (2) RGD-peptides antagonize internalization of fibronectin-coated (i.e. RGD-containing) but not uncoated microspheres *in vitro*; (3) RGD-peptides modulate the generation of ROS in the injured nerve *in situ*; and (4) RGD-peptides modulate axonal regeneration *in situ*. These results support two key conclusions. First, they implicate RGD-dependent mechanisms as vital to the axonal repair process and, second, they indentify endoneurial phagocytes as one of the potential targets of RGD-peptides in the axonal regeneration process.

Our results identify RGD-dependent processes as a key regulator of the *Lymnaea* endoneurial phagocyte response to nerve injury and make a case for the involvement of one or more RGD-binding integrins and RGD-presenting ligands. Our findings share intriguing parallels with those recently reported by Milner et al. (Milner et al., 2007), who implicated RGD-binding integrins α5β1 and αvβ5 and their RGD-containing ligands fibronectin and vitronectin in the regulation of microglial activation in a mouse model of experimental autoimmune encephalomyelitis (EAE). Although we are not yet in the position to identify the integrin homologues involved in the effects presented here, recent data emerging from a collective effort to sequence a *Lymnaea* brain-derived expression sequence tag (EST) library indicates that *Lymnaea*, like other invertebrates, expresses at least one integrin β-subunit with the hallmarks of an RGD-binding integrin homologue (information from '*Lymnaea stagnalis* Sequence Consortium', www.Lymnaea.org, personal communication W.C.W.). Thus, while more work is needed to settle this issue, *Lymnaea* seems to be no exception to other invertebrates in that it expresses at least one integrin receptor of the RGD-binding subfamily [see Burke (Burke, 1999) or Hughes (Hughes, 2001) for discussion of the evolution of integrins).

Previously we demonstrated that, *in vitro*, the pharmacological actions of RGD-peptides in *Lymnaea* are consistent with the existing literature in that they only interfere with cell adhesion to fibronectin substrates but not with adhesion to unmodified laminin, collagen or artificial poly-anionic substrates (Wildering et al., 1998). Thus, it is reasonable to postulate that, reminiscent of the role of fibronectin and vitronectin in the activation of microglia in a mouse EAE model (Milner and Campbell, 2003; Milner et al., 2007), treatment with RGD-peptides interferes with the ability of *Lymnaea* endoneurial phagocytes to bind fibronectin *in vivo*. However, it should be noted that while the fibronectin/integrin ligand/receptor pair constitutes the archetype of RGD-mediated cell adhesion, fibronectin is not the only ECM protein containing RGD epitopes. In fact, while fibronectin has only one RGD epitope, other ECM proteins like for example collagen type IV may have as many as 11 RGD-containing epitopes. However, in most cases these epitopes are unavailable for receptor binding and therefore biologically inactive, i.e. they are referred to as matricryptic RGD-sites (Davis et al., 2000). A growing body of literature suggests that reconfiguration of the parent molecule through proteolysis or other means may uncover these matricryptic RGD-sites and thereby dramatically alter the biological activity of the molecule (Davis et al., 2000; Platt et al., 2003; Schenk and Quaranta, 2003). In the case of the injury response of the mammalian PNS such a scenario is quite probable. First of all several of the collagens, including collagen type IV, are abundantly present in the basal membrane and therefore quite common in the PNS. Moreover, PNS injury has been shown to trigger the proteolytic activity of several members of the family of matrix metalloproteinases including those capable of digesting collagens (La Fleur et al., 1996; Platt et al., 2003; Shubayev and Myers, 2000). Thus it is conceivable that matricryptic RGD-epitopes that normally do not interact with RGD-selective integrins are involved in the activation of phagocytic cell types during inflammatory- or injury-induced events in the mammalian PNS. Although we have no detailed information about the variety and distribution of ECM proteins expressed in the *Lymnaea* nervous system the major ECM glycoproteins, including collagens, appeared early in the evolution of metazoans and appear broadly represented in all modern metazoan phyla including gastropod molluscs (Exposito et al., 2002; Garrone, 1998; Miller and Hadley, 1991; Moroz et al., 2006; Müller and Müller, 2003; Serpentini et al., 2000). Hence, future efforts to reveal the identity of the endogenous integrin ligand(s) involved in RGD-dependent injury-induced regulation of endoneurial phagocyte activity should, in addition to fibronectin, also consider proteins containing matricryptic RGD-containing epitopes as possible ligands of interest.

