

# Current Biology

## 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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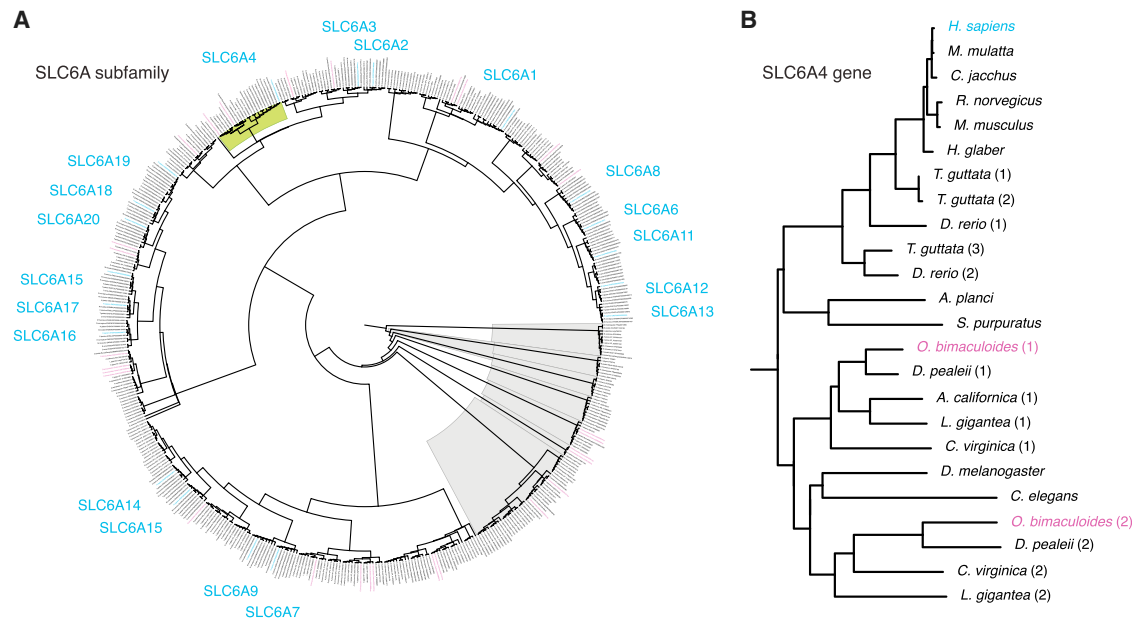
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## SUMMARY

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.

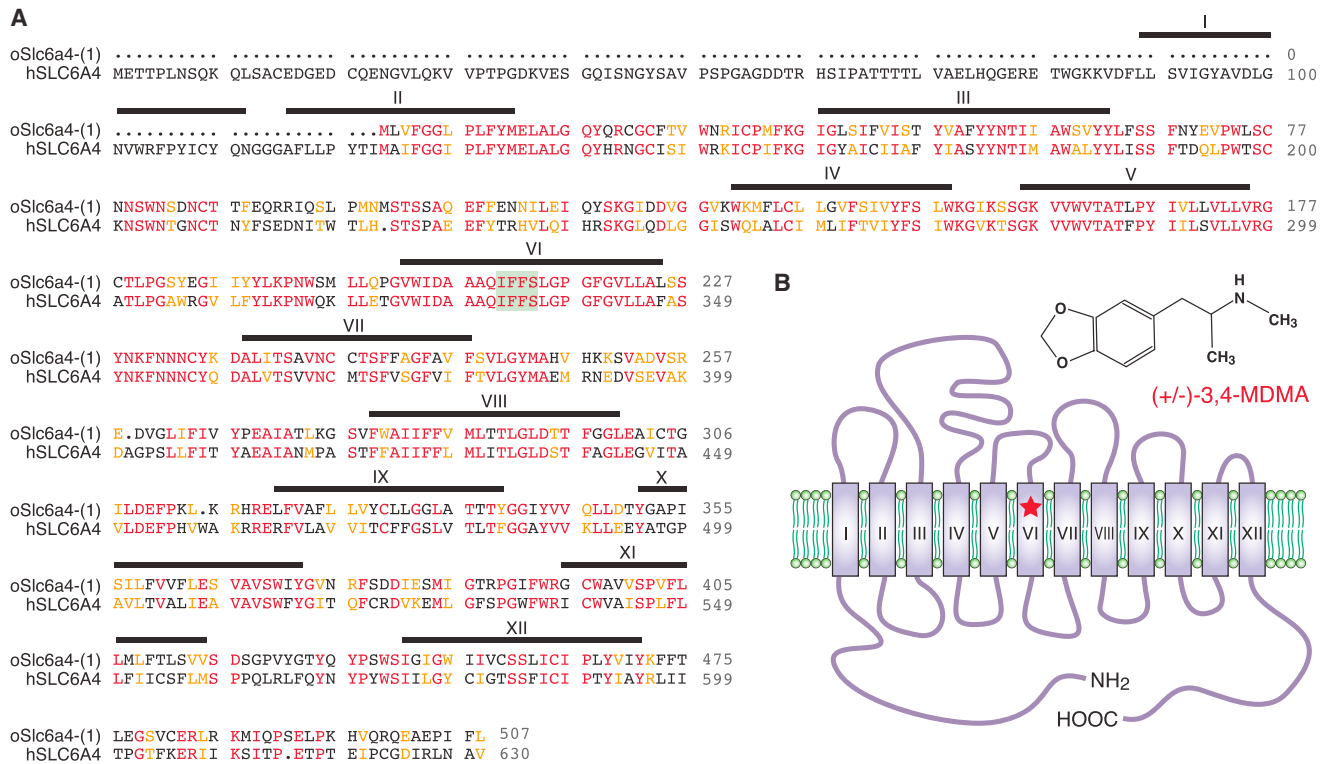
deuterostomes. These patterns may reflect differential loss of SLC6A4 after two rounds of vertebrate genome duplication [24], with a single copy in the ancestral vertebrate, and losses of three copies in mammals, two copies in fish, and one copy in birds (Figure 1B).

