Statistical characterization of social interactions and collective behavior in medicinal leeches

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METHODS

Animals and preparations. Adult leeches (H. verbana) obtained from Ricarimpex (Eysines, France) were kept at 10°C in tap water dechlorinated by aeration for 24 h. Leeches were anesthetized with an 8% ethanol in leech saline solution (in mM: 116 NaCl, 1.8 CaCl₂, 4 KCl, 1.5 MgCl₂, 10 glucose, 10 Tris maleate buffered to pH 7.4), and two colored beads with a diameter of ~3 mm were glued onto the dorsal side of the leeches with 3M Vetbond Tissue Adhesive (World Precision Instruments, Berlin, Germany) near their head and tail. Leeches were allowed to recover from anesthesia and then were transferred to the observation tank where their motion was tracked in real time. Leeches were kept in either a large (top diameter 28 cm; bottom diameter 18 cm; height 15 cm) or a small (top diameter 18 cm; bottom diameter 14 cm; height 10 cm) circular tank filled with dechlorinated tap water kept at 10–15°C. Observation tanks were illuminated with a dim, diffuse light with no abrupt spatial and/or temporal gradients. No additional sensory stimuli such as chemical, mechanical, or visual inputs were intentionally applied. A transparent...
plastic disk was used to keep leeches inside the tank. We used two leech populations, referred to as “hungry” and “satiated”: satiated leeches were those arriving from the supplier and kept in the laboratory unfed for <2 mo; hungry leeches were those kept unfed for >8 mo.

Delivery of neuromodulators. Some leeches were injected with specific amounts of DA or 5-HT. A 4 mM DA (Sigma-Aldrich)-containing saline solution was freshly prepared every day, stored in dark conditions at 4°C, and used within 1 h. A 1 mM 5-HT (Sigma-Aldrich)-containing saline solution was stored at −20°C in 500-µl aliquots and defrosted in 30 min at room temperature. Immediately before use, an aliquot of the DA or 5-HT stock solution was diluted in leech saline solution to its final concentration. We injected 300 µl of 5-HT or DA solutions at different concentrations, using 1-ml insulin syringes with 29-gauge, 15-mm-long needles. Each leech was gently held in one hand, and the needle was inserted under the skin of the dorsal side, along its dorsal axis at a depth of ~10% of the body thickness. We assume that the injected solution was diluted into the extracellular space roughly 25–30% of the leech volume, usually 3–4 ml. Therefore, the final drug concentration injected in the leeches is approximately one-fourth of the injected drug concentration.

The body volume of an adult leech is 3–4 ml, while the overall blood volume is ~8–9% of the body mass, corresponding to 240–360 µl (Wenning and Meyer 2007). To determine the relation between the internal level of a neuromodulator and the associated behavior, we dissolved different amounts of 5-HT or DA varying from 50 to 800 µM in the same amount of leech saline (300 µl). As a control for the effects of diluting the leech’s own neuromodulators, we also monitored the behaviors of leeches injected with 300 µl of leech saline without any added drug. Changes of body volume are well tolerated by leeches; because they can increase their initial weight by 8–11 times during feeding (Dickinson and Lent 1984), it is likely that an increase of the body volume (~10% of its body mass) caused by the injection did not impair the animal’s movements.

Imaging. We used a color charge-coupled device (CCD) camera (640 × 480 pixels of image size, model DFK 21BF04; The Imaging Source Europe) to image leeches from above to monitor their movements (Fig. 1A). The camera was connected via the FireWire output to a frame grabber (PCI-1394; Texas Instruments), installed on a personal computer, able to process images in real time. Colored beads glued onto the backs of the leeches were tracked at 15 Hz with software developed in Matlab language (MathWorks, Natick, MA). Images were directly acquired in the hue/saturation/lightness color space (Fig. 1B) and converted into red, green, and blue (RGB) values (Fig. 1C).

