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A Conserved Role for Serotonergic Neurotransmission in Mediating Social Behavior in Octopus

Highlights

- Phylogenetic analysis revealed clear octopus orthologs of human *SLC6A4*
- *SLC6A4* protein alignment revealed conservation of the MDMA binding site in octopuses
- A novel assay was developed for quantification of octopus social behaviors
- Behavioral analysis revealed conservation of prosocial function of MDMA in octopuses

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In Brief

Edsinger and Dölen identify clear octopus orthologs of the human serotonin transporter gene, *SLC6A4*. This finding is paralleled by conservation of the *SLC6A4* binding site and acute prosocial functions of MDMA in octopuses. These data provide evidence for the evolutionary conservation of serotonergic signaling in the regulation of social behaviors.



A Conserved Role for Serotonergic Neurotransmission in Mediating Social Behavior in Octopus

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SUMMARY

Human and octopus lineages are separated by over 500 million years of evolution [1, 2] and show divergent anatomical patterns of brain organization [3, 4]. Despite these differences, growing evidence suggests that ancient neurotransmitter systems are shared across vertebrate and invertebrate species and in many cases enable overlapping functions [5]. Sociality is widespread across the animal kingdom, with numerous examples in both invertebrate (e.g., bees, ants, termites, and shrimps) and vertebrate (e.g., fishes, birds, rodents, and primates) lineages [6]. Serotonin is an evolutionarily ancient molecule [7] that has been implicated in regulating both invertebrate [8] and vertebrate [9] social behaviors, raising the possibility that this neurotransmitter's prosocial functions may be conserved across evolution. Members of the order Octopoda are predominantly asocial and solitary [10]. Although at this time it is unknown whether serotonergic signaling systems are functionally conserved in octopuses, ethological studies indicate that agonistic behaviors are suspended during mating [11–13], suggesting that neural mechanisms subserving social behaviors exist in octopuses but are suppressed outside the reproductive period. Here we provide evidence that, as in humans, the phenethylamine (+/–)-3,4-methylenedioxymethamphetamine (MDMA) enhances acute prosocial behaviors in *Octopus bimaculoides*. This finding is paralleled by the evolutionary conservation of the serotonin transporter (SERT, encoded by the *Slc6A4* gene) binding site of MDMA in the *O. bimaculoides* genome. Taken together, these data provide evidence that the neural mechanisms subserving social behaviors exist in *O. bimaculoides* and indicate that the role of serotonergic neurotransmission in regulating social behaviors is evolutionarily conserved.

RESULTS

Recently, we completed whole-genome sequencing and assembly in *Octopus bimaculoides* [14]. Because octopuses are thought to be the most behaviorally advanced invertebrates, this resource enables testing of molecular homology for complex behaviors, despite anatomical differences in brain organization across evolutionarily distant lineages [15]. In this context, it is interesting to note that in vertebrates (humans and rodents), the phenethylamine (+/–)-3,4-methylenedioxymethamphetamine (MDMA) is known for its powerful prosocial properties [16–18]. Furthermore, the sixth trans-membrane domain (TM6) of the serotonin transporter (SERT), encoded by the *SLC6A4* gene [19], has been identified as the principle binding site of MDMA [20–22]. To determine whether this binding site is conserved in *O. bimaculoides*, we performed a molecular phylogenetic analysis of the solute carrier (SLC) 6A subfamily of neurotransmitter transporter proteins [23] across selected diverse taxa (Tables S1 and S2). Blasting a reference gene set against 21 proteomes resulted in a final set of 503 sequences. These sequences were used to generate a maximum-likelihood phylogenetic tree (RAxML's "best tree") for SLC6A across all 21 species (Figure 1A; Figures S1, S2B, and S2C), along with 156 bootstrap trees and a bootstrap "consensus tree" (Figure S2A). The "best tree" included the full set of human SLC6A genes and no additional human genes from other SLC families (Figure 1B; Figures S2B and S2C; Table S3). Expected SLC6A4 family members were found for the fruit fly (*Drosophila melanogaster*), the worm (*Caenorhabditis elegans*), and all vertebrate species tested, although the "best tree" (Figure 1B) and the "consensus tree" (Figure S2A) differed slightly for the worm. Surprisingly, two copies of SLC6A4 were found in mollusks, including *O. bimaculoides*. One copy in octopuses, named here *Slc6a4-(1)* (protein Ocbimv22009795m.p; gene model Ocbimv22008529m.g) was part of a clade that contained only mollusks. The second copy, named here *Slc6a4-(2)* (protein Ocbimv22009795m.p; gene model Ocbimv22009795m.g), was in a group that included diverse species, like the fly and worm, but no mammals, vertebrates, or other deuterostomes. It is unclear whether this duplication is a molluscan innovation or has more ancient origins in the Lophotrochozoa. Two and three copies of SLC6A4 were present in the zebrafish (*Danio rerio*) and zebra finch (*Taeniopygia guttata*), respectively, but only a single copy was present in mammals and in basal-branching