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 diagramed in Figure 3A, a glass aquarium partitioned into three equally sized chambers containing a novel object or a



**Figure 2. Protein Alignment of *O. bimaculoides* Slc6a4-(1) to Human SLC6A4**

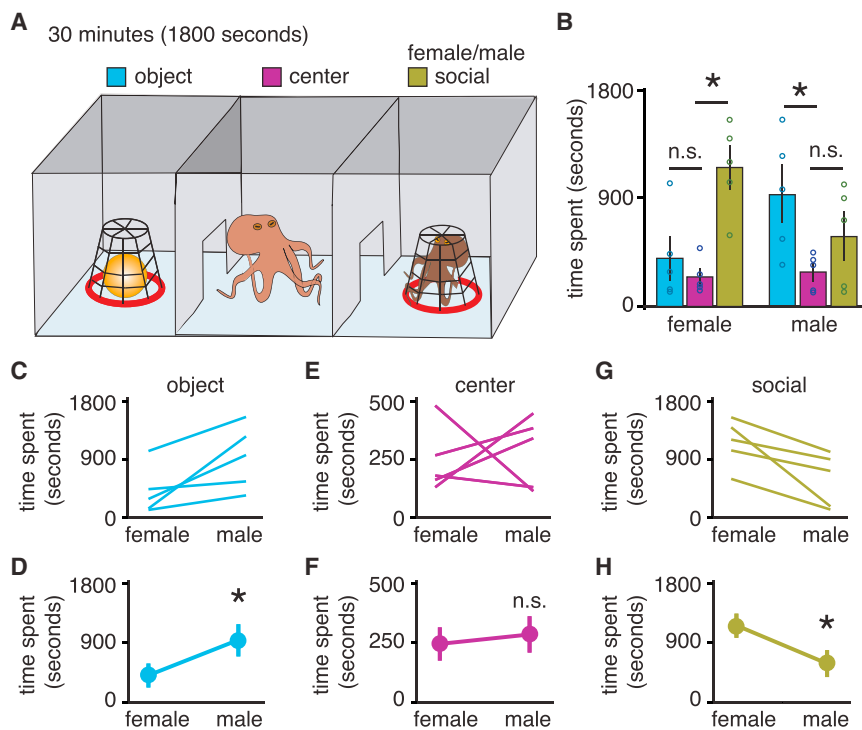
(A) Pairwise protein alignment. Human SLC6A4 transmembrane domain 1–12 annotations are based on Uniprot Topology annotation of protein UniProt: P31645 (SLC6A4\_HUMAN). The binding pocket region that overlaps serotonin and MDMA binding (green) is based on [20]. Alignment of annotated human SLC6A4 to octopus Slc6a4-(1) was done by pairwise alignment in Geneious.

(B) Diagram illustrating the structure of MDMA, transmembrane domains of the SLC6A4 protein, and the MDMA binding site (red star). Surprisingly, domain 1 was absent or largely absent in each of the two octopus paralogs (see also [Figure S4](#)); however, the domain (annotate all paralogs directly) was present in both paralogs for the four other mollusks, suggesting that its absence in octopuses might be a sequencing or assembly artifact of the genome).

See also [Figures S1–S3](#) and [Tables S1–S3](#).

novel conspecific (male or female social object) in each of the lateral chambers, as well as an empty center chamber, served as the test arena. Social-object animals were restrained by a perforated plastic container that allowed bidirectional access to visual, tactile, and chemosensory cues, which are thought to convey social information in octopuses [10, 12, 29]. The amount of time subject animals spent freely exploring each chamber was recorded during 30-min test sessions. As shown in Figure 3B, when the social object was a female, male and female subject animals spent significantly more time in the social chamber compared to the center chamber, whereas when the social object was a male, subject animals spent significantly more time in the object chamber compared to the center chamber. Comparisons between conditions revealed that the time spent with the novel object was significantly increased (Figures 3C and 3D) and time spent with the social object was significantly decreased (Figures 3G and 3H) when the social object was a male versus a female. The time spent in the center chamber was unchanged across conditions (Figures 3E and 3F). These data provide the first quantification of social approach behavior in *O. bimaculoides* and demonstrate a significant preference for interactions with female versus male social objects.

We next sought to test the functional conservation of MDMA's effects in *O. bimaculoides*. The strongest test of functional conservation would be to determine whether MDMA induces social approach to a social object that is normally aversive. Thus, we again used the three-chambered social approach task described above, but this time measured approach to a male social object before and after treatment with MDMA. As diagrammed in [Figures 4A](#) and [4B](#), baseline social approach behaviors were tested in drug naive subjects in an arena containing a novel object and a male social object for 30 min (pre-trial). 5 to 24 hr later, subject animals were placed in a bath containing MDMA for 10 min, followed by a 20-min saline wash, and were then tested again for 30 min (post-trial). As shown in [Figure 4C](#), during the pre-trial, subject animals spent significantly more time in the object chamber compared to the center chamber, whereas in the post-trial, subject animals spent significantly more time in the social chamber compared to the center chamber. Comparisons between pre- versus post-MDMA conditions revealed that the time spent with the social object was significantly increased after MDMA treatment ([Figures 4H](#) and [4I](#)), whereas the time spent in the object and center chambers was not significantly different across conditions ([Figures 4D–4G](#)). Quantification of the number of transitions between chambers indicated no