The real-time algorithm is able to track up to six beads of different colors. Because the tank and the leeches have almost the same dark blackish color, the algorithm easily detects the colored beads. Dark pixels corresponding either to the background or to the leech bodies were first detected by a simple threshold procedure. The RGB color distribution of used beads was measured before the experiment. As the beads are homogeneously colored, their color distribution is Gaussian and each bead is characterized by its mean color vector and the correspondent covariance matrix. Subsequently, a Bayesian classifi-
categorization of colored pixels was performed in the RGB feature space. If \( v_i = [r, g, b] \) is the color vector of the \( i \)-th pixel, \( m_i \) is the mean vector and \( \Sigma_i \) the covariance matrix, respectively, of the \( i \)-th detected colored region \((k = 1 \ldots L; L = N + 1; \text{where } N \text{ is the number of the beads})\). Each colored bead represents a single class that can be visualized as a covariance ellipsoid in the feature space (Fig. 1C). The most probable class or color to which the \( i \)-th pixel belongs is obtained by minimizing the Mahalanobis distance (Mahalanobis, 1936) \( d_{ik} = (v_i - m_i)' \Sigma_i^{-1} (v_i - m_i) \). This criterion represents a quadratic Bayesian classifier that can be implemented to work in real time. After classification, the algorithm produces \( N \) binary images, one for each bead, in which the ON bit represents the pixel belonging to it. Then the algorithm searches for a cluster of at least 10 ON pixels and calculates the center of mass of its coordinates (Fig. 1D). The software stores only the coordinates of detected beads so that it is possible to monitor leech behavior for several hours. In experiments requiring the use of a goldfish, the algorithm was tuned to recognize the color of the goldfish’s body and the color of the leeches’ body.

**Computation of velocity.** From the time series describing the coordinates of the head and tail bead we computed the head \([V_{\text{head}}(n)]\) and tail \([V_{\text{tail}}(n)]\) velocity. On other occasions we computed the leech center of mass as the midpoint between the head and tail beads (see Fig. 2), and its velocity was computed. Centers of mass, head, and tail velocities were computed by the convolution of the corresponding time series of the \( x \) and \( y \) coordinates with the derivative of a Gaussian function. Similarly, in experiments requiring the use of a goldfish, the fish velocity \( v_f \) was calculated by convolving the \( x \) and \( y \) coordinates of the center of mass with the derivative of a Gaussian function and the average velocity vector from the leech population \( \langle \rangle \) was calculated by convolving the \( x \) and \( y \) coordinates of the center of mass of each detected leech—or group of leeches—with the derivative of a Gaussian function. In all the calculations we used a Gaussian function with a standard deviation of 1–2 s.

**Classification of behavior of leeches.** Leeches were monitored for 3–12 h. The corresponding time series usually comprised \( >100,000 \) samples. We adopted an automatic classification method to analyze behavior based on the kinematics and spectral content of the recorded time series as previously described (Mazzoni et al. 2005). Briefly, leech movements were classified into five different categories (Fig. 1): exploratory movements (Fig. 1E), crawling (Fig. 1F), swimming (Fig. 1G), pseudoswimming (which is likely to be ventilation) (Fig. 1H), and stationary (i.e., not moving). At each time \( n \) of the recorded time series, different criteria were used to discriminate each behavior. The stationary state was detected by computing the speed of the head \( V_{\text{head}}(n) \) and tail \( V_{\text{tail}}(n) \). If both speeds were <1 pixel/s, the state was considered stationary. The other behavioral categories were identified by computing the following parameters: 1) elongation \( e(n) \), i.e., the head distance from head to tail; 2) maximal speed \( V_{\text{max}}(n) \) of the head over a time window of width \( T \) centered in \( n \) (with \( T \) ranging from 20 to 40 s); and 3) dominant frequencies \( f_d(n) \) of the spectrum \( S(f) \) of \( e(n) \), computed with a Hamming window of length 30 s and with 96.7% overlap. Oscillatory behaviors (such as swimming, pseudoswimming, and crawling) could be reliably identified by analyzing the pairs of values \([V_{\text{head}}^2(n), f_d(n)]\): swimming episodes were characterized by \( f_d(n) \sim 1 \text{ Hz} \) and \( V_{\text{max}}^2(n) \sim 20 \text{ pixels/s} \), whereas crawling was associated to \( f_d(n) \sim 0.08 \text{ Hz} \) and \( V_{\text{max}}^2(n) \sim 30 \text{ pixels/s} \). It should be noted that all definitions of behavior are based on units of pixels; in our recording system, 1 pixel corresponds to 0.4 mm.