Alternative targets for RGD-peptides

Although our data identify endoneurial phagocytes as one of the targets of RGD-peptides in axonal regeneration in *Lymnaea* nerves they are probably not the only targets. Many studies have implicated integrins and/or several of their ligands in axonal growth cone extension and navigation (Condic, 2001; Condic and Letourneau, 1997; Gardiner et al., 2005; Guan et al., 2003; Ivins et al., 2000; Kiryushko et al., 2004; Tucker et al., 2005; Vogelezang et al., 2001) (reviewed by McKerracher et al., 1996; Nakamoto et al., 2004). *In vitro* studies demonstrated that *Lymnaea* RPA neurons (i.e. the same neurons involved in the current study) utilize RGD-dependent adhesion mechanisms to adhere to a fibronectin substrate (Wildering et al., 1998). Although we demonstrated in that study that RGD-peptides do not interfere with brain conditioned medium-induced neurite outgrowth of these neurons *in vitro,* we currently do not know whether this evidence can be extrapolated to the organ-cultured brain. It is therefore conceivable that part of the effects shown here involve direct actions of RGD-peptides on RPA neurons themselves.

RGD-dependent and -independent processes

We have shown here that RGD-peptides interfere with various aspects of endoneurial phagocyte activation (i.e. the spreading response and the production of ROS) as well as with the process of phagocytosis itself. Similar effects of RGD-peptides have been demonstrated in circulating immune effector cells in a variety of species ranging from insects to mammals, indicating that RGD-dependent, integrin-mediated processes are an evolutionarily conserved feature in the regulation of metazoan cellular host defence systems (Ballarin and Burighel, 2006; Ballarin et al., 2002; Davids and Yoshino, 1998; Gresham et al., 1989; Hanayama et al., 2002; Moita et al., 2006; Pech and Strand, 1995; Plows et al., 2006).

Our results indicate that particle internalization by activated *Lymnaea* endoneurial phagocytes involves at least one RGD-dependent and one RGD-independent pathway. That is, our data show that RGD-peptides interfere with the uptake of microspheres coated with RGD-containing proteins but do not interfere with phagocytosis of uncoated microspheres. In this context is it intriguing that a novel opsonin, called granularin, consisting of a single von Willebrand factor (vWF) type C domain has recently been isolated in *Lymnaea* (Smit et al., 2004). This peptide, secreted by cells in the connective tissue surrounding the CNS, opsonizes zymosan particles for phagocytosis by haemocytes (i.e. blood-borne phagocytes). Granularin does not contain RGD-motifs. At present we do not know whether granularin interacts with endoneurial phagocytes. However, considering our data indicating that *Lymnaea* endoneurial phagocytes also utilize RGD-independent internalization mechanism, such a possibility should not be ignored.

Multiple integrins, multiple phagocyte populations

Although both linear and circular RGD-peptides significantly antagonize endoneurial phagocyte spreading, a substantial proportion of the cells still display the characteristic morphology of an activated phagocyte. Yet, our results also show that RGD-peptides can interfere with internalization of fibronectin-coated microspheres in the latter group of cells. There are various scenarios that could explain these observations. For instance, as suggested in a recent review (Humphries and Yoshino, 2003), one plausible explanation is that we are dealing with multiple phagocyte populations differing with respect to their integrin phenotype. In this scenario, one or more of these populations relies on RGD-dependent pathways for activation and at least one population uses RGD-independent pathways for the purpose of activation but utilizes RGD-dependent mechanisms for internalization of microspheres exposing RGD-containing epitopes. This idea is consistent with the observation that several phagocytic cell types can express multiple integrins with different ligand affinities (Hynes, 1992; Moita et al., 2006; Milner et al., 2007). These observations may also provide an explanation for one of the most puzzling features of our results, i.e. the biphasic, concentration-dependent effects of the circular RGD-peptide cG**RGD**SPA on endoneurial phagocyte activation and activity *in vitro*, as well as on RPA neuron axonal regeneration and injury-induced ROS production in the organ-cultured brain. In theory, these results could arise from the actions of two integrins that have differential affinities for cG**RGD**SPA and that are coupled to pathways with opposing effects on phagocyte function. Alternatively, although the molecular basis of these effects is often not well understood, there is evidence suggesting that depending on their configuration, presentation and/or density, integrin ligands may have variable effects on their receptor's ligand-binding, adhesive and signalling states and may trigger a range of biological responses (Eid et al., 2001; Gaudet et al., 2003; Hynes, 1992; Legler et al., 2001; Rajagopalan et al., 2004; Schense and Hubbell, 2000; Schense et al., 2000). In this context, it is important to note that this is not the first time we observed concentration-dependent, non-linear biological effects of cG**RGD**SPA. In a previous study, we showed that depending on its concentration this peptide can either enhance or suppress high voltage-activated Ca^{2+} currents in *Lymnaea* neurons (Wildering et al., 2002).

In summary, our results provide pharmacological support for the significance of RGD-mediated mechanisms in axonal regeneration of gastropod molluscs, a phylum known for the superior regenerative capacity of their nervous systems. Our data implicate RGD-dependent mechanisms in the regulation and execution of the *Lymnaea* cellular immune response triggered by nerve injury, an area that has thus far received little consideration in the study of nerve repair. In more general terms, our results suggest that further investigation into the interactions between RGD-binding proteins and their receptors is worthwhile for advancing our understanding of the management of inflammatory responses in tissue injury and repair.

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