**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

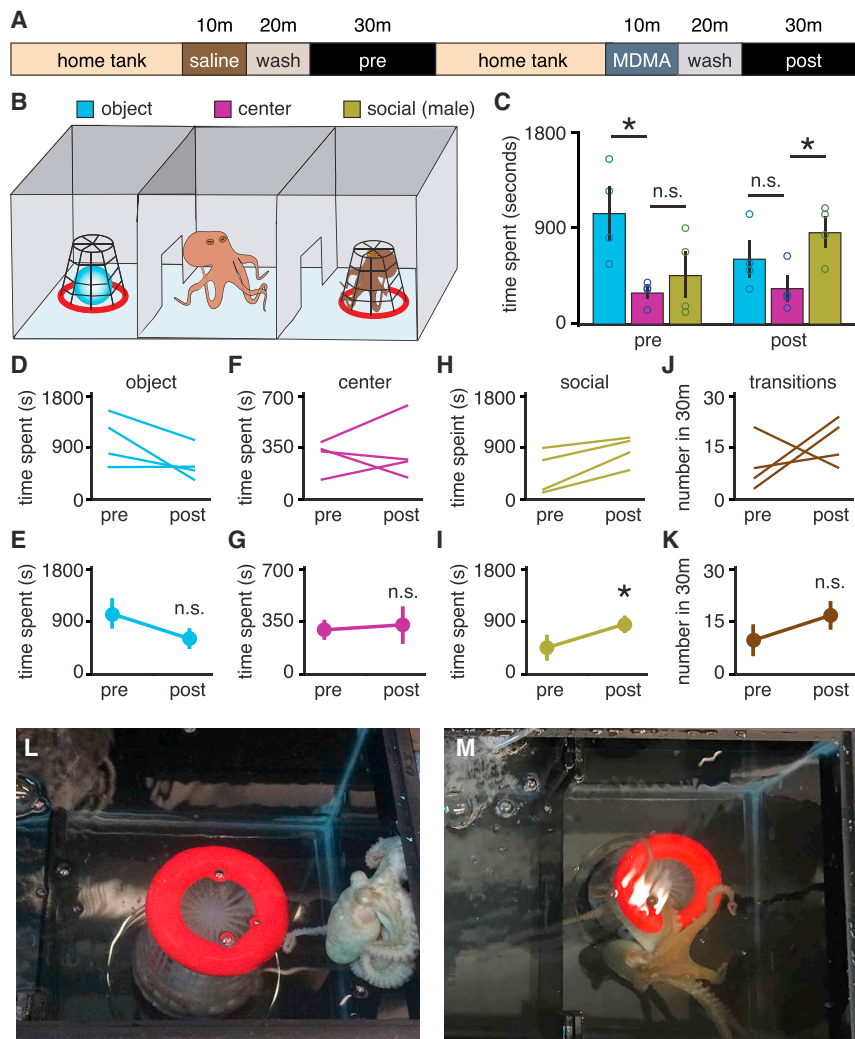
The current studies are the first to examine deep evolution of SLC6A, monoamine transporters, and the SLC6A4 gene family across the animal tree of life and across diverse animal genomes (Figure 1; Figures S1 and S2). Monoamine transporters, including human SERT, DAT, and NET, appear to be a bilaterian 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-

[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:

- KEY RESOURCES TABLE
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  - Behavioral Analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Experimental design
- 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](mailto: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](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-HPG 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>.

**Current Biology, Volume 28**

**Supplemental Information**

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

**Eric Edsinger and Gül Dölen**



Taxa

- Human

Vertebrata

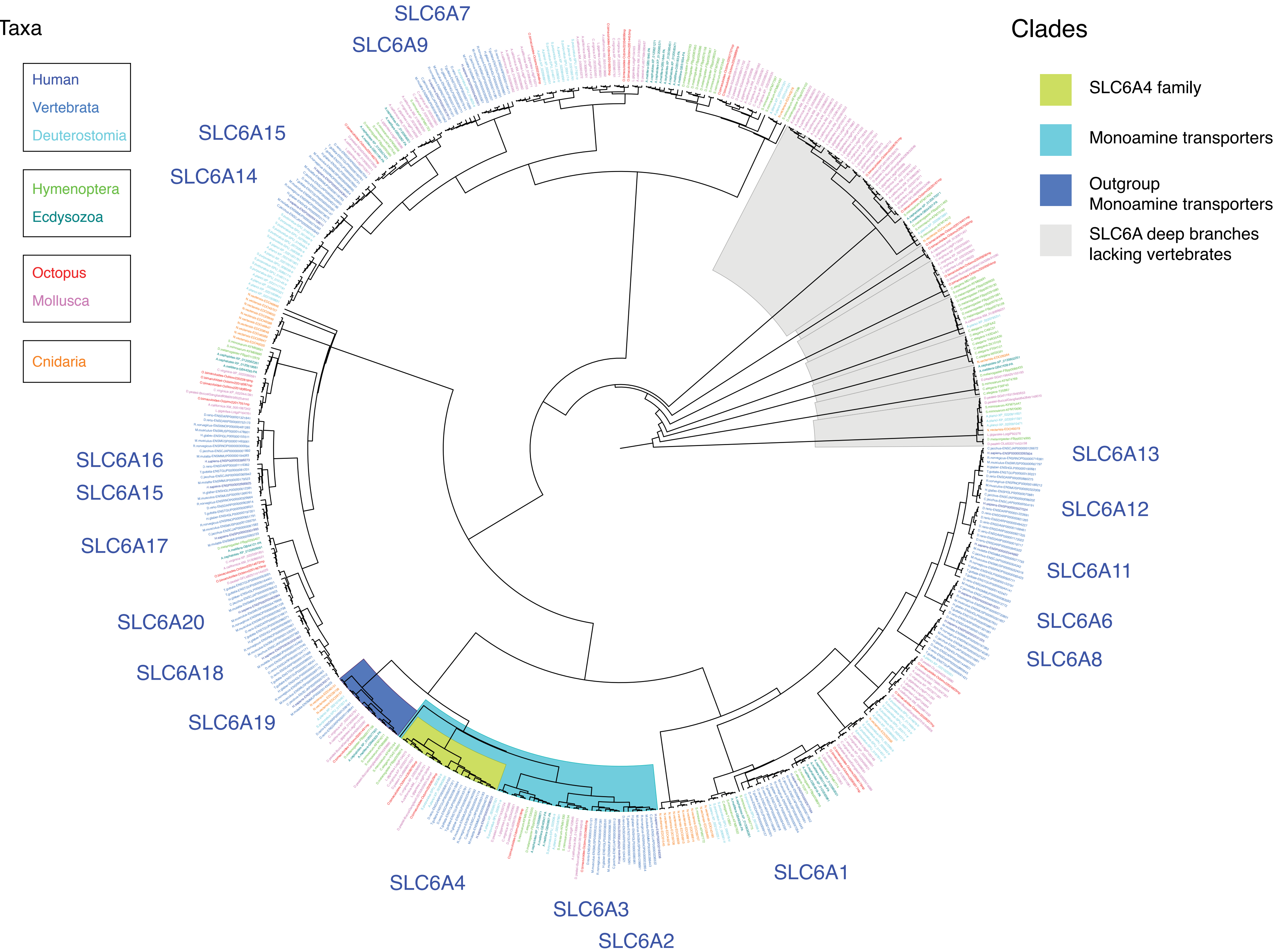
Deuterostomia
- Hymenoptera

Ecdysozoa
- Octopus

Mollusca
- Cnidaria

Clades

- SLC6A4 family
- Monoamine transporters
- Outgroup  
Monoamine transporters
- SLC6A deep branches  
lacking vertebrates



**Figure S1. A larger version of Figure 1A with major animal groups highlighted by color. Related to Figure 1.** Monoamine transporters, including SLC6A4, have undergone complex patterns of evolution in diverse animal groups. Two copies are found in octopus and all mollusc genomes examined, except the sea slug (*Aplysia californica*), which lacked Slc6a4-(2), an absence that may reflect incompleteness of its current genome assembly. Spider proteins were often on long-branches and placed to the outside of clades, or were embedded within non-arthropod clades, throughout the SLC6A tree (**Figure 1A, S1, and S2**), and their highly divergent sequences remain difficult to interpret phylogenetically. Still, there are likely monoamine transporters in spider, even if their exact affinity or origins remains unresolved. Non-SLC6A4 monoamine transporters appear to have duplicated in the ancestral bilaterian. One copy was lost in vertebrates (Clade A) but the second copy (Clade B) was duplicated in vertebrates (Clades C (SLC6A2) and D (SLC6A3)) perhaps through gene retention following the first round of vertebrate genome duplication. Consistent with previous reports [S1] zebra finch lacked DAT orthologs. A number of deep branches lacking vertebrate orthologs clustered at the base of the SLC6A tree and may include non-SLC6A proteins but this requires further analysis of SLC evolution.



**Figure S2. SLC6A monoamine transporters subtree to highlight species composition per clade and branch lengths. Related to Figure 1. (A)** Maximum-likelihood tree. The depicted subtree contains monoamine transporters plus their outgroup as found in the maximum likelihood tree of **Figure 1A**. The tree was re-formatted to highlight species composition per clade in FigTree. **(B)** Maximum-likelihood tree. The monoamine transporter subtree of **Figure S3A** is re-formatted to highlight branch lengths. Turquoise arrows indicate branch tips for proteins in the SLC6A4 gene family and include sea urchin and spider. Blue arrows indicate branch tips for proteins in the outgroup to monoamine transporters and include squid and spider. **(C)**

A

SLC6A Monoamine Transporters and Outgroup

Phylogenetic Taxa

- Human

Vertebrata

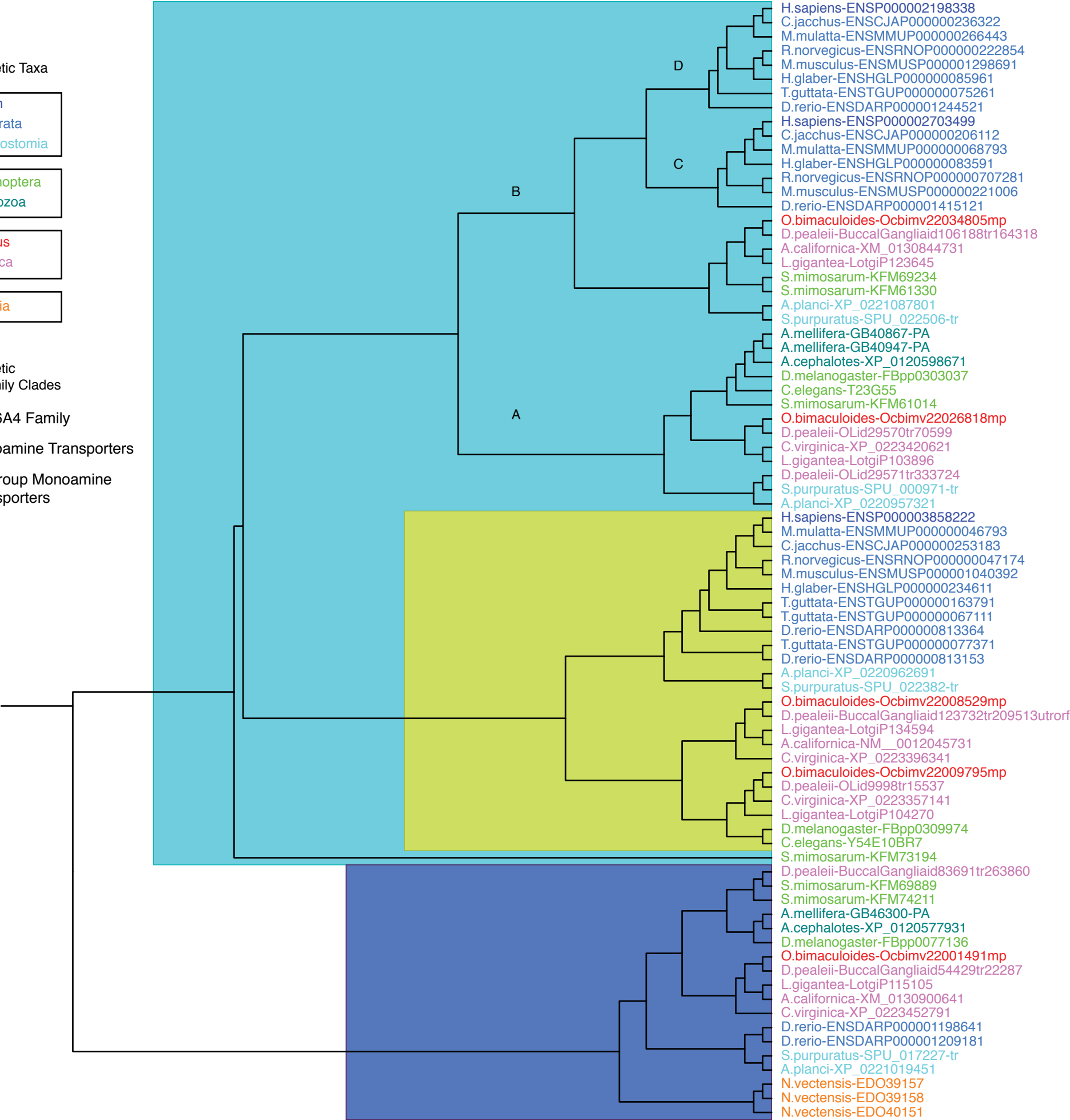
Deuterostomia
- Hymenoptera

Ecdysozoa
- Octopus

Mollusca
- Cnidaria

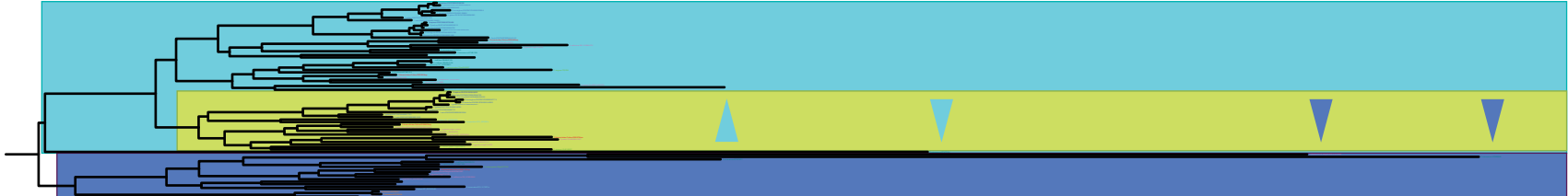
Phylogenetic Gene Family Clades

- SLC6A4 Family
- Monoamine Transporters
- Outgroup Monoamine Transporters



B

SLC6A Monoamine Transporters and Outgroup: Branch lengths





**Figure S3. Consensus bootstrap tree for the SLC6A4 gene family. Related to Figure 1. (A)** Maximum likelihood bootstrap “consensus tree”. The “consensus tree” was made based on 156 bootstrap trees generated by RAxML for the maximum likelihood “best tree” of the SLC6A gene family (**Figure 1A**).

[illegible]

### Human SLC6A4 Octopus SLC6A4-A

Domain

Domain	Human SLC6A4	Octopus SLC6A4-A
1	0.0%	
2	60.0%	
3	57.7%	
4	36.8%	
5	60.0%	
6	69.6%	
7	65.0%	
8	91.0%	
9	40.0%	
10	31.8%	
11	25.0%	
12	47.6%	

### Human SLC6A4 Octopus SLC6A4-B

Domain

Domain	Human SLC6A4	Octopus SLC6A4-B
1	0.0%	
2	75.0%	
3	57.7%	
4	42.1%	
5	85.0%	
6	95.7%	
7	65.0%	
8	77.3%	
9	40.0%	
10	45.5%	
11	35.0%	
12	52.4%	

**Figure S4. Details of protein and domain alignments for *O. bimaculoides* Slc6a4-(1) and Slc6a4-(2) to human SLC6A4. Related to Figure 2. (A)** Protein and domain pairwise alignments. Human SLC6A4 transmembrane domain 1-12 annotations are based on Uniprot Topology annotation of protein UniProt: P31645 (SC6A4\_HUMAN). Annotated Human SLC6A4 was aligned to octopus Slc4a4-(1) and Slc6a4-(2) using Geneious. Aligned regions were then extracted per domain. **(B)** Pairwise protein alignment. Human SLC6A4 transmembrane domain 1-12 annotations are based on Uniprot Topology annotation of protein UniProt: P31645 (SC6A4\_HUMAN). The binding pocket region that overlaps serotonin and MDMA binding (green) is based on [S2]. Alignment of annotated human SLC6A4 to octopus Slc6a4-(2) was done by pairwise alignment in Geneious. Identical amino acids are indicated in red, while positive (physiochemically similar) amino acids are indicated in orange.

<b>Species</b>	<b>Uniprot Identifier</b>	<b>Gene Name</b>
<i>Homo sapiens</i>	UniProt: A0A024R2G0	SLC6A1
<i>Homo sapiens</i>	UniProt: P23975	SLC6A2
<i>Homo sapiens</i>	UniProt: Q01959	SLC6A3
<i>Homo sapiens</i>	UniProt: P31645	SLC6A4
<i>Homo sapiens</i>	UniProt: Q9Y345	SLC6A5
<i>Homo sapiens</i>	UniProt: A0A087WY96	SLC6A6
<i>Homo sapiens</i>	UniProt: E5RJL1	SLC6A7
<i>Homo sapiens</i>	UniProt: P48029	SLC6A8
<i>Homo sapiens</i>	UniProt: P48067	SLC6A9
<i>Homo sapiens</i>	UniProt: P48066	SLC6A11
<i>Homo sapiens</i>	UniProt: P48065	SLC6A12
<i>Homo sapiens</i>	UniProt: Q9NSD5	SLC6A13
<i>Homo sapiens</i>	UniProt: Q9UN76	SLC6A14
<i>Homo sapiens</i>	UniProt: Q9H2J7	SLC6A15
<i>Homo sapiens</i>	UniProt: Q9GZN6	SLC6A16
<i>Homo sapiens</i>	UniProt: Q9H1V8	SLC6A17
<i>Homo sapiens</i>	UniProt: Q96N87	SLC6A18
<i>Homo sapiens</i>	UniProt: Q695T7	SLC6A19
<i>Homo sapiens</i>	UniProt: Q9NP91	SLC6A20
<i>Drosophila melanogaster</i>	UniProt: A0A0B4LHJ6	SerT (SLC6A4)
<i>Drosophila melanogaster</i>	UniProt: Q6NND1	GAT (SLC6A1)
<i>Drosophila melanogaster</i>	UniProt: Q1RKX2	CG5549 (SLC6A5)
<i>Drosophila melanogaster</i>	UniProt: B5RIX7	ntl (SLC6A7)
<i>Drosophila melanogaster</i>	UniProt: A0A0B4K7V4	CG43066 (SLC6A19)
<i>Drosophila melanogaster</i>	UniProt: B7Z122	CG10804 (SLC6A19)
<i>Caenorhabditis elegans</i>	UniProt: Q963F3	mod-5 (SLC6A4)
<i>Caenorhabditis elegans</i>	UniProt: G5EF43	snf-11 (SLC6A1)
<i>Caenorhabditis elegans</i>	UniProt: G5EBN9	snf-3 (SLC6A)

**Table S1. SLC6A reference gene set for phylogenetic analysis. Related to Figure 1.** A mapping of species to Uniprot identifier and gene symbol for the reference gene set is provided.



Name	Genus species	NCBI Identifier	Proteome File Name / Proteome version	Proteome URL
western honey bee	<i>Apis mellifera</i>	NCBI Taxonomy: 7460	Arthropoda_Apis_mellifera.Amel_4.5.pep.all.fa	<a href="https://metazoa.ensembl.org/Apis_mellifera/Info/Index">https://metazoa.ensembl.org/Apis_mellifera/Info/Index</a>
leafcutter ant	<i>Atta cephalotes</i>	NCBI Taxonomy: 12957	Arthropoda_Atta_cephalotes.Attacep1.0.pep.all.fa	<a href="http://metazoa.ensembl.org/Atta_cephalotes/Info/Index">http://metazoa.ensembl.org/Atta_cephalotes/Info/Index</a>
fruit fly	<i>Drosophila melanogaster</i>	NCBI Taxonomy: 7227	Arthropoda_Drosophila_melanogaster.BDGP6.pep.all.fa	<a href="https://metazoa.ensembl.org/Drosophila_melanogaster/Info/Index">https://metazoa.ensembl.org/Drosophila_melanogaster/Info/Index</a>
African social velvet spider	<i>Stegodyphus mimosarum</i>	NCBI Taxonomy: 407821	Arthropoda_Stegodyphus_mimosarum.Stegodyphus_mimosarum_v1.pep.all.fa	<a href="http://metazoa.ensembl.org/Stegodyphus_mimosarum/Info/Index">http://metazoa.ensembl.org/Stegodyphus_mimosarum/Info/Index</a>
white-tufted ear marmoset	<i>Callithrix jacchus</i>	NCBI Taxonomy: 9483	Chordata_Callithrix_jacchus.C_jacchus3.2.1.pep.all.fa	<a href="https://useast.ensembl.org/Callithrix_jacchus/Info/Index">https://useast.ensembl.org/Callithrix_jacchus/Info/Index</a>
zebrafish	<i>Danio rerio</i>	NCBI Taxonomy: 7955	Chordata_Danio_rerio.GRCz10.pep.all.fa	<a href="https://useast.ensembl.org/Danio_rerio/Info/Index">https://useast.ensembl.org/Danio_rerio/Info/Index</a>
naked mole rat (female)	<i>Heterocephalus glaber</i>	NCBI Taxonomy: 10181	Chordata_Heterocephalus_glaber_female.HetGla_female_1.0.pep.all.fa	<a href="https://useast.ensembl.org/Heterocephalus_glaber_female/Info/Index">https://useast.ensembl.org/Heterocephalus_glaber_female/Info/Index</a>
human	<i>Homo sapiens</i>	NCBI Taxonomy: 9606	Chordata_Homo_sapiens.GRCh38.pep.all.fa	<a href="https://useast.ensembl.org/Homo_sapiens/Info/Index">https://useast.ensembl.org/Homo_sapiens/Info/Index</a>
Rhesus monkey	<i>Macaca mulatta</i>	NCBI Taxonomy: 9544	Chordata_Macaca_mulatta.Mmul_8.0.1.pep.all.fa	<a href="https://useast.ensembl.org/Macaca_mulatta/Info/Index">https://useast.ensembl.org/Macaca_mulatta/Info/Index</a>
mouse	<i>Mus musculus</i>	NCBI Taxonomy: 10090	Chordata_Mus_musculus.GRCm38.pep.all.fa	<a href="https://useast.ensembl.org/Mus_musculus/Info/Index">https://useast.ensembl.org/Mus_musculus/Info/Index</a>
rat	<i>Rattus norvegicus</i>	NCBI Taxonomy: 10116	Chordata_Rattus_norvegicus.Rnor_6.0.pep.all.fa	<a href="https://useast.ensembl.org/Rattus_norvegicus/Info/Index">https://useast.ensembl.org/Rattus_norvegicus/Info/Index</a>
zebra finch	<i>Taeniopygia guttata</i>	NCBI Taxonomy: 59729	Chordata-Taeniopygia_guttata.taeGut3.2.4.pep.all.fa	<a href="https://useast.ensembl.org/Taeniopygia_guttata/Info/Index">https://useast.ensembl.org/Taeniopygia_guttata/Info/Index</a>
anemone	<i>Nematostella vectensis</i>	NCBI Taxonomy: 45351	Cnidaria_Nematostella_vectensis.ASM20922v1.pep.all.fa	<a href="https://metazoa.ensembl.org/Nematostella_vectensis/Info/Index">https://metazoa.ensembl.org/Nematostella_vectensis/Info/Index</a>
crown of thorns seastar	<i>Acanthaster planci</i>	NCBI Taxonomy: 133434	Echinodermata_Acanthaster_planci.GCF_001949145.	<a href="https://www.ncbi.nlm.nih.gov/genome/7870?genome">https://www.ncbi.nlm.nih.gov/genome/7870?genome</a>

			1_OKI-Apl_1.0_protein.faa	<a href="#">assembly_id=301786</a>
sea urchin	<i>Strongylocentrotus purpuratus</i>	NCBI Taxonomy: 7668	Echinodermata_Strongylocentrotus_purpuratus.Spur_3.1.pep.all.faa	<a href="https://metazoa.embl.org/Strongylocentrotus_purpuratus/Info/Index">https://metazoa.embl.org/Strongylocentrotus_purpuratus/Info/Index</a>
oyster	<i>Crassostrea virginica</i>	NCBI Taxonomy: 6565	Mollusca_Crassostrea_virginica.GCF_002022765.2_C_virginica-3.0_protein.faa	<a href="https://metazoa.embl.org/Crassostrea_gigas/Info/Index">https://metazoa.embl.org/Crassostrea_gigas/Info/Index</a>
snail	<i>Lottia gigantea</i>	NCBI Taxonomy: 225164	Mollusca_Lottia_gigantea.Lotgi1.pep.all.faa	<a href="https://metazoa.embl.org/Lottia_gigantea/Info/Index">https://metazoa.embl.org/Lottia_gigantea/Info/Index</a>
octopus	<i>Octopus bimaculoides</i>	NCBI Taxonomy: 37653	Mollusca_Octopus_bimaculoides.PRJNA270931.pep.all.faa	<a href="https://metazoa.embl.org/Octopus_bimaculoides/Info/Index">https://metazoa.embl.org/Octopus_bimaculoides/Info/Index</a>
worm	<i>Caenorhabditis elegans</i>	NCBI Taxonomy: 6239	Nematoda_Caenorhabditis_elegans.WBcel235.pep.all.faa	<a href="https://metazoa.embl.org/Caenorhabditis_elegans/Info/Index">https://metazoa.embl.org/Caenorhabditis_elegans/Info/Index</a>
squid	<i>Doryteuthis pealeii</i>	NCBI Taxonomy: 1051067	LPealei.Buccalganglion.Annotated.transcriptome.v1.0.fasta	<a href="http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html">http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html</a>
squid	<i>Doryteuthis pealeii</i>	NCBI Taxonomy: 1051067	LPealei.GFL.Annotated.transcriptome.v1.0.fasta	<a href="http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html">http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html</a>
squid	<i>Doryteuthis pealeii</i>	NCBI Taxonomy: 1051067	LPealei.OL.Annotated.transcriptome.v1.0.fasta	<a href="http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html">http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html</a>
squid	<i>Doryteuthis pealeii</i>	NCBI Taxonomy: 1051067	LPealei.SG.Annotated.transcriptome.v1.0.fasta	<a href="http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html">http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html</a>
squid	<i>Doryteuthis pealeii</i>	NCBI Taxonomy: 1051067	LPealei.VerticalLobe.Annotated.Transcriptome.v1.0.fasta	<a href="http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html">http://ivory.idyll.org/blog/2014-loligo-transcriptome-data.html</a>
sea slug	<i>Aplysia californica</i>	NCBI Taxonomy: 6500	GCF_000002075.1_AplCal3.0_protein.faa	<a href="https://www.ncbi.nlm.nih.gov/genome/?term=txid6500[orgn]">https://www.ncbi.nlm.nih.gov/genome/?term=txid6500[orgn]</a>

**Table S2. Species and proteome sources for phylogenetic analysis. Related to Figure 1.** A mapping of species names and their NCBI identifiers to proteome sources is provided.