**Test for independence between behavioral processes.** In the time domain, transitions among behaviors can be described by a Markov process, the structure of which (allowed states and transitions) is highly conserved across individuals (Garcia-Perez et al. 2005). We thus estimated the transition matrix of a hidden Markov model by the

![Fig. 2. A and B: schematics of the experiments. Before the light was turned on and leech motion recording began, leeches were kept in the same tank (A) or were transferred into a new tank (B). In the latter case, we observed an initial phase during which leeches explored and searched the new tank, followed by a second phase in which leeches were not moving. C: time evolution of the displacement (in pixels) of a leech center of mass from the axis origin (corresponding to the lower left corner of the camera-acquired frames). To define the duration of the initial searching phase, we calculated the center of mass displacement (C) and considered episodes where its velocity exceeded 3 pixels/s (D, red area): the length of this episode—occurring during the first 90 min—was taken as the duration of the searching phase. E: comparison of the duration of the searching phase in the 2 experimental conditions illustrated in A and B (Student’s t-test, *\( P < 0.05 \)). F: histogram of the duration of the searching phase in the new tank for experiments illustrated in B. Data from 13 solitary leeches are shown. G: joint density of the normalized Cartesian coordinates \( P(X, Y) \), estimated from the trajectory of solitary leeches during their searching phase. G–I: joint density of the radial and angular coordinates \( P(R, \phi) \), during both the searching phase (G) and the following phase in which leeches were not moving (H). The radial density is in essence flat, but the radial density has 2 peaks corresponding to the upper and lower rim. During the postexploratory phase, no spatial preference is evident (I).
maximum likelihood estimator implemented in Matlab (function hm-
estimate) on 20-min time windows. These windows were used to
guarantee stationarity. Let \( \{X_n\} \) and \( \{Y_n\} \) be two Markov chains
modeling the leech X and Y behavior in a time window of 20 min, with
their transition matrices \( \Pi^X \) and \( \Pi^Y \), with \( T = 66 \text{ ms} \), and \( n \) an positive
integer. Let \( P^X \) and \( P^Y \) be the maximum likelihood estimates \( \Pi^X \) and \( \Pi^Y \),
respectively. The statistical test for independence (H0) between
two binary Markov chains is obtained by a likelihood-ratio test or a
\( \chi^2 \)-test (Anderson and Goodman 1957; Sandland 1976). By adopting
a simplifying procedure (Solow et al. 1995), the final result consists of
a 5 x 5 matrix reporting the conditional significance level for all
possible pairs of behaviors (see Fig. 6).

In experiments with more than five leeches, only one bead was
attached to each animal: under these conditions it was possible to
measure and quantify their relative distance but not their behavior. To
quantify the emergence of collective behaviors we focused our attention
on the swimming behavior because it is easy to recognize and not
very frequent (Fig. 3C). By visually inspecting acquired movies, we
identified all the times \( t_{B} \) and \( t_{T} \) when \( k \) leeches begin and stop
swimming, respectively. In the time interval between \( t_{B} \) and \( t_{T} \), \( k \) leeches swim with \( k \) varying from 1 to \( L \), i.e., the total number of
leeches in the observation tank. We then estimated the probability \( p_{sw} \) that a leech swims as:

\[
p_{sw} = \sum_{n=1}^{N} \left( \frac{(t_n^T - B_n^T) \cdot k_n}{LT} \right)
\]

where \( N \) is the total number of observed swimming episodes and \( T \)
is the duration of the experiment. We have computed the probability
of observing the simultaneous swimming of \( k \) leeches (regardless of the
behavior of the other \( L - k \) leeches) \( f_k \) for \( k = 1, \ldots, L \). If leeches
swim independently of each other, \( f_k \) is expected to be proportional to
\( f_1 \) (where \( f_1 = p_{sw} \)). The probability \( f_k \) that exactly \( k \) leeches swim
follows a binomial distribution.

Experimental probability density estimates were compared with the
binomial equation, and a Kolmogorov-Smirnov test was performed to
determine the statistical significance of the deviation. A statistically
significant deviation indicates the emergence of a collective behavior
and social interactions.

Fig. 3. Identification of the different leech behaviors. A: the leech length was recovered from the bead coordi-
ates and normalized to its maximal observed length. Leech behavior was classified into the 5 stereotyped
behaviors most commonly observed: swimming, pseudo-
swimming, crawling, exploratory, and static. B: en-
larged view, showing the first 10 min of the trace in A. C: percentage of behavior. Each bar represents the
percentage of each behavior during the searching (col-
ored bars) and static (colored patterned bars) phases. During the searching phase the leech crawls along the
rims of the tank, with sporadic swimming bouts,
whereas in the following static phase it does not move or
probes the environment with exploratory head move-
ments (Student’s \( t \)-test, *\( P < 0.05 \)). D: comparison of the
duration of the searching phase for leeches in the
small and large tanks. The searching phase duration is
longer in the large tank (Student’s \( t \)-test, *\( P = 0.037 \)). E:
comparison of behavior percentage of leeches in the
small and large tanks. F: