

Serotonin and MAOA enable the organizer and tip dominance in Dictyostelium

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Abstract

The embryonic organizer is essential to determine one or more developmental polarities during chordate early development^{1,2}. Functionally similar organizers also occur in more ancient animals³, and even in some protozoans such as *Dictyostelium*, in which the tip of the multicellular mound acts as an organizer⁴, establishing the main developmental axis, and regulating the size of the fruiting body⁵. However, our understanding of how the *Dictyostelium* organizer arises, and functions, is limited. Here we show that monoamine oxidase A (maoA), which degrades serotonin, confers the fate of an organizer to the *Dictyostelium* tip. Conversely, once a tip has formed, serotonin contributes to tip dominance. It inhibits further tip formation, and thus ensures the mound retains the size specified during an earlier developmental stage. Reducing the expression of maoA through RNA interference or by adding MAO specific inhibitors suppresses tip formation. Conversely, adding human MAOA enzyme, or an antagonist or antibodies against serotonin, restores tip formation in maoA knockdowns. Overexpression of maoA or adding a serotonin antagonist to the wildtype induces multiple tips from a single mound in a dose dependent manner. Using an array of genetic and molecular techniques, we show that serotonin's inhibition of cAMP signalling and cell-cell adhesion is the basis of its regulation of tip formation. Our study demonstrates that serotonin, recently appreciated for its developmental roles in widespread phyla⁶, also has a novel and ancient role in the formation and function of an organizer.

Main Text

Our understanding of many fundamental questions of development has benefited from the study of *Dictyostelium discoideum*^{7,8}, a forest-floor-dwelling social amoeba with the rare feature of its morphogenesis occurring almost entirely in the absence of cell division. Cellular differentiation and morphogenetic redistributions can therefore be conveniently followed. *D. discoideum*'s multicellular, developmental phase begins when up to 10⁵ free-living cells (amoebae) aggregate together, a process initiated, when the supply of the food source, soil bacteria, is depleted. The amoebae move up a concentration gradient of extracellular cAMP and form a multicellular mound, within which an axis of polarity becomes macroscopically evident when a tip arises from the centre of the mound. The mound next transforms into a motile, slug-like body that can migrate through the soil, or leaf litter, in response to light and other cues; after culmination, it finally forms an aerial, spore-filled fruiting body⁹. For all but the last stage of development, the cells of the original tip act in similar ways to the classical organizers of chordate development: the tip cells remain the same, and regulate the organisation of tissues along one of the organism's axes; additionally, they exert control upon the overall size of all later stages^{5,10}. If a tip from one slug is transplanted onto another, the second slug will transform into two smaller, but properly organized slugs within four hours⁴. As to the identity of the persistent signal emanating from the tip that maintains tip dominance, adenosine has been suggested, on the basis of the smaller slugs formed when endogenous levels of adenosine are reduced¹¹. But size regulation in *D. discoideum* is also influenced by processes that act prior to tip formation¹², and it is possible that adenosine's contribution is restricted to the earlier, aggregation stage. Here, we chose to investigate whether tip dominance involves serotonin,

previously unreported in *D. discoideum*, but which, in addition to its well-known role as a neurotransmitter, is an important developmental regulator in organisms across many phyla¹³⁻¹⁵. We present multiple lines of evidence that indicate that serotonin does contribute to tip dominance in *D. discoideum*, and that the breaking of this dominance, and the enablement of the mound's original tip, involves monoamine amine oxidase A, which degrades serotonin.

MAOA and serotonin in *D. discoideum*

To our knowledge, neither monoamine oxidases (MAOs) nor serotonin have been previously reported in *D. discoideum*, nor more widely, in protozoans and slime molds, respectively. According to its annotated genome, however, *D. discoideum* is predicted to encode four different isoforms of monoamine oxidase¹⁶ of which, *maoA* expression (DDB_G0288541) is particularly high during development¹⁷ (Extended Data Fig. 1a, b). Also, MAOA of *D. discoideum* has the conserved amine oxidase domain (Extended Data Fig. 2a) and its predicted tertiary structure is similar to that of humans MAOA (Extended Data Fig. 2b). The different levels of MAO activity in wildtype, [RNAi]*maoA* and *maoA* overexpressing clones confirm amine oxidase activity in *D. discoideum* (Extended Data Fig. 1c, d).

In spite of the absence of the gene, aromatic amino acid decarboxylase (AAAD), responsible for serotonin production¹⁸, *D. discoideum* cells produce serotonin, as ascertained by liquid chromatography-mass spectrometry (LC-MS) (Extended Data Fig. 3a, b). Tryptophan, a precursor for serotonin synthesis, when tagged with a methyl group, is expected to have a higher mass. MS confirmed that cells cultured with alpha-methyl tryptophan synthesize alpha-methyl serotonin (Fig. 1a), suggesting a novel serotonin biosynthesis pathway in *D. discoideum*. Serotonin levels in the wildtype mound were found to be 0.321 ng/10⁸ cells (Extended Data Fig. 3c).

MAOA enables the tip organizer

To understand the function of MAOA, an [RNAi]*maoA* line was generated (Extended Data Fig. 4a, b). Development was arrested at the mound stage, and these mounds did not form tips (Fig. 2b and Supplementary Video 2). This phenotype could be mimicked by adding MAO specific inhibitors (isocarboxazid or nialamide) to wildtype cells (Fig. 2c, d and Supplementary Video 3, 4). Thus, inhibiting MAOA activity or reducing the level of MAOA blocks tip formation. Tip formation in [RNAi]*maoA* clones could be restored by adding human MAOA (hMAOA) enzyme directly onto them, and each mound then proceeded to form a slug (Fig. 2e). Conversely, when *maoA* was overexpressed in the wildtype (Extended Data Fig. 4c), each mound had multiple tips (Fig. 2f). Taken together, these results suggest that the increased level of *maoA* activity has a role in tip enablement.

Serotonin blocks tip formation

To examine whether the function of MAOA is related to its degradation of serotonin, [RNAi]*maoA* lines were treated with either methiothepin, a serotonin antagonist, or antibodies against serotonin. In each case, the wildtype phenotype was restored and [RNAi]*maoA* mounds were transformed into slugs (Fig.

3a). Further, when *maoA*^{OE} lines (which normally form multiple tips) were treated with 1 mM serotonin, development was arrested at the mound stage (Extended Data Fig. 5d); a lower dose (500 μ M) had no effect (Extended Data Fig. 5c). Conversely, the development of wildtype cells treated with 500 μ M methiothepin was arrested at the mound stage (Extended Data Fig. 5b). These results suggest that serotonin's normal role might be to block tip formation in the mound. Indeed, when the wildtype was treated with the serotonin antagonist, methiothepin, this resulted in multiple tips emerging from each mound, a phenotype similar to *maoA* overexpressing clones (Fig. 3b). When applied at 15 μ M (minimal inhibitory dose) this gave rise to 1.34 ± 0.09 tips/ mound, and increasing the dose to 20 μ M resulted in 2.17 ± 0.02 tips/mound (Fig. 3c). Thus, MAOA and serotonin appear to have opposite roles in both the enabling of the tip within a mound, and in suppressing the formation of any further tips, that is, tip dominance.

Serotonin and MAOA act on cAMP and cell-cell adhesion

To investigate the mechanisms by which MAOA/serotonin could regulate tip formation, we began by looking at whether extracellular serotonin affects cAMP levels. cAMP in *D. discoideum* plays a role in tip formation and cell fate determination^{11,19}, and extracellular serotonin is known to trigger intracellular cAMP signalling in neurons²⁰. LC-MS analysis of concentrated conditioned media (CM) (Fig. 1b), and the rescue of [RNAi]*maoA* by either extracellular hMAOA enzyme or by serotonin antibodies, are all consistent with the secretion of serotonin in *D. discoideum*. Further, in the *maoA* RNAi lines (which have high serotonin), the levels of adenylyl cyclase A gene expression (which converts ATP to cAMP) were high, as were the total cAMP levels (Fig. 4a, b). Also, adding the *aca* specific inhibitor SQ22536, rescues the mound arrest phenotype of the *maoA* RNAi lines (Fig. 4d), which also supports the idea that increased serotonin inhibits tip formation by raising cAMP levels.

As to what might occur downstream, cAMP has previously been shown to regulate tip formation by modulating PKA activity, adenosine signalling and morphogenetic cell movements^{11,21,22}. To ascertain which of these three pathways is significantly affected by the observed serotonin-induced cAMP increase, the following compounds were each applied to [RNAi]*maoA* developmentally-arrested mounds: an activator of PKA (8-Br-cAMP, cAMP) or its inhibitor (H89); or adenosine deaminase; or adenosine. Each treatment failed to restore tip formation (Extended Data Fig. 6, 7), suggesting that PKA and adenosine signalling act independently or upstream of serotonin.

In investigating whether it is morphogenetic cell movements that are affected by serotonin, our starting point was that their contribution to normal tip formation is known to depend upon chemotaxis and cell-cell adhesion²³. Surprisingly, in [RNAi]*maoA* mounds, we found that chemotaxis is not affected (Extended Data Fig. 8c) but cell-cell adhesion is drastically reduced (Extended Data Fig. 8a). Thus, increased cAMP in [RNAi]*maoA* mounds possibly decreases cell-cell adhesion and thus impairs wave propagation, leading to inhibition of tips. In support of this, when [RNAi]*maoA* mounds were treated with the *aca* inhibitor, SQ22536 (which, as shown above, rescues developmental arrest) cell-cell adhesion was higher (Extended Data Fig. 8b). Further, we compared²² collective cell movements in the wildtype and [RNAi]*maoA* lines: in

the former, the wave propagation pattern was spiral, and towards the tip, while in the knockdown, it was circular (Supplementary Video. 5, 6).

To now probe the impact of MAOA/serotonin on wave propagation, we treated [RNAi]*maoA* mounds with caffeine or IBMX (PDE4 inhibitor), both of which are known to induce tips²⁴. Adding either one of the compounds to [RNAi]*maoA* mounds resulted in a cAMP wave propagation pattern that was closer to that of the wildtype, and restored tip formation to a more normal pattern (Fig. 4e, f and Supplementary Video. 7, 8). Interestingly, caffeine is known to reduce intracellular cAMP²⁵ whereas PDE4 inhibitor increases extracellular cAMP²⁶.

A model for tip enablement and dominance

Considering all the results together, we can propose the following model for the involvement of MAOA/serotonin in normal development of *D. discoideum*: (1) Prior to tip enablement, serotonin levels are uniformly high throughout a mound. High serotonin leads to decreased levels of extracellular cAMP (perhaps relative to intracellular cAMP), thus reducing cell-cell adhesion and blocking spiral wave propagation. Tip formation is thus inhibited. (2) This inhibition is subsequently released, but only at the site of future tip formation, by a localized increase in MAOA which degrades serotonin locally; and (3) In a mound, serotonin contributes to tip dominance, helping the mound to retain the size specified by processes that act during the earlier, aggregation stage²⁷. One would expect, however, that another diffusible factor, with a tip-high concentration gradient, must also be involved. It is possible this second factor is adenosine, operating independently of MAOA/serotonin, and thus adding to its earlier role in size regulation, during the late aggregation stage^{11,28}. Apart from size regulation, serotonin is likely to affect the cell type ratio along with free Ca^{2+} and cAMP^{29,30}. Differences in serotonin levels between the tip and the rest of the mound possibly affect serotonylation driving differences in gene expression thereby establishing the prestalk-prespore identity.

The roles we have ascribed to serotonin and MAOA in *D. discoideum* are also indirectly supported by their developmental roles in other organisms. Serotonin has been specifically shown to regulate some features of animal morphogenesis, particularly gastrulation, via its effects on cell-cell adhesion^{31,32}; and, more generally, differential cell adhesion is considered an important physical cue in the generation of self-organising structures³³. *MaoA* is also expressed during gastrulation in many vertebrates and it is likely that it helps to regulate morphogenesis in these organisms³⁴⁻³⁶ (Extended Data Fig. 9a-c), perhaps also by its dampening of serotonin's effects. MAO is also present in various ascomycota³⁷ (spore forming fungi) and colony forming bacteria (Extended Data Fig. 10), where it could perhaps play a conserved role in regulating group or colony size.

The synthesis of serotonin in *D. discoideum* was surprising given the absence of the AAAD gene required for serotonin synthesis in humans. *Entamoeba histolytica*, a pathogenic amoeba, synthesizes and secretes serotonin³⁸ without the canonical AAAD gene and thus, both *D. discoideum* and *E. histolytica* possibly synthesize serotonin through similar non-canonical pathways. While we have demonstrated the

synthesis of serotonin, a BLAST analysis indicated the absence of the established serotonin receptors in *D. discoideum*. However, adding serotonin receptor specific antagonists confers the expected phenotype suggesting the presence of novel, but structurally overlapping, receptor(s). Such receptors are likely to be conserved across protozoa that infect humans, and identifying these novel serotonin receptors may point to useful drug targets.

Methods

The culturing of *Dictyostelium* and the developmental assays including chemotaxis and cell adhesion assays were carried out as described by Nassir *et al*³⁹ or Lam *et al*⁴⁰. Cells used in this study were from Ax2 background that were grown in modified maltose-HL5 medium (Formedium, UK).

Bioinformatic analysis. The amine oxidase domain within the *Dictyostelium* MAOA was identified by the online tools, SMART and BLAST^{41,42}. To model the 3D structure of MAOA, we used the hhPRED online database⁴³ and images was viewed using PyMol. Docking of MAOA protein with serotonin was carried out using AutoDock Vina⁴⁴. The phylogenetic tree of MAOA was constructed using MEGA5 software⁴⁵. The gene expression patterns of *maoA* during mouse and human gastrulation were respectively obtained from the databases (marionilab.cruk.cam.ac.uk/MouseGastrulation2018/ and human-gastrula.net/).

Monoamine oxidase assay. MAO activity was ascertained using a monoamine oxidase assay kit as per the protocol of the manufacturer (Sigma-Aldrich, USA).

Plasmid constructs. *MaoA* RNAi lines were generated as described by Rosen and Kimmel⁴⁶. For generating the RNAi vector construct, two *maoA* cDNA fragments of 900 and 1200 bp were PCR amplified and cloned in pDNeo vector. For *maoA* overexpression, a full length (1241bp) *Dictyostelium maoA* cDNA (NBRP Nenkin, Japan) was cloned in pDXA-3C vector. The plasmid constructs were transfected to *Dictyostelium* Ax2 cells by electroporation (BTX ECM830, Harvard Apparatus; 300 V, 2 ms, 5 square wave pulses with 5 s interval) and antibiotic-resistant clones (G418, 15µg/ml) were handpicked.

Sample preparation for mass spectrometry. Ax2 cells were developed till the mound stage on KK2 agar plates or harvested as amoeba, washed and resuspended in 100 µl of ice-cold KK2 buffer and stored at -80 °C. After 2 h, the cell suspension was transferred to 37 °C for 2 min and subjected to vigorous vortexing for 1 h at RT. To this extract, 0.5 ml of methanol was added, stored in ice for 15 min, then 1 ml chloroform was added and centrifuged at 13000 RPM. The two phases were collected separately, lyophilized and resuspended in 100 µl of 50% acetonitrile before performing liquid chromatography-mass spectrometry.

Liquid chromatography - mass spectrometry analysis. HPLC analysis was carried out either by Agilent 1290 Infinity UHPLC or Shimadzu UHPLC Nexera X2. For Agilent 1290 Infinity UHPLC, C18 column of size 2.1×100 mm, 1.8µm (Acquity, USA), 10 mM ammonium acetate in water (A) and acetonitrile (B) was used as mobile phase at a flow rate of 0.2 ml min⁻¹. Isocratic gradient was maintained at 0-3 min: 10% B, 3-8

min: 10-50% B, 8-9 min: 50-100% B, 9-10 min: 100% B, 10-10.1 min: 100-10% B, 10.1-15 min: 10% B. The temperature of the column was maintained at 40 °C and 10 µl of the sample was injected. A slightly modified protocol was followed when using the Shimadzu UHPLC Nexera X2.

MS analysis. To confirm the presence of serotonin and alpha-methyl serotonin, Thermo Fisher- Orbitrap Elite (High Resolution Accurate Mass Spectrometry) was used. For subsequent analysis, the triple quadrupole MS instruments (Shimadzu - LCMS-8050 or Thermo Fisher TSQ Vantage) were used. All the analyses were performed in +ve ionization mode.

Chemical genetics studies. MAO inhibitors, isocarboxazid and nialamide, were independently mixed with wildtype cells at a concentration ranging from 2-200 µM and the minimal inhibitory dose affecting tip formation was found to be 200 µM. The mound arrest of [RNAi]*maoA* was rescued by 10 units of human MAOA enzyme. The serotonin antagonist, methiothepin was mixed with [RNAi]*maoA* cells while plating and the optimal concentration (tested 1-5 µM) for rescue of mound arrest was found to be 2 µM. Methiothepin at a concentration from 2-20 µM was tested directly onto wildtype mounds and the minimal dose that gave a prominent multi-tipped phenotype was found to be 15 µM. The serotonin antibody (Invitrogen, USA) was added to [RNAi]*maoA* cells in a 1:40 ratio of antibody: phosphate buffer respectively. Serotonin was tested from 100 nM to 1 mM on wild-type cells and 500 µM concentration was found to be the effective dose in causing the mound arrest phenotype. The concentrations of SQ22536 (2 mM), H89 (50 µM), 8-Br-cAMP (5 mM), cAMP (0.2 mM), c-di-GMP (1 mM), DIF (200 nM), adenosine (2 mM), adenosine deaminase (5U), caffeine (2 mM) and IBMX (0.5mM) treatment were based on the literature^{24,39,47,48}. All the fine chemicals were from Sigma-Aldrich, USA except as indicated.

Microscopy. A Nikon SMZ-1000 stereo zoom microscope with epifluorescence optics, Nikon 80i Eclipse upright microscope or a Nikon Eclipse TE2000 inverted microscope equipped with a Digital Sight DS-5MC camera (Nikon) were used for microscopy. Images were processed with NIS-Elements D (Nikon) or Image J.

Statistical tools. Microsoft Excel (2016) was used for data analyses. Paired or unpaired, one-tailed Student's t-tests (GraphPad Prism, version 6) were used to determine the statistical significance.

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Declarations

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Author contributions

R.M., and R.B. conceptualized the work. R.M. performed the experiments. R.M., G.J.H. and R.B. analysed the data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Materials & Correspondence should be addressed to R.B.

Figures

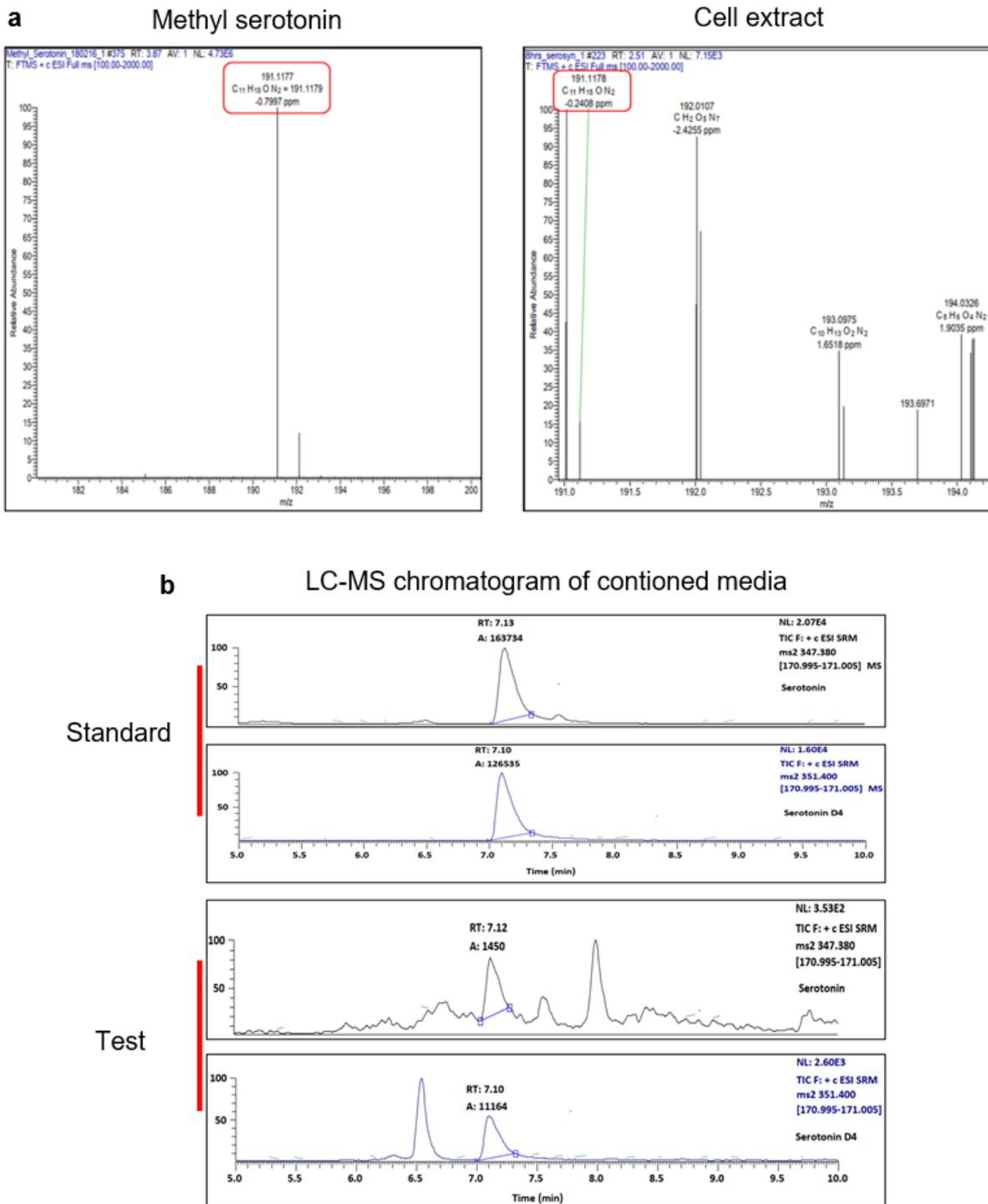


Figure 1

Identification of synthesis and secreted serotonin in Dictyostelium. a, Dictyostelium cells incubated with alpha methyl tryptophan (20 mM) convert them to alpha methyl serotonin as detected by High-Resolution Accurate Mass Spectrometry. The mass value of alpha methyl serotonin (191.1177) standard matched

with that of our test. Comparison between theoretical and observed mass value shows less than 0.25 ppm difference indicating a high mass accuracy (n=2). b, Conditioned media (CM) was prepared by starving the Dictyostelium cells in KK2 buffer for 20 h. Using a triple quadrupole LC-MS instrument, the presence of serotonin was detected in CM (n=2).

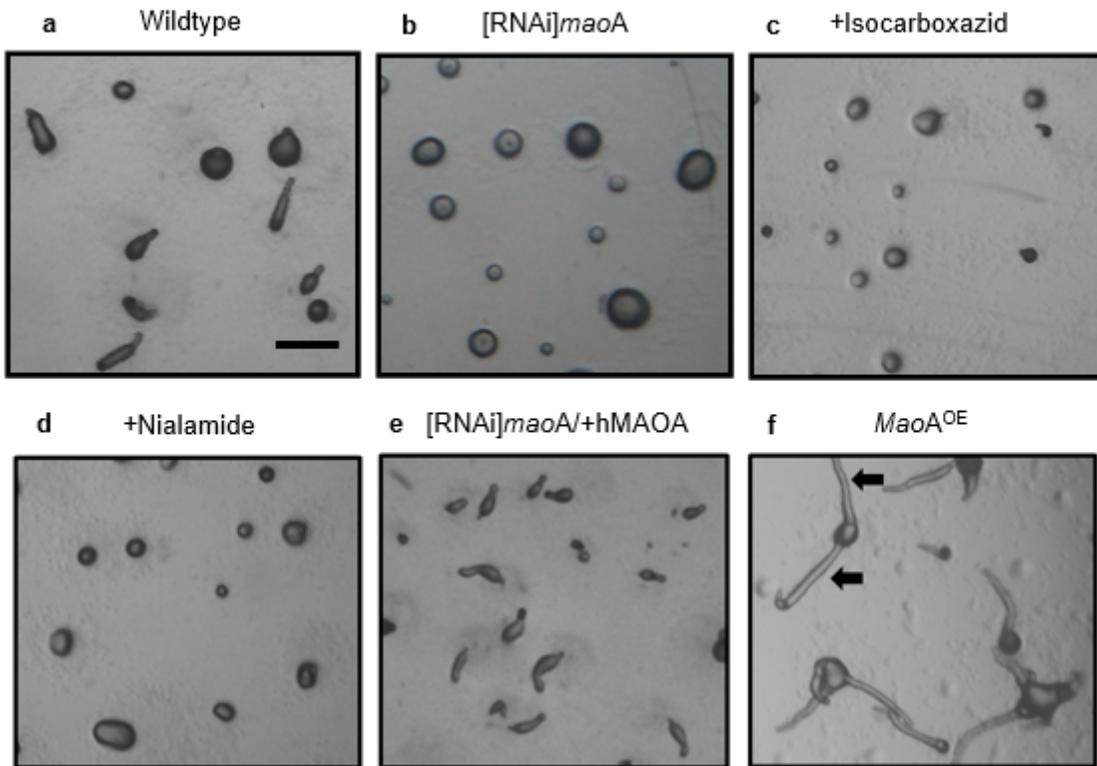


Figure 2

MaoA enables tip formation in Dictyostelium. a-b, [RNAi]maoA (b) and wildtype cells (a) were developed on non-nutrient KK2 agar and observed after 15 h. c-d, Wildtype cells were developed after mixing with 200 μ M isocarboxazid and nialamide and imaged after 15 h. e, [RNAi]maoA mounds at 14 h were treated with 10 units of hMAOA enzyme and observed after 4 h. f, MaoA OE clones were developed and photographed at the mound stage. Scale bar, 0.5 mm.

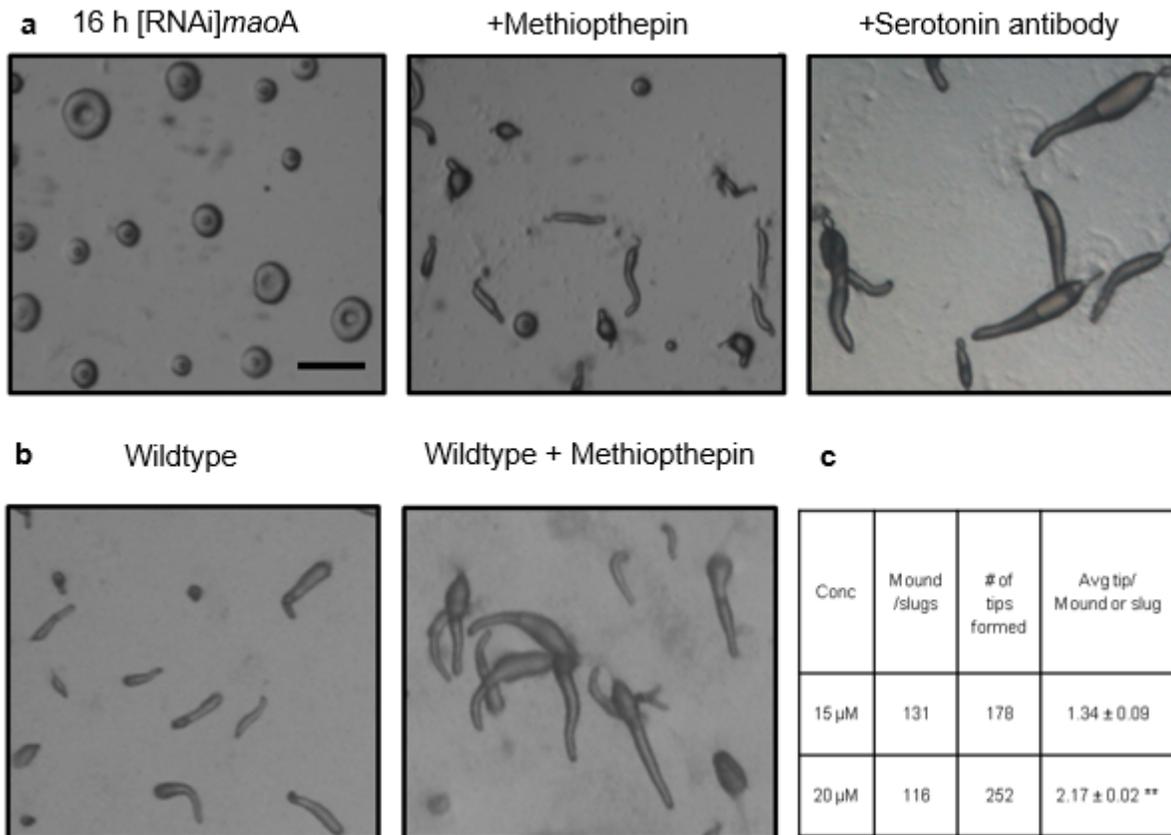


Figure 3

Serotonin enables tip dominance in *Dictyostelium*. a, [RNAi]*maoA* cells were mixed with either 2 μM of methiothepin or 1 in 40 dilutions of the antibodies against serotonin. The treated cells were plated on non-nutrient phosphate buffered agar along with the [RNAi]*maoA* control and observed at 16 h. b, 15 μM methiothepin was added onto wildtype mounds and imaged after 4 h. Scale bar, 0.5 mm. c, Average number of tips that emerged with increasing dosage of serotonin antagonist on wildtype mounds. **P< 0.01.

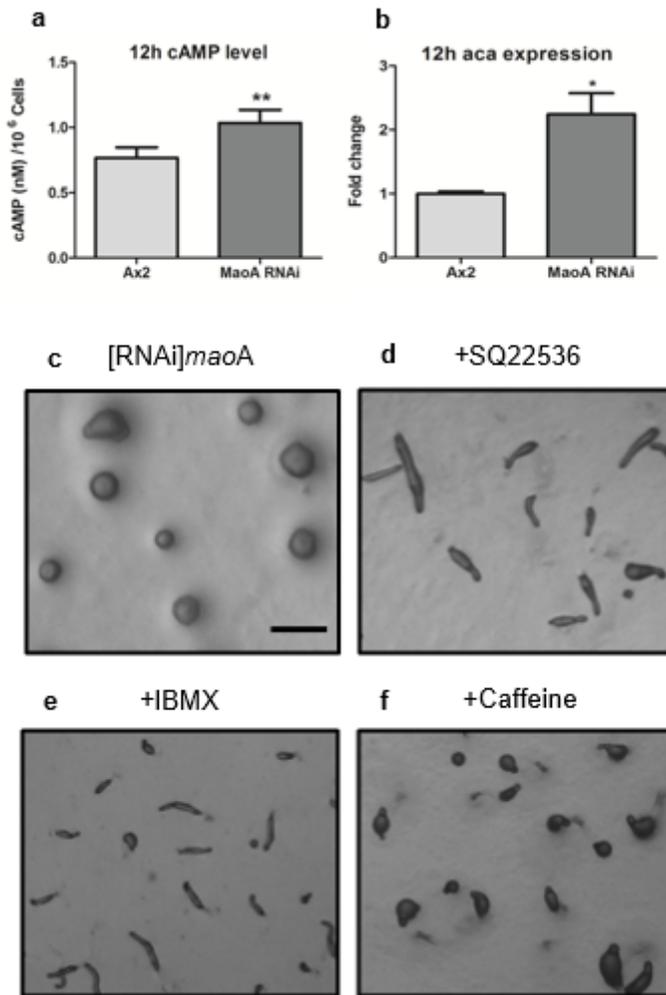


Figure 4

Mound arrest of [RNAi]*maoA* lines are due to altered cAMP signalling. a, Wildtype and [RNAi]*maoA* cells were developed till 14 h on phosphate buffered agar and lysed with 100 μ l of 1X lysis buffer. Total cAMP was quantified using cAMP-XP assay kit (Cell Signalling, USA). ** $P < 0.01$. b, Total RNA from wildtype and [RNAi]*maoA* mounds were isolated at 12 h and converted to cDNA using a commercial kit (Thermo Fisher, USA). Using a specific set of primers, *aca* expression was quantified using qRT-PCR. Error bars represent the mean and SEM ($n=3$). * $P < 0.05$. c, [RNAi]*maoA* mounds. d, *MaoA* knockdown mounds developed on non-nutrient agar were treated with 2 mM *aca* inhibitor. e-f, 0.5 mM IBMX (e) and 2 mM caffeine (f) were added on top of [RNAi]*maoA* mounds. Images were taken 2-4 h after treatment. Scale bar, 0.5 mm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryVideo.pptx](#)
- [ExtendedDataFigures.docx](#)