Genus species	SLC6A4 Tree Identifier	SLC6 Tree Identifier	Genome Protein Identifier
<i>Homo sapiens</i>	H.sapiens	H.sapiens-ENSP000003858222	ENSP000003858222
<i>Macaca mulatta</i>	M.mulatta	M.mulatta-ENSMUMP000000046793	ENSMUMP000000046793
<i>Callithrix jacchus</i>	C.jacchus	C.jacchus-ENSCJAP0000000253183	ENSCJAP0000000253183
<i>Rattus norvegicus</i>	R.norvegicus	R.norvegicus-ENSRNOP0000000047174	ENSRNOP0000000047174
<i>Mus musculus</i>	M.musculus	M.musculus-ENSMUSP000001040392	ENSMUSP000001040392
<i>Heterocephalus glaber</i>	H.glaber	H.glaber-ENSHGLP0000000234611	ENSHGLP0000000234611
<i>Taeniopygia guttata</i>	T.guttata (1)	T.guttata-ENSTGUP0000000163791	ENSTGUP0000000163791
<i>Taeniopygia guttata</i>	T.guttata (2)	T.guttata-ENSTGUP0000000067111	ENSTGUP0000000067111
<i>Taeniopygia guttata</i>	T.guttata (3)	T.guttata-ENSTGUP0000000077371	ENSTGUP0000000077371
<i>Danio rerio</i>	D.rerio (1)	D.rerio-ENSDARP0000000813364	ENSDARP0000000813364
<i>Danio rerio</i>	D.rerio (2)	D.rerio-ENSDARP0000000813153	ENSDARP0000000813153
<i>Acanthaster planci</i>	A.planci	A.planci-XP_0220962691	XP_0220962691
<i>Strongylocentrotus purpuratus</i>	S.purpuratus	S.purpuratus-SPU_022382-tr	SPU_022382-tr
<i>Crassostrea virginica</i>	C.virginica (1)	C.virginica-XP_0223396341	XP_0223396341
<i>Crassostrea virginica</i>	C.virginica (2)	C.virginica-XP_0223357141	XP_0223357141
<i>Aplysia californica</i>	A.californica	A.californica-NM_0012045731	NM_0012045731
<i>Lottia gigantea</i>	L.gigantea (1)	L.gigantea-LotgiP134594	LotgiP134594
<i>Lottia gigantea</i>	L.gigantea (2)	L.gigantea-LotgiP104270	LotgiP104270
<i>Doryteuthis pealeii</i>	D.pealeii (1)	D.pealeii-BuccalGangliaid123732tr209513 utrorf	BuccalGangliaid123732tr209513 utrorf
<i>Doryteuthis pealeii</i>	D.pealeii (2)	D.pealeii-OLid9998tr15537	OLid9998tr15537
<i>Octopus bimaculoides</i>	O.bimaculoides (1)	O.bimaculoides-Ocbimv22008529mp	Ocbimv22008529mp
<i>Octopus bimaculoides</i>	O.bimaculoides (2)	O.bimaculoides-Ocbimv22009795mp	Ocbimv22009795mp
<i>Drosophila melanogaster</i>	D.melanogaster	D.melanogaster-FBpp0309974	FBpp0309974
<i>Caenorhabditis elegans</i>	C.elegans	C.elegans-Y54E10BR7	Y54E10BR7

**Table S3. Mapping of species to their SLC6A4 gene and tree identifiers. Related to Figure 1.** A mapping is provided to link each species depicted in the trees of **Figure 1** to their protein and tree identifiers.

Animal	Sex	Weight (Kg)	Exp 1-female social object	Exp 1-male social object	Exp 2-Pre	Exp 2-Post
			Feb 10, 2018 AM	Feb 10, 2018 PM	Feb 10, 2018 PM * Feb 13, 2018 6PM	Feb 11, 2018 PM * Feb 13, 2018 11PM
1	M	0.132	Social object = 2 (female) Novel object = Chewbacca-Pot	Social object = 5 (male) Novel object = Stormtrooper-Pot	Social object = 5 (male) object = Stormtrooper-Pot	Social object = 5 (male) object = Stormtrooper-Pot
2	F	0.156	N/A	N/A	N/A	N/A
3	M	0.081	Social object = 2 (female) Novel object = Chewbacca-Pot	Social object = 5 (male) Novel object = Stormtrooper-Pot	N/A	N/A
4	F	0.176	Social object = 2 (female) Novel object = Chewbacca-Pot	Social object = 5 (male) Novel object = Stormtrooper-Pot	Social object = 5 (male) object = Stormtrooper-Pot	Social object = 5 (male) object = Stormtrooper-Pot
5	M	0.118	N/A	N/A	* Social object = 7 (male) object = Stormtrooper-bottle	* Social object = 7 (male) object = Stormtrooper-bottle
6	M	0.145	Social object = 2 (female) Novel object = Chewbacca-Pot	Social object = 5 (male) Novel object = Stormtrooper-Pot	N/A	N/A
7	M	0.152	Social object = 2 (female) Novel object = Chewbacca-Pot	Social object = 5 (male) Novel object = Stormtrooper-Pot	Social object = 5 (male) object = Stormtrooper-Pot	Social object = 5 (male) object = Stormtrooper-Pot

**Table S4. Experimental animals used for behavioral analysis. Related to Figures 3 and 4.**  
Weight, sex, age and experimental date information is provided for each animal.



### **Supplemental References:**

[S1]. Lovell, P. V., Kasimi, B., Carleton, J., Velho, T.A., and Mello, C. V. (2015). Living without DAT: Loss and compensation of the dopamine transporter gene in sauropsids (birds and reptiles). *Sci. Rep.* 5, 1–12.

[S2]. Field, J., Henry, L., and Blakely, R. (2010). Transmembrane domain 6 of the human serotonin transporter contributes to an aqueously accessible binding pocket for serotonin and the psychostimulant 3,4-methylene dioxymethamphetamine. *J. Biol. Chem.* 285, 11270–80.