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New evidence of serotonin involvement in the neurohumoral control of crinoid arm regeneration: effects of parachlorophenylanine and methiothepin

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As well as acting as a neurotransmitter, serotonin is also involved in morphogenetic signalling during crucial phases of many developmental and regenerative processes such as cleavage, migration and differentiation. Echinoderms display nerve-dependent regenerative phenomena and serotonin is one of the main neural regulatory factors that have been identified in them. The present work was designed to investigate the broad-spectrum involvement of this molecule in echinoderm regeneration, focusing on arm regeneration in the crinoid Antedon mediterranea. *We carried out specific* in vivo *exposure experiments to selected anti-serotonergic drugs (a synthesis inhibitor: parachlorophenylalanine and a receptor antagonist: methiothepin) and explored their possible effects on arm regeneration. Drug exposure appeared to affect regeneration, causing a general delay of regenerative growth and several histological anomalies mainly in the muscles of the stump. In addition, exposure to the antagonist produced a marked reduction of cell proliferation in both the regenerate and the stump. Our results provide further evidence that serotonin has wider functions than those related to interneuronal communication and that it may be a critical signalling molecule in the main processes of regeneration such as proliferation, morphogenesis and differentiation.*

Keywords: invertebrates, neuroendocrine signal, regenerative development, differentiation, proliferation

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INTRODUCTION

Regenerative phenomena are typical developmental processes which, although not representing an accelerated version of ontogenetic events (Candia Carnevali & Bonasoro, 2001; Candia Carnevali, 2006), involve analogous problems in all animal models and often superficially resemble embryonic processes in terms of basic mechanisms and events such as cell proliferation, identity and positioning (Davidson *et al.*, 1998). It is therefore not surprising that the nervous system plays a crucial role by directing the correct progression of regenerative events in most animal models. It is well established that it can supply regulatory mitogenic or morphogenic factors that regulate the basic developmental processes of regeneration. Among these molecules, serotonin has been cited often as a multifunctional neurohumoral agent (Franquinet, 1979; Lauder, 1988; Whitaker-Azmitia *et al.*, 1996; Buznikov *et al.*, 2001). During regenerative events in planarians, serotonin is a critical regulatory factor which stimulates, via adenylate cyclase, RNA and DNA synthesis (Franquinet & La Moigne, 1979). Moreover, flatworms exposed to antiserotonergic drugs display a retarded regeneration rate (Saitoh *et al.*, 1996).

Echinoderms are well known for their capacity to regenerate many body parts and organs following traumatic or self-induced mutilation (autotomy) (Wilkie, 2001). Moreover, their regenerative processes are also nerve-dependent (for review see Thorndyke & Candia Carnevali, 2001), and serotonin is one of the main neural regulatory factors identified so far (Bonasoro *et al.*, 1995; Candia Carnevali *et al.*, 1996, 2001). Previous results obtained in the crinoid *Antedon mediterranea* (Lamarck, 1816), a common featherstar endemic in the Mediterranean Sea (Figure 1), showed that the process of arm regeneration is characterized by changes in the tissue localization and concentration of serotonin (Bonasoro *et al.*, 1995; Candia Carnevali *et al.*, 1989, 1996, 1999, 2001). In particular, immunocytochemical techniques revealed variations in serotonin distribution during the different regenerative phases, in comparison with the standard pattern of positive reactions displayed by the cellular elements of the different nervous system components (brachial nerve and basiepithelial plexuses) in non-regenerating arms. It is notable that during regeneration labelling was found both in neural structures, where it was appreciably enhanced, and at the level of non-neural elements, such as migrating amoebocytes, coelothelial cells and differentiating myocytes. Quantitative HPLC data confirmed the involvement of serotonin in regenerative

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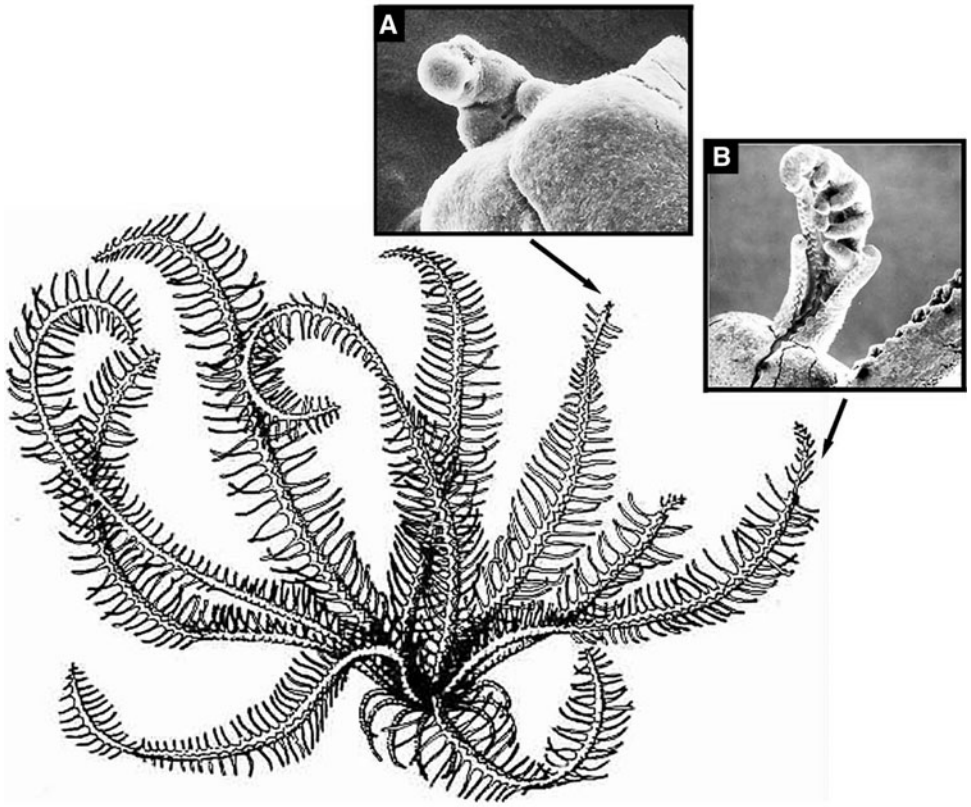

Fig. 1. The featherstar *Antedon mediterranea* (Lamarck, 1816): different stages of the regeneration process are in progress in different arms (A and B: SEM images).

events and showed fluctuations in serotonin concentration during the crucial phases. These data showed a clear variability in absolute values, an initial peak being detectable in the early regenerative stages (24–72 hours post amputation (p.a.)) when most proliferative phenomena occur, and a further concentration increase being measurable at the onset of advanced regenerative stages (72 hours–4 weeks) characterized by early cell and tissue differentiation (Candia Carnevali *et al.*, 2001).

The immunocytochemical and biochemical results obtained so far thus suggest that serotonin is involved in crinoid regenerative processes. In the present work we employed neuropharmacological compounds that interfere with serotonin uptake or metabolism. Treatment of other animal models with serotonin receptor antagonists or with serotonin synthesis inhibitors induces appreciable anomalies in both the embryonic and regenerative development (Franquinet, 1979; Palén *et al.*, 1979; Lauder, 1988; Saitoh *et al.*, 1996; Buznikov *et al.*, 2001). The aim of the present investigation was to explore the possible role of serotonin in crinoid arm regeneration using the serotonin synthesis inhibitor, parachlorophenylalanine, and the serotonin receptor antagonist, methiothepin.

MATERIALS AND METHODS

Animals

Adult specimens of *Antedon mediterranea* (Figure 1) were collected by SCUBA divers from the Tyrrhenian Sea (Giglio Island, 42°21′57″96N 10°54′7″20E) and immediately transferred to our laboratory in Milan. The experimental animals were maintained at 14–16°C in artificial seawater tanks (Instant Ocean; salinity 37‰) provided with chemical and biological filters and water circulation and aeration systems, and were fed once a week with drops of an artificial diet (Tetramin). The following parameters were monitored daily: temperature, water pH, nitrite and nitrate levels, animal mortality and health (general aspects, behaviour and vital reactions). Experiments were started after animals had been acclimatized for a few days.

All the experiments were carried out according to the current laws of Italy.

Experimental amputation and regeneration

Arm regeneration was experimentally induced as described previously (see Candia Carnevali *et al.*, 1993): each specimen was subjected to the amputation of two different arms by cutting at the level of autotomy planes (syzygies) about half way down the length of the arm. The experimental animals were then left to regenerate for a predetermined time and treated according to different protocols. Exposure to anti-serotonergic compounds (see below) in 4 l tanks started immediately after experimental amputation.

The regenerating samples of early stages were prepared for light microscopy as described in detail by Candia Carnevali *et al.* (1993, 1995). Briefly, collected samples were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours, washed overnight in the same buffer and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer. After standard

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dehydration in ethanol and propylene oxide, samples were embedded in Epon–Araldite 812. Semithin sections (about 1 μm thick) were cut with a Reichert Ultracut E, stained with crystal violet-basic fuchsin and observed under a Jenaval light microscope. Some samples were processed for specific immunocytochemical (ICC) methods for monitoring cell proliferation (BrdU labelling; see below) using previously established protocols (Candia Carnevali *et al.*, 1995, 1997).

Regenerating specimens at advanced stages were observed under a stereomicroscope (Leica Wild M3C Planapo) and photographed using a Leica digilux zoom 18 102 E-type digital camera.

BrdU labelling

Cell proliferation was monitored with the thymidine analogue, 5-bromo-deoxyuridine (BrdU), localized by a monoclonal mouse antibody against BrdU (Amersham: cell proliferation kit). Animals were immersed in a BrdU and FldU (5-fluoro-2′-deoxyuridine; an inhibitor of thymidine syhthetase) solution (0.05% in seawater; BrdU:FdU = 10:1) for the final 2 hours prior to regenerate collection and processing for optical microscopy. The standard BrdU-immunocytochemistry protocol for paraffin was modified for use on semithin Epon–Araldite sections, as described previously (Candia Carnevali *et al.*, 1995, 1997). After a brief treatment (2 minutes) with a resin-remover mixture (10 ml methanol, 5 ml propylene oxide and 2 g KOH), sections were rinsed with methanol (2 minutes), then with PBS (3 × 5 minutes) and incubated overnight at 4°C with anti-BrdU antibody diluted (1:100) in an aqueous solution containing nuclease (Amersham: cell proliferation kit). Pre-treatment for 20 minutes with 0.3% H_2O_2 in PBS was performed to exclude potential endogenous peroxidase activity. After washing in PBS (3 × 10 minutes) samples were incubated (3 hours) with a peroxidase anti-mouse IgG (1:70; Amersham: cell proliferation kit) at room temperature and, after further washing in PBS (3 × 10 minutes), incubated (5 minutes) with 0.05% 3,3′-diaminobenzidine (DAB) in PBS and then washed in distilled water. In order to amplify the peroxidase reaction product, a cobalt and nickel intensifier supplied with the kit was employed. Control reactions omitted the primary antibody.

Exposure experiments

Two different anti-serotonergic drugs were tested:

- parachlorophenylalanine (pCPA) (SIGMA): a potent inhibitor of tryptophan-hydroxylase, one of the key enzymes involved in serotonin biosynthesis;
- methiothepin (as the mesylate salt; SIGMA): a serotonin receptor antagonist which has a high affinity for most invertebrate serotonin receptors (Tierney, 2001).

Optimal concentrations and treatment times were determined in initial tests by examining the effect of the above agents on samples that had been regenerating for one week. The developmental stage after one week is well defined and characterized in terms of growth and differentiation in standard regenerating samples (Candia Carnevali & Bonasoro, 2001; Candia Carnevali, 2006). During this period the regenerating animals were daily exposed to different drug concentrations for prefixed times as shown in Tables 1 & 2. Daily treatment for a few hours was preferred to continuous exposures in order to minimize neurotoxic effects, since the selected drugs are likely to affect all serotonin-dependent nervous functions, i.e. both neurotransmission and any possible broader spectrum neuroendocrine functions.

Table 1. Effect of different pCPA concentrations. Exposure times are given as hours/day.

Tested concentration	Exposure time	Number of animals	Observations
1 mM	6	3	100% mortality→ total disarticulation within 24 hours
0.1 mM	6	3	100% mortality→ total disarticulation within 24 hours
1 μM	6	4	Atypical posture and increased fragility after 48 hours
0.5 μM	5	4	Atypical posture and increased fragility after 72 hours
0.1 μM	3	6	No observable effects

In order to avoid differences in physicochemical parameters, the exposure solutions for all the experimental groups were prepared by dissolving appropriate amounts of the substances in autoclaved seawater drawn from the maintenance aquarium. During the daily neuropharmacological treatment the animals were temporarily transferred to exposure tanks (4 l) provided with an aeration system. At the end of the daily treatment/exposure time they were returned to their original maintenance aquarium, which was subdivided into compartments in order to guarantee the same maintenance conditions for all the experimental groups.

After these initial tests and once the optimal concentrations and exposure times had been established, two sets of definitive experiments were performed on different regenerative phases.

Early regenerative phase (48–72 hours)

In the initial tests, samples at this stage remained healthy only in 0.5 μM methiothepin for 5 hours/day (Tables 1 & 2). This treatment was therefore repeated using seven experimental animals and regenerating arms were collected at 48 and 72 hours p.a. and processed as described above. Since in the initial tests pCPA treatment resulted in high mortality, extreme fragility or lack of observable morphological

Table 2. Effect of different methiothepin concentrations. Exposure times are given as hours/day.

Tested concentration	Exposure time	Number of animals	Observations
15 μM	5	3	100% mortality→ total disarticulation within 24 hours
3 μM	5	3	Atypical posture and increased fragility after 72 hours
0.5 μM	5	3	Regenerative anomalies

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alterations (Table 1), no further experiments on the early regenerative stages were carried out with this compound.

Advanced regenerative phase (1 week)

The second set of experiments was performed as follows: amputated animals, randomly subdivided in three different groups of ten individuals each, were daily exposed to: (1) 0.5 μM methiothepin for 5 hours/day; (2) 0.2 μM pCPA for 5 hours/day; and (3) artificial seawater for 5 hours/day (control group). The intermediate pCPA concentration was selected on the basis of the results of the initial tests (see Table 1), which showed that 0.5 μM was too toxic and 0.1 μM had no detectable effect. At the end of the daily exposure, animals from all the experimental groups were placed again in the maintenance aquarium. After treatment for 1 week, regenerating samples were collected, processed and photographed as described above. Photographs were used for measuring regenerate lengths by employing the computer program ArcView 3.2 (GIS). The homogeneity of variance of the obtained data was verified by a Levene test and a one-way ANOVA test was performed.

RESULTS

The results of the initial tests are summarized in Tables 1 & 2. The highest concentrations of pCPA (1 mM and 0.1 mM) were extremely toxic, causing the death of all animals within the first 24 hours of treatment. Lower pCPA concentrations (1 μM and 0.5 μM) also appeared to cause general damage. In all specimens exposed to these concentrations the jointed appendages (arms, pinnules and cirri) displayed extreme fragility, i.e. they fragmented at preformed autotomy planes. On the other hand, at a lower concentration (0.1 μM) and exposure time (3 hours), there were no observable morphological anomalies.

Although a lethal effect was observed with the highest methiothepin concentration (15 μM; 5 hours/day), the specimens exposed to the lowest concentration (0.5 μM; 5 hours/day) had regenerative abnormalities, yet showed no increase in mortality or fragility (Table 2). We therefore selected the latter concentration and exposure time for the subsequent tests with methiothepin. The intermediate methiothepin concentration (3 μM) induced the same tendency to break at autotomy planes as did pCPA.

Early regenerative phase (48–72 hours p.a.)

Figure 2 gives a schematic view of a regenerating arm (in sagittal section) at an early regenerative phase (48–72 hours p.a.). In standard conditions these stages are characterized by proliferation and migration phenomena which contribute to new arm formation (Candia Carnevali *et al.*, 1995, 1997).

Exposure to 0.5 μM methiothepin produced obvious anomalies in microscopic anatomy indicating dysfunctions in early regenerative development. The morphological analysis of treated samples showed significant malformations at the cellular and tissue levels in both the regenerate and the stump. The regenerate showed a general delay in regenerative growth starting from the 48 hour-stage. The blastemal bud, which is usually easily recognizable in normal regenerating arms at this stage (Figure 4), was replaced by a flattened and poorly

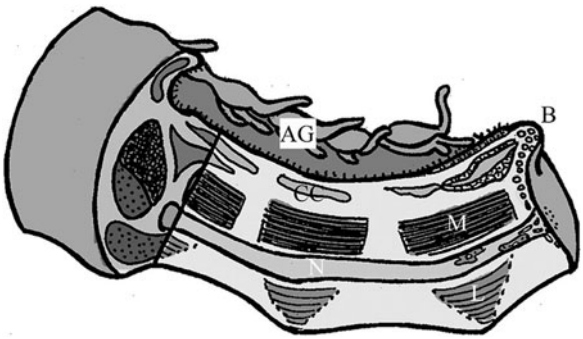

Fig. 2. Schematic reconstruction showing the main anatomical components of an arm at early regenerative phase (72 hours post amputation). AG, ambulacral groove; B, blastema; CC, coelomic canals, L, ligament; M, muscle; N, nerve.

defined blastemal area. In addition, at the level of the amputation region, the re-growing coelomic canal was abnormally flattened and bent aborally, following the general flat shape of the regenerate. The apparent growth inhibition displayed by the exposed samples was confirmed by BrdU–ICC results. As observed in Figure 5A, in standard regenerating samples the BrdU reaction was particularly evident at the level of the regenerative bud, where both coelothelial and blastemal cells were labelled, the latter representing presumptive stem elements. At the level of the stump (Figure 5C), in addition to labelling of the coelothelium, a strong BrdU reaction was shown by scattered cells of the brachial nerve, particularly non-neural satellite elements in the cortex (amoebocytes). In contrast, in methiothepin treated samples, the blastemal region was less immunoreactive (Figure 5B). Reduced labelling was even more evident in the stump where only a very small number of elements were immunoreactive (Figure 5D).

At the level of the stump, treated samples also displayed an unusual rearrangement of differentiated tissues and cells, specifically involving the muscles, in which marked dedifferentiation and/or transdifferentiation processes were frequently detectable. Muscle bundles in treated samples lost their normal compact structure (Figure 6A) and their individual myocytes underwent intensive reorganization and progressive dedifferentiation. These myocytes showed progressive disorganization of their contractile apparatus, particularly in terms of filament arrangement (Figure 6B, C & D). This phenomenon was very evident in the periphery of the muscle bundles, particularly in the areas close to the coelomic canals. Observation of serial sections indicated that the de-differentiating myocytes migrated towards the coelomic cavity (Figure 6B, D).

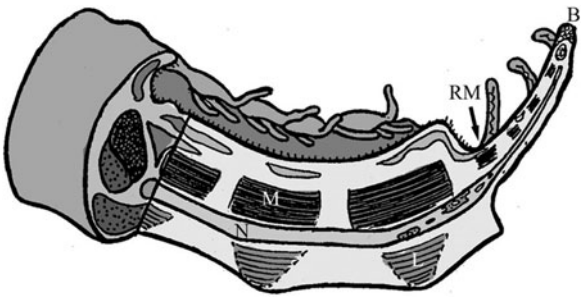

Fig. 3. Schematic reconstruction showing the main anatomical components of an arm at advanced regenerative phase (1 week). B, blastema; L, ligament; M, muscle; N, nerve; RM, regenerating muscle.

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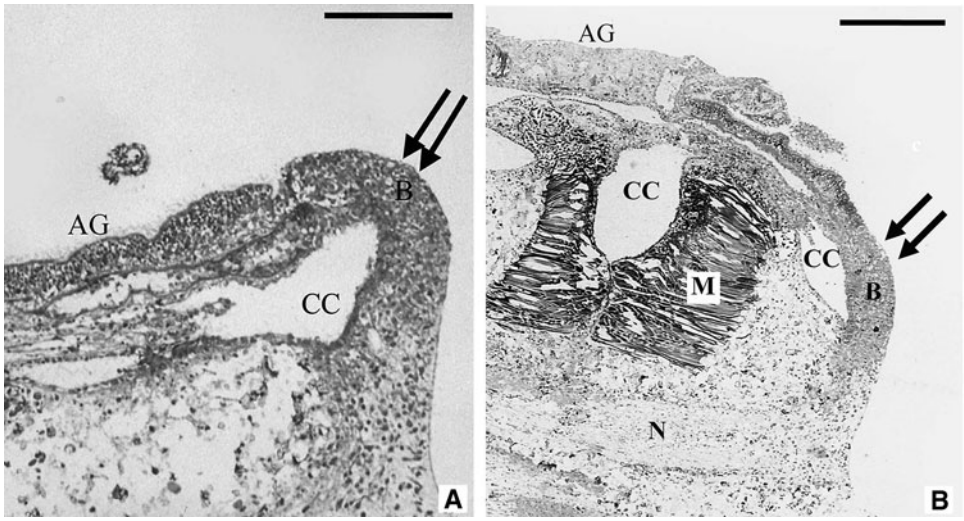

Fig. 4. Light microscopy view of the effects of methiothepin post-amputation (p.a.) treatment (0.5 μM; 5 hours/day) on regenerative growth at early regenerative stages. Histological sagittal sections. (A) Standard regenerate at 72 hours p.a. showing a protruding regenerative bud (double arrow). Bar = 100 μm; (B) sagittal section of a treated regenerating sample (72 hours p.a.). The blastema (double arrow) is scarcely developed and rather flat in comparison with standard regenerating bud. Bar = 200 μm. B, blastema; CC, coelomic canals; M, muscle; N, nerve.

Advanced regenerative phase (1 week p.a.)

Figure 3 shows a schematic view of a standard regenerating arm (in sagittal section) at an advanced regenerative phase (1 week p.a.). Both methiothepin and pCPA produced a statistically significant decrease in regenerate growth (expressed in terms of regenerate length) in comparison with the control group (ANOVA test: $P < 0.001$; Tukey and Bonferroni post-hoc tests: $P < 0.001$ for both compounds). Moreover, methiothepin inhibited growth more strongly than pCPA ($P < 0.05$) (Figure 7).

DISCUSSION

In echinoderms, and most other animals, interactions between specific neurochemicals and cellular elements are necessary for the successful course of most developmental phenomena, including regenerative processes. Several authors have suggested that many common neurotransmitters, such as serotonin, are broad-spectrum messengers responsible for promoting and controlling embryonic and regenerative developmental processes in many animal models (Franquinet, 1979; Lauder, 1988; Whitaker-Azmitia *et al.*, 1996; Buznikov *et al.*, 2001; Moiseiwitsch *et al.*, 2001). In agreement with this hypothesis, our previous (Candia Carnevali *et al.*, 1989, 1996; Thorndyke & Candia Carnevali, 2001) and present results suggest that serotonin is also involved in the overall process of arm regeneration in the crinoid *Antedon mediterranea*, influencing morphogenesis, differentiation and growth. In addition, our present and past data are consistent with the idea that in echinoderms serotonin functions both as a specific neurotransmitter and as a neurohumoral factor influencing basic developmental processes such as cell proliferation and differentiation (Candia Carnevali *et al.*, 2001; Thorndyke & Candia Carnevali, 2001). The involvement of serotonin in arm regeneration was indicated previously by the finding of significant differences in its distribution, localization and concentration between normal and regenerating samples at different regenerative stages (Candia

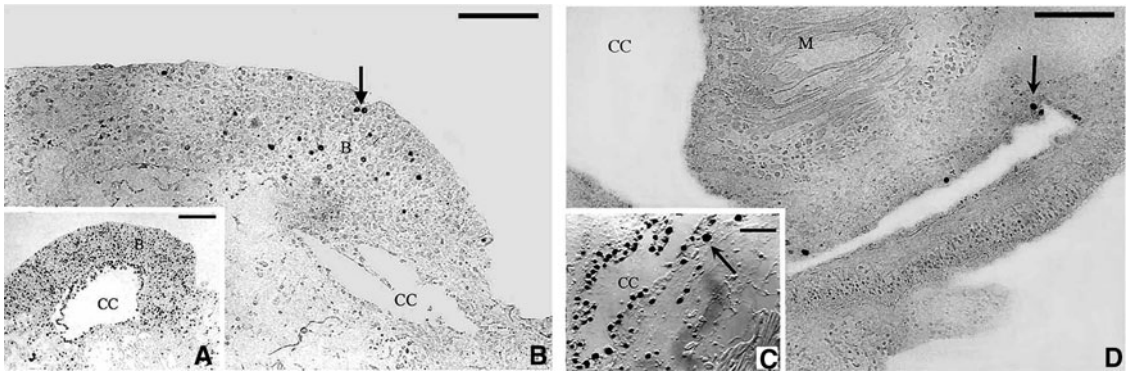

Fig. 5. Light microscopy details of the effects of post-amputation (p.a.) methiothepin treatment (0.5 μM; 5 hours/day) on cell proliferation. ICC–BrdU method. Sagittal sections of regenerating samples at 72 hours p.a. (A) Normal regenerating blastema showing many immunolabelled blastemal cells. Bar = 70 μm; (B) blastema of a treated sample. Only few cells (arrow) are immunoreactive for BrdU. Bar = 60 μm; (C) coelomic canals of a normal regenerate. Arrow = labelled epithelial cells. Bar = 20 μm; (D) coelomic canals of treated sample: labelling (arrow) is localized in few cells. Bar = 60 μm. CC, coelomic canal; M, muscle. (Note: in order to highlight the immunolabelling, the semithin-sections were not counter-stained and the image contrast was slightly enhanced using the computer program Adobe Photoshop 7.0.)

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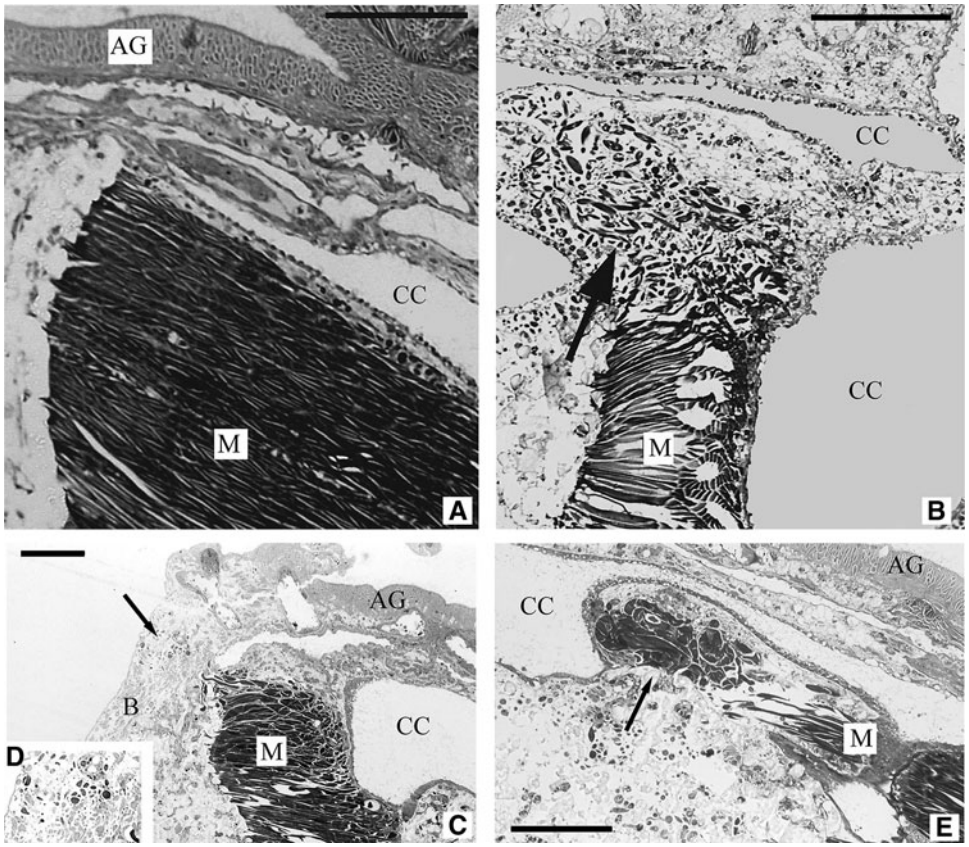

Fig. 6. Light microscopy details of the effects of methiothepin on the muscles of the stump. Sagittal sections. (A) Muscle bundle of a normal regenerating arm at 72 hours showing the classic compact arrangement of the fibres. Bar = 80 μm; (B) muscle bundle of a treated regenerating sample at 72 hours post amputation (p.a.) showing an anomalous histological pattern. The muscle fibres have lost their defined structure and numerous dedifferentiating myocytes (arrow) appear to detach from the bundle and move towards the coelomic cavity. Bar = 60 μm; (C) muscle of a treated sample at 48 hours p.a.: some myocytes (arrow) seem to be migrating toward the blastema. Bar = 100 μm; (D) detail of (C); (E) ectopic mass of dedifferentiating myocytes (arrow) in the muscle bundle of a treated sample. Bar = 100 μm. B, blastema; CC, coelomic canals; M, muscle.

Carnevali *et al.*, 1989, 1996; Thorndyke & Candia Carnevali, 2001). Our present results showed that repeated treatment with neuropharmacological compounds interfering with serotonin synthesis or action induced appreciable anomalies in regenerative development. Although more tests are needed to definitively confirm the specificity of the antiserotonergic treatments employed, our present results provide further evidence to support the view that serotonin-regulated processes are crucial to the correct course of regeneration. Different cellular processes appear to be affected by these neuropharmacological treatments, suggesting that serotonin is involved at different levels and with different functions. This is in agreement with the well known multifunctional role proposed for serotonin in early development (Whitaker-Azmitia *et al.*, 1996), where this molecule has been reported as a mitogen, morphogen and migration-inducing substance (Gustafson & Toneby, 1970; Moiseiwitsch & Lauder, 1995a, b; Fanburg & Lee, 1997; Moiseiwitsch, 2000; Bhasin *et al.*, 2004).

Our results suggest that serotonin influences cell proliferation and muscle differentiation/maintenance.

Proliferation

Serotonin involvement in cell division can be inferred from the remarkable decrease in the number of proliferating cells at the blastemal and coelothelial levels following 48 hours of antiserotonergic treatment (Figure 5). An inhibited proliferation rate could explain the reduced regenerative regrowth observable in all the exposed samples at advanced stages (Figure 7). These data are consistent with the often reported role of serotonin in promoting mitosis in a wide range of cultured mammalian cell types (Nemecek *et al.*, 1984; Zachary *et al.*, 1987; Fanburg & Lee, 1997).

Although the *in vivo* approach employed here does not help to identify the specific signalling pathway of serotonin or its interactions with other regulatory factors, the reduced proliferation rate observed after antiserotonergic treatment indicates that it probably influences cell division during regenerative phenomena.

Muscle differentiation/maintenance

Our previous work on *A. mediterranea* arm regeneration showed that there is a prominent localization of serotonin at the level of the differentiating muscle bundles and individual muscle fibres in the new arm (Bonasoro *et al.*, 1995; Candia Carnevali *et al.*, 1996, 2001; Thorndyke & Candia Carnevali, 2001). This localization led us to suggest that serotonergic innervation might be relevant for the development and maintenance of the motor components (Candia Carnevali *et al.*, 1999). The present work appears to provide further support for this idea. In contrast to the rather good histological preservation and apparent morphological integrity of most structures/tissues of the arm stump (e.g. epithelial tissues, brachial

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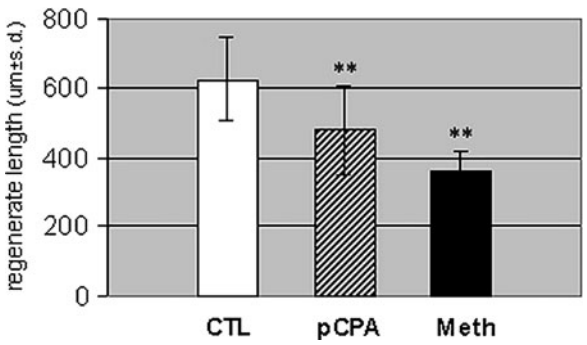

Fig. 7. Effects of para-chlorophenylalanine (pCPA: 0.2 μM; 5 hours/day) and methiothepin (0.5 μM; 5 hours/day) on regenerative growth. Advanced regenerative stages. Both neuropharmacological treatments caused highly significant reductions on regenerate length after 1 week of treatment (**$P <$ 0.001, ANOVA and Tukey post hoc tests) in comparison with controls (CTL), but the receptor antagonist methiothepin displayed more evident effects than pCPA ($P < 0.05$).

nerve, skeletal components, etc.), the muscles appeared to be selectively targeted by the neuropharmacological treatment. In fact, the receptor antagonist methiotephin produced an intensive and extensive rearrangement of myocytes in the muscle bundles, particularly in the oral portion adjacent to the coelomic canal (Figure 6B, D). These muscular elements had lost their typical elongate structure, though retaining their normal cytochemical properties, as indicated by heavy staining. These cytological features have been described at the ultrastructural level (Candia Carnevali & Bonasoro, 2001; Candia Carnevali *et al.*, 2001; Candia Carnevali, 2005) and are also seen less frequently in normal muscle bundles implying that they represent a basal rate of physiological cell turn-over. The anomalous increase in the number of muscle fibres apparently in progressive stages of dedifferentiation, which was detected after methiothepin exposure, suggests a loss of balance in the intrinsic mechanism of muscle maintenance. Serotonin may act as a specific signal for motor components, playing an important role in both their development and maintenance. As stated above, in the regenerating arm this molecule is associated specifically with differentiating myocytes (as demonstrated by its widespread and intense localization on the surface of these elements during the advanced regenerative phase: Candia Carnevali *et al.*, 1996, 2001) and its presence might be critical for the correct anatomical and functional development of the regenerating muscles. In the stump, decreased serotonin leads to an extensive disorganization of myocytes and their consequent dispersion into the pool of migratory cells recruited for regeneration (Figure 6B, C & D). In other words, the presumptive neurotrophic function of serotonin in muscles may not be restricted to development, but may also be important for adult muscles: as long as the serotonergic signal is present, muscle cells may retain their normal morphology and motor functions; whereas a deficiency in serotonin may lead to a progressive rearrangement of individual muscle fibres and detachment of peripheral dedifferentiating myocytes from the bundle. It is perhaps relevant that serotonin has been reported to modulate CAM (cellular adhesion molecules) expression, therefore influencing cell adhesion and, consequently, interactions between cells (Peter *et al.*, 1994; Zhu *et al.*, 1994).

Current knowledge of crinoid molecular biology is very limited. However, we anticipate that future research based on a genetic approach to the selective blocking of serotonin receptors will help to elucidate the specific role of this monoamine. In addition, an *in vitro* model is currently being developed (*A. mediterranea* cell culture; Parma *et al.*, 2006), which could help to clarify the mechanisms of action of serotonin on crinoid cells. Such information, integrated with *in vivo* results, will provide a fuller picture of the role of serotonin in *A. mediterranea* arm regeneration.

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