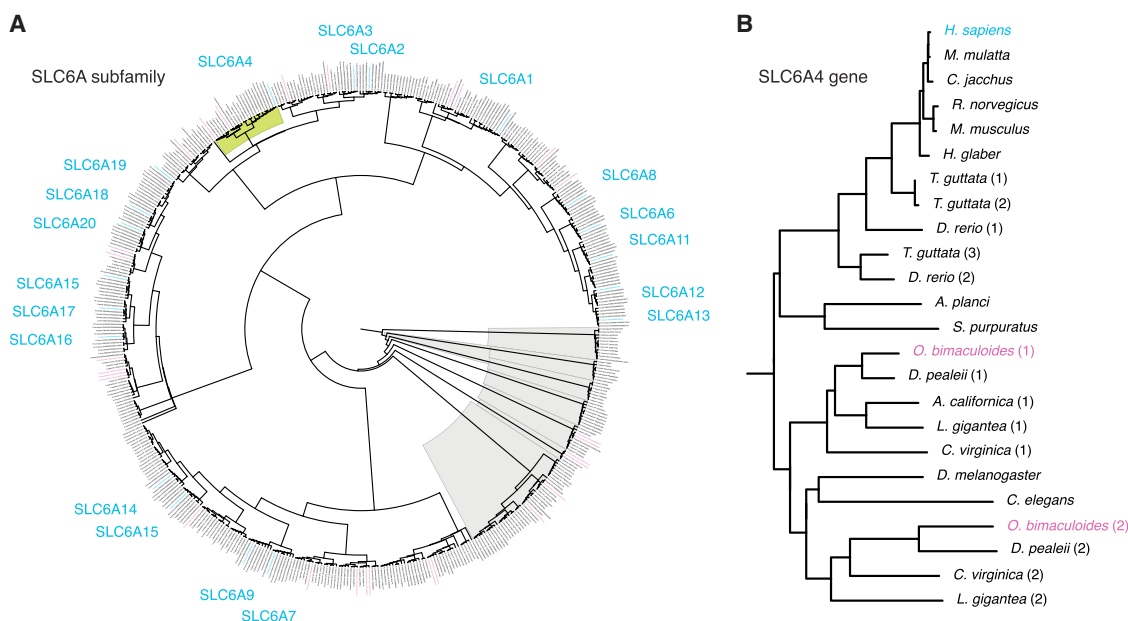


Figure 1. Phylogenetic Trees of SLC6A and SLC6A4 Gene Families

(A and B) Maximum-likelihood trees of SLC6A transporters (A) and SLC6A4 serotonin transporters (B) in select taxa. Species are mapped to tree and protein identifiers in Table S3. For a larger version of (A), see Figure S1.

(A) A maximum-likelihood “best tree” for the SLC6A gene family. The maximum-likelihood tree produced by RAxML includes 503 proteins and 21 species, with tree building based on a MAFFT alignment of full-length sequences.

(B) The SLC6A4 gene family, a subtree of the maximum-likelihood “best tree” in (A).

See also Figures S1–S3 and Tables S1–S3.

deuterostomes. These patterns may reflect differential loss of SLC6A4 after two rounds of vertebrate genome duplication [24], with a single copy in the ancestral vertebrate, and losses of three copies in mammals, two copies in fish, and one copy in birds (Figure 1B).

In contrast to the octopus, the honeybee (*Apis mellifera*), leaf cutter ant (*Atta cephalotes*), spider (*Stegodyphus mimosarum*), and anemone (*Nematostella vectensis*) all lacked SLC6A4 orthologs. To understand these losses, we next examined all monoamine transporters, including human SLC6A2 (DAT), SLC6A3 (NET), and SLC6A4 (SERT), along with their outgroup, a branch that includes the fruit fly inebriated (*Ine*) gene (Figures S1 and S2). Anemones had multiple paralogs within the *Ine* clade. Zebrafish and more basal-branching deuterostomes were also present, but other vertebrates, including humans, were absent. In contrast, the anemone was absent in SLC6A4 and throughout the monoamine transporter clade. Thus, monoamine transporters may represent an ancient innovation that arose early in bilaterian evolution, with various ancient and more recent duplications in different lineages. Representing ecdysozoans, the honeybee, leaf cutter ant, fruit fly, and worm all had one or more monoamine transporter proteins outside the SLC6A4 family, whereas only the fruit fly and worm had proteins within the family, suggesting that SLC6A4 has been lost in hymenopteran insects (ants, bees, wasps, and sawflies). Interestingly, although many of the invertebrates lacking SLC6A4 orthologs are eusocial, this adaptation is not ubiquitous across all eusocial species, since the SLC6A4 gene is conserved in the naked mole rat (*Heterocephalus glaber*), which is a eusocial vertebrate. Taken

together, these studies underscore the complexity of monoamine transporter evolution in animals and identify clear orthologs of human SLC6A4 in octopuses.

The binding pocket of SLC6A4 is formed by a subset of 12 transmembrane domains, including TM6 [19]. Previous studies have revealed an especially important role for the region of TM6 that spans amino acids 333 to 336 (indicated in green in Figure 2A), as it provides an overlapping binding pocket for MDMA and serotonin [20]. Furthermore, residue Ser336 has been implicated in the MDMA-induced conformational change not observed with serotonin [20]. Significantly, for both octopus orthologs within this region, there is 100% percent identity when aligned to human SLC6A4 (Figure 2; Figure S4A). More generally, many of the domains forming the binding pocket are highly conserved in comparison to other domains and to the full-length protein (Figure S4B). For instance, TM6 in Slc6a4-(1) has 95.7% identity to human SLC6A4, compared to 53.4% for the whole protein, and TM8 in Slc6a4-(2) has 91.0% identity to human SLC6A4, compared to 39.0% for the whole protein (Figure S4B). This work further demonstrates the remarkable conservation of transmembrane domains forming the MDMA and serotonin binding pocket in octopus SLC6A4 paralogs, including complete conservation of amino acids implicated in MDMA binding in humans.

In order to experimentally quantify octopus social behaviors, next we adapted the three-chambered social approach assay routinely used in rodents [25, 26] for use in *O. bimaculoides* [27, 28]. As diagrammed in Figure 3A, a glass aquarium partitioned into three equally sized chambers containing a novel object or a

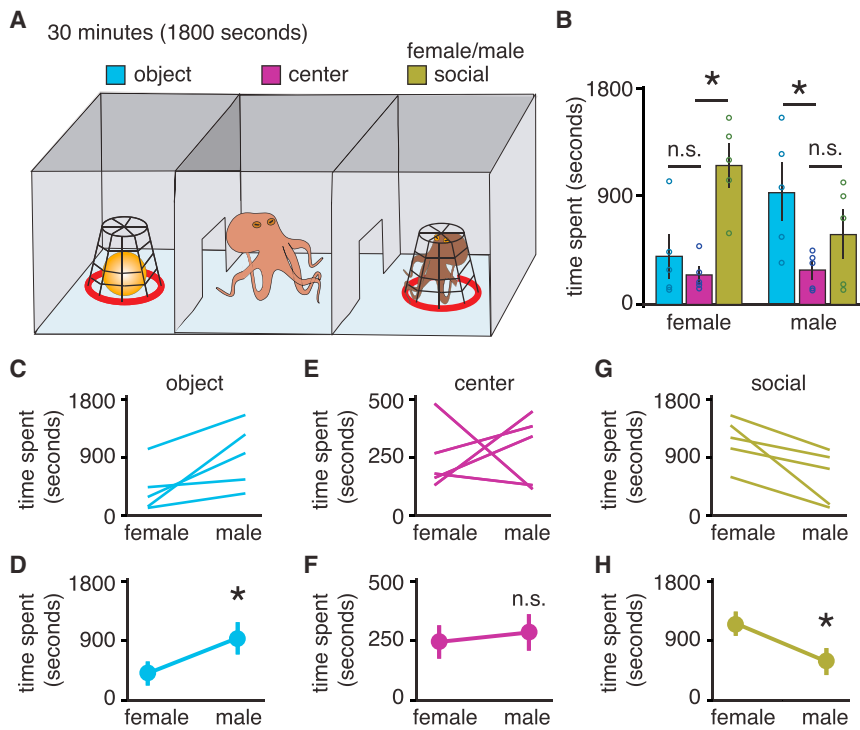


Figure 3. Social Approach Behaviors toward Male and Female Conspecifics in *O. bimaculoides*

(A) Diagram illustrating the three-chambered social approach assay.

(B) Quantification of time spent in each chamber during 30-min test sessions ($n = 5$; two-way repeated-measures ANOVA: $p = 0.0110$; post hoc unpaired t test female social object, social versus center $p = 0.0009$, object versus center $p = 0.4031$; male social object, social versus center $p = 0.1710$, object versus center $p = 0.0224$).

(C–H) Comparisons between female versus male social object conditions for each chamber (paired t test female versus male: novel object chamber $p = 0.0386$; social object chamber $p = 0.0281$; center chamber $p = 0.7544$).

Error bars represent the SEM. See also Table S4.

significant increase in locomotor activity (Figures 4J and 4K). Finally, in addition to the quantitative increase in the amount of time spent in the social chamber after MDMA, we also observed qualitative changes in social interactions. Specifically, as shown in Figure 4L, under saline conditions, even when subject animals spent time in the chamber containing the social object, direct contact between animals was limited, usually to one extended arm. In contrast, as shown in Figure 4M, after MDMA treatment, social interactions were characterized by extensive ventral surface contact, which appeared to be exploratory rather than aggressive in nature. Taken together, the present studies demonstrate that the acute prosocial effects of MDMA are conserved in *O. bimaculoides* and suggest that this pharmacological manipulation releases extant, but normally suppressed, neural mechanisms sub-serving social behaviors.

DISCUSSION

The current studies are the first to examine deep evolution of SLC6A, monoamine transporters, and the SLC6A4 gene family across the animal tree of life and across diverse animal genomes (Figure 1; Figures S1 and S2). Monoamine transporters, including human SERT, DAT, and NET, appear to be a bilaterian innovation, suggesting a possible ancient evolutionary role in nervous system centralization and elaboration, both hallmarks of the Bilateria, and the families have undergone complex patterns of gene duplication and loss throughout the clade over time. Phylogenetic analysis revealed clear orthologs of human SLC6A4 in octopuses, as well as high levels of conservation in the transmembrane domain and amino acid region critical to MDMA binding [20]. Interestingly, we found that SLC6A4 is broadly conserved in the fruit fly, the worm, and most other bilat-

erian animals but is surprisingly absent in both of the eusocial hymenopteran insects, the honeybee and leaf cutter ant (Figure 1; Figures S1 and S2). This absence raises the possibility that in these eusocial invertebrates, sociality evolved convergently utilizing other neurotransmitters or peptide hormones [30, 31]. Alternatively, it could be that loss of SLC6A4 is a permissive mutation for eusociality; however, the conservation of this gene in the eusocial naked mole rat (*H. glaber*) argues against this interpretation.

The current studies are also the first to experimentally quantify social approach behaviors in *O. bimaculoides* (Figures 3A–3K) and demonstrate that, consistent with previous ethological descriptions [11], this species shows no preference for social approach to a novel male conspecific (Figures 3A–3H). Nevertheless, somewhat surprisingly, both male and female subjects did exhibit social approach to a novel female conspecific (Figures 3A–3H), a finding that may reflect an adaptation of laboratory raised animals or an incomplete ethological description of the full repertoire of social behaviors in the wild [28]. Although we cannot rule out the possibility that the female versus male social object preference effect is governed by relative size differences between subject and social objects, we think this is unlikely since we observed aversion to a male social object both when the subject was greater and smaller in size (Table S4). Because the current study design allowed access to visual, tactile, and chemosensory cues, the selective contribution of these sensory systems to detecting male and female social cues is unknown. Based on previous reports that have implicated each of these sensory systems in the detection of social cues in octopuses [10, 12, 29], it seems likely that under conditions of selective sensory deprivation, the absence of one modality would be readily compensated by the presence of others.

Finally, the current studies provide the first functional evidence that the prosocial effects of MDMA [32] are evolutionarily conserved in *O. bimaculoides* (Figure 4). Although we did not observe a significant increase in locomotor activity after MDMA, this finding is consistent with the complex locomotor

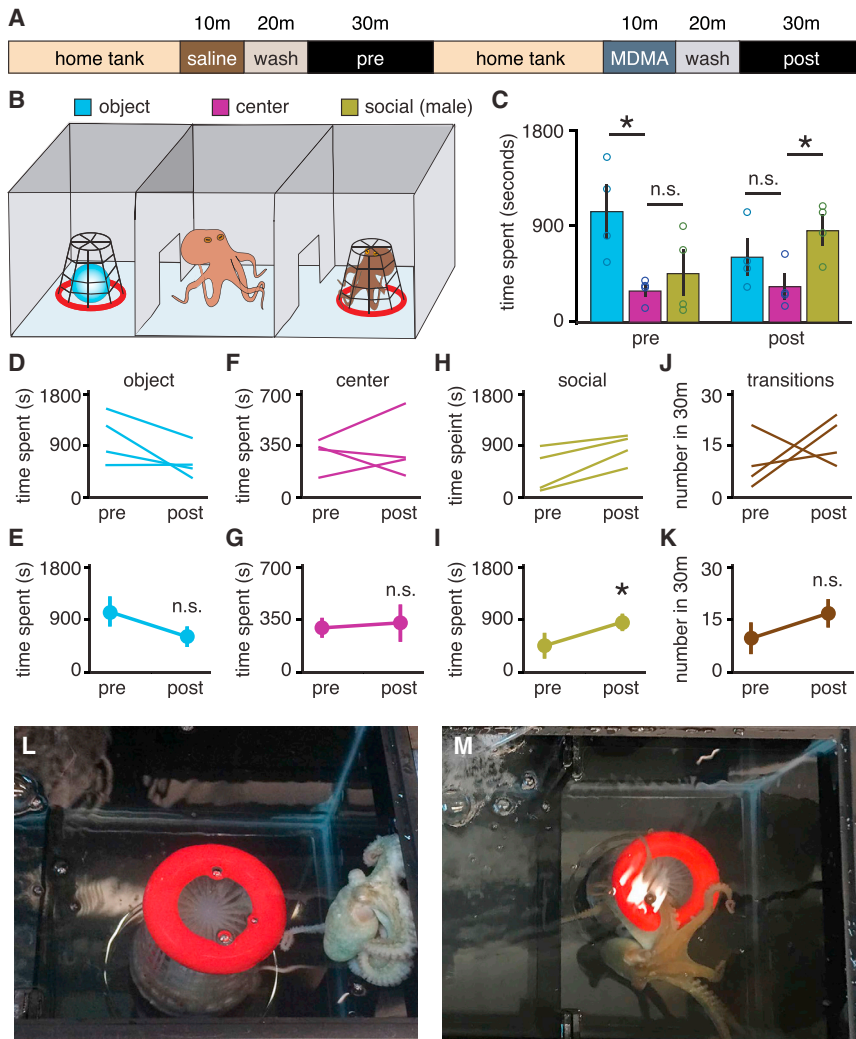


Figure 4. Prosocial Effects of MDMA in *O. bimaculoides*

(A and B) Diagrams illustrating timeline (A) and experimental protocol (B) for three-chambered social approach assay.

(C) Quantification of time spent in each chamber during 30-min test sessions ($n = 4$; two-way repeated-measures ANOVA: $p = 0.0157$; post hoc unpaired t test pre, social versus center $p = 0.4301$, object versus center $p = 0.0175$; post, social versus center $p = 0.0190$, object versus center $p = 0.1781$).

(D–K) Comparisons between pre- versus post-MDMA-treatment conditions (paired t test pre versus post, social time $p = 0.0274$; object time $p = 0.1139$; center time $p = 0.7658$; transitions $p = 0.3993$).

(L) Photograph of social interaction under the saline (pre) condition.

(M) Photograph of social interaction under the MDMA (post) condition.

Error bars represent the SEM. See also Table S4.

profile of MDMA previously reported in rodents [18, 33]. In addition, MDMA appeared to induce changes in the quality of social interactions, which are consistent with previously reported “entactogenic” properties of MDMA [34, 35]. Future quantification of these aspects of the behavioral response to MDMA in octopuses will be informative. Based on our ability to induce prosocial approach behaviors by manipulating serotonergic signaling, it is tempting to speculate that in octopuses, sociality is a latent state outside ethologically relevant periods, such as mating, and is manifested in response to MDMA treatment. Additionally, the current studies establish the first drug delivery protocols for behavioral pharmacology experiments in octopuses and indicate that effective doses of MDMA are in the same range as those described for humans and rodents. Beyond their utility for the current studies, development of this experimental infrastructure fulfills an unmet need for the field and will enable future mechanistic studies in octopuses. Moreover, this work demonstrates that medications currently in use or under investigation in humans [23, 36–38] target a homologous binding site in *O. bimaculoides* (Figure 2; Figure S4) and provides important proof-of-concept data supporting further

development of octopuses as model organisms for translational research.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article online at <https://doi.org/10.1016/j.cub.2018.07.061>.

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AUTHOR CONTRIBUTIONS

G.D. and E.E. designed the study, interpreted results, and wrote the paper. G.D. and E.E. performed behavioral and pharmacological experiments. E.E. performed phylogenetic analysis and octopus culturing. G.D. performed statistical analysis. Both authors edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
(+/-)-3,4-MDMA	Organix (Woburn, MA)	(+/-)-3,4-MDMA
Instant Ocean Sea Salt	PetCo	SKU77763

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gül Dölen (gul@jhu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Animals

Octopus bimaculoides, the California Two-Spot Octopus, was used for all behavioral experiments. A wild female octopus brooding developing embryos was commercially collected in the Los Angeles, CA area in accordance with all applicable California State and US Fish and Wildlife regulations, and live shipped to the Marine Biological Laboratory in Woods Hole, MA. After hatching, animals were housed together for roughly 2-3 weeks in tanks containing hundreds of other animals. Following this brief period in early development, each animal was housed in social isolation for a period of 7 months prior to testing. 7 siblings from the cohort were used in experiments here. [Table S4](#) details the sex and weight for each animal, as well as which animals were used for each experiment.

Housing and Husbandry

Animals were housed in a custom-built partially recirculating aquaria system, with natural heated seawater (20-23°C) pooled in a sump tank and shared between individual 18 and 36 L tanks. Each tank exchanged 100 L of recirculating seawater or more per hour, while the system exchanged 500 L or more of fresh seawater per day. Tank sides were covered $\frac{3}{4}$ their length with translucent plasticboard to allow animals to self-isolate visually from neighboring tanks. Early hatchling stages were group cultured and select sibling juveniles moved to individual tanks after a month. Each animal was housed individually (but with shared seawater) for the remainder of the project, outside the experiments. The room housing the animals maintained seasonal light cycles (12:12 or 14:10 light:dark cycles). Tank environments were enriched with clay pot dens and sand or rock substrate. Post-hatching stages were fed a daily diet of live crabs and snails. Sexually mature animals at around 9 months post-hatching were shipped to Johns Hopkins University for behavioral pharmacology experiments. Animals were maintained at Johns Hopkins University for several days in buckets with artificial seawater (Instant Ocean), air, dens, live food, and with water changes made twice daily. For the current studies we did not continuously monitor organic waste products because previous pilot studies—conducted in animals that were in the same weight range, living in buckets identical to those used in the current study, and had been fed 4 hr before—have shown that pH, nitrites, and nitrates did not change appreciably over a 12 hr period (which is the longest interval the animals in the current studies went without a water change). In one animal, ammonia levels did change from 0 to 2 ppm (on a scale of 0-8), but this amount was deemed acceptable for short term culturing. Monitoring was conducted with a Marine Saltwater Master Test Kit (API Marine). Room lighting at Johns Hopkins meant that animals had around 9 hr of dark each night and room temperature was 22-23°C. Animals were inspected hourly or more for evidence of wounds or disease, and their behavior generally monitored for signs of stress and agitation. Respiration rates and signs of excess agitation or decline were closely monitored during and after drug treatments. Aggressive interactions were also closely monitored for when more than one animal shared the experimental tank.

Ethical Considerations

Care of invertebrates, like *O. bimaculoides*, does not fall under United States Animal Welfare Act regulation, and is omitted from the PHS-NIH “Guide for the Care and Use of Laboratory Animals.” Thus, an Institutional Animal Care and Use Committee, a Committee on Ethics for Animal Experiments, or other granting authority does not formally review and approve experimental procedures on and care of invertebrate species, like *O. bimaculoides*, at the Marine Biological Laboratory. However, in accordance with Marine Biological Laboratory Institutional Animal Care and Use Committee guidelines for invertebrates, our care and use of *O. bimaculoides* at the Marine Biological Laboratory and at Johns Hopkins University generally followed tenets prescribed by the Animal Welfare Act, including the three ‘Rs’ (refining, replacing, and reducing unnecessary animal research).

METHOD DETAILS

SLC6 and SLC6A4 phylogenetic analysis

To identify orthologs of SLC6A4 in octopus, and to broadly characterize monoamine transporter evolution in animals, a SLC6 reference gene set was made using nineteen SLC6 proteins from human [23], in addition to six from fly and three from worm based on all fly and worm orthologs identified for the human genes in Ensembl (version 91). A more recent phylogenetic analysis of SLC6A in human lists 21 SLC6A genes [38]. However, the study also indicates SLC6A21 is a pseudogene. Furthermore, the study includes SLC6A10 but Ensembl lists SLC6A10 is a pseudogene, and neither appear to be part of the current proteome for human. Thus, we focus on the original 19 genes indicated by Kristensen 2011 [23]. All reference sequences were downloaded from UniProt (version 2018_01; Table S1). Twenty-one species representing molluscan diversity or that are models for studying sociality and that have published genomes were identified and their proteomes downloaded from Ensembl (version 91) or National Center for Biotechnology Center (NCBI) Genome (February 2018) (Table S2). Isoforms were collapsed to the longest protein per gene using a custom Python script, and local Blast databases were built per species proteome using makeblastdb in the BLAST+ package (version 2.7.1). In addition, five published transcriptomes of different brain regions in squid [39], which still lacks a publicly available genome, were downloaded from the Living in an Ivory Basement blog (<http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html>), combined into a single transcriptome, and translated to protein sequence with removal of shorter isoforms per default settings of EvidentialGene (<http://arthropods.eugenics.org/EvidentialGene/>). To determine BLAST+ blastp settings that best capture the diversity of SLC6A4 genes, while minimizing the introduction of excess genes from outside the SLC6 family, a series of blasts jobs were run under different settings for ‘-maxhits’ and ‘-e-val’ (5, e10-5; 10, e10-5; 25, e10-5; 25, e-25; 100, e10-5; 100, e-25). The output was analyzed to see if the complete human SLC6A gene set was returned and how many unique hits were found across all proteomes, assuming that both a full human SLC6A gene set with no additional human genes and a minimal number of total hits was ideal. For optimized blastp settings (-maxhits 25 and -eval e10-5), inspection of human sequences revealed 21 proteins, two more than expected for the human SLC6A gene subfamily. However, two proteins (ENSP000004795971 and ENSP000004800791) placed with other human SLC6A proteins (SLC6A3 and SLC6A8, respectively) in initial phylogenetic trees, and while the genes were similarly annotated as SLC6A3 and SLC6A8 by Ensembl, they were also flagged as potential assembly artifacts (no ungapped mapping and no stable id for the genes in GRCh37). Based on these findings, the two sequences were removed from the gene set prior to final alignment. The final set of 503 sequences identified under optimized blastp settings was aligned using Geneious (version 11.03) MAFFT (version 7.338; algorithm auto, gap open penalty 1.53, offset value 0.123, gap matrix BLOSUM 62) [40]. Alignments were run through GBlocks (version 0.91b; online server http://molevol.cmima.csic.es/castresana/Gblocks_server.html; default settings except allow smaller final blocks yes, allow gap positions within final blocks yes, and allow less strict flanking positions yes) to restrict alignments to highly conserved regions [41]. Models of protein evolution were determined for full-length and GBlock alignments using ModelTest-NG (version 0.1.3; default RAxML settings) and the best model used in tree building. RAxML-HPC Blackbox (version 8.2.10; default settings except Protein Substitution Matrix LG, empirical base frequencies yes, find best tree using maximum likelihood search yes) [42] on the CIPRES Science Gateway [43] was used to build trees for different sets of species and with or without GBlocks to explore the robustness of different trees and datasets. A consensus tree of resulting bootstrap trees was made using Geneious (version 11.03) Consensus Tree Builder (Support Threshold 0%). Resulting trees were visually assessed in Geneious (version 11.03) and FigTree (version 1.4.3). The tree built from full-length alignment and the gene set using blastp settings of -maxseq 25 and -eval e10-5 was used for the final analysis and all figures. For domain analysis, sequence annotation and pairwise alignments of proteins and domains were made using Geneious (version 11.03).

Octopus SLC6A4 comparative analysis

Domain annotation of human SLC6A4 was based on Uniprot and NCBI Conserved Domains CDSEARCH/cdd v3.16 [44]. Pairwise protein alignments of annotated human SLC6A4 were made to each of the octopus SCL6A4 genes using Geneious. Each domain alignment was then extracted and the percent identify per protein and per domain determined.

Behavioral Analysis

The social testing apparatus, illustrated in Figure 3A, was a rectangular, glass aquarium (76 X 30 X 30 cm, 68.4 L), separated into three equal sized chambers using black Plexiglas dividing walls, with small circular openings (3.5 cm in diameter) allowing access into each chamber. The outer walls of the chamber were covered with blackout window film (Window Whirl). Artificial seawater (Instant Ocean) was prepared per instructions from the manufacturing company, and the social testing apparatus filled to several centimeters from the top. Seawater osmolarity was measured using a micro osmometer (Precision Systems), and adjusted to 925 milliosmoles/liter (mOsm/L). Fresh seawater was prepared for each experiment, with the tank scrubbed in deionized water per seawater change. A single air stone was placed to the side of the center of the tank and generated mild local currents. Otherwise, water in the tank was stagnant.

Octopuses were tested in the social approach and MDMA experiments described below. For the social approach task (Figures 3 and 4), the subject octopus was first placed in the center chamber inside a 946 mL perforated screw top plastic cylindrical bottle (Pinnacle Mercantile), weighted down with a 450 g C-shaped lead flask ring (VWR) secured with Wet-Surface & Underwater Setting Epoxy (Hardman). After a two-minute habituation period, subject animals were released from the screw top container, and allowed to freely explore all three chambers for 30-min test sessions. The 30-min interaction time was determined empirically in pilot studies,

which revealed that octopuses, like mice, exhibit maximal exploratory behavior in the first 15–20 min, followed by increasing levels of quiescence (as measured by a decline in the number of chamber transitions). An unfamiliar octopus was placed in one of the side chambers (social object). The social object was enclosed in a weighted orchid pot or screw top plastic bottle, which allowed contact, but prevented fighting. Novel objects consisted of multiple configurations of 4 objects: 1) plastic orchid pot with red weight, 2) plastic bottle with green weight, 3) Galactic Heroes ‘Stormtrooper’ figurine, and 4) Galactic Heroes ‘Chewbacca’ figurine. In all cases the novel object included either the orchid pot or the bottle in order to keep the overall size of objects constant across experimental conditions. These containers contained one of the two figurines, which were selected to be maximally different in terms of color (dark brown versus white), but were the same size (50 mm height, 25 mm width) and material (plastic, containing no shiny or metallic parts). No obvious novel object preference bias was observed. The amount of time spent in each chamber and the number of entries into each chamber were manually scored by a single observer sitting at a central position, within one meter of the tank. The observer was visible to the subject animal only from the top of the tank, since all other walls were covered with a light-blocking sheet. The test arena was located in the corner of a larger laboratory space, so care was taken to restrict access to the immediate area (5–10 m) surrounding the test arena during behavioral testing. Entry was defined as both eyes and the mantle in one chamber.

Pharmacology

For subject animals 1, 4, and 7 the interval between the pre- and post-test was 24 hr. For the fourth subject (animal number 5), the interval between pre- and post-test was 5 hr. This difference was due to the fact that animal number 5 was used as the social object for other experiments, and was not tested under ‘pre’ conditions until all other non-drug experiments were concluded. Subject animal 5’s behavior was not appreciably different from animals 1, 4, and 7 with respect to either pre (saline) or post (MDMA) response, and formal quantification using the Shapiro-Wilk test revealed no statistical justification for excluding this animal (data normally distributed, no outliers).

The phenethylamine, (+/–)-3,4-methylenedioxymethamphetamine (MDMA) was obtained from Organix Inc (Woburn, MA, Gift of Rick Doblin, Multidisciplinary Association For Psychedelic studies, MAPS). Previous reports indicate that MDMA’s effects are maximally manifest 30–60 min following drug application [18, 45], therefore MDMA was delivered for 10 min, followed by a 20 min washout period before beginning the 30 min test session. Subject animals weighed between 130 to 200 g (Table S4), and were administered MDMA, by placing the animals in a beaker containing MDMA dissolved in 600 mL artificial seawater. Drug delivery by submersion is an established protocol for marine species including octopuses [46], whereby the drug enters the bloodstream directly through the gills [47]. Since the gills in a marine animal are analogous to the lungs in a land animal, a 10-min MDMA submersion for octopus would theoretically be the equivalent of continuously inhaled MDMA for 10 min in humans. In ongoing human clinical trials, MDMA is delivered orally (*per os*, p.o.) in the range of 0.67–2 mg/Kg (assuming an average adult human weighs 60 Kg). Since drug absorption following the p.o. route of administration is less efficacious than the inhaled route, for the current studies octopuses were given MDMA submersion doses between 0.5–0.005 mg/Kg, which corresponds to the low end of the oral dose range effective in humans. In addition, pilot studies in 3 animals indicated that higher submersion doses of MDMA (ranging from 10–400 mg/Kg) induced severe behavioral changes (e.g., hyper or depressed ventilation, traveling color waves across the skin or blanching, as well as catatonia or hyper-arousal/vigilance) and these animals were excluded from further analysis. In rodents, previous studies have demonstrated that the pharmacokinetics of MDMA are nonlinear, such that its metabolites interfere with the body’s ability to metabolize MDMA, resulting in additive effects that can lead to hepatotoxicity even with low-concentration repetitive dosing [48, 49]. Thus, here we only examined the acute effects of MDMA in drug naive animals, and each subject only received a single dose of MDMA during the course of the experiments. Animals were returned to the aquarium system at the Marine Biological Laboratory following the experiments described here and were used in other unrelated research projects.

QUANTIFICATION AND STATISTICAL ANALYSIS

Global comparisons between experimental manipulations were made using 2x3 2-factor ANOVA with repeated-measures on both factors. The 2-factor repeated-measures ANOVA was chosen because it is robust to violations of normality, and we are unaware of any existing non-parametric test that can be used with a 2-factor study design. Furthermore, in order to determine the appropriateness of using parametric statistics for this dataset, we have formally calculated whether our data meet statistical criteria for normal distribution using the Shapiro-Wilk test. Out of 14 experimental groups, the data is normally distributed for all groups, and contained no outliers. For post hoc comparisons, two-tailed, Student’s t test (paired or unpaired, as appropriate) were used. For all statistical tests, a p value < 0.05 was considered significant. Statistical parameters (n values, SEM, p values) are reported in the Figure legends.

Experimental design

In order to maximize data robustness, behavioral quantification was restricted to objective measures including time spent in chamber and number of transitions and strict scoring criteria were applied (e.g., chamber transition defined as both eyes and mantle passing through the opening). Because the scoring was carried out by an unblinded observer, care was taken to extensively cross-validate the scorer’s inter-rater reliability against automated measures (e.g., infrared beam breaks, Med Associates) in mice. Since mice exhibit greater locomotor activity compared to octopuses, and are therefore more difficult to score, we are confident in the observer’s ability to accurately score the time spent in each chamber for octopus. An animal was excluded from analysis if it failed to enter all three chambers at least once during the defined test period. In order to control for any side preference

bias, the social object and novel object positions were held constant within an experiment, and only counterbalanced between experiments. No obvious side preference bias was observed. For qualitative descriptions of social behaviors, photographic examples were provided, and clinical criteria were applied [34, 35]. Sample sizes were estimated based on previously published results using this assay in mice.

DATA AND SOFTWARE AVAILABILITY

Sequences, alignments, and tree text files and the data used for statistical analyses of behavior are all available in Mendeley Data at <https://doi.org/10.17632/z9t3x4p5kk.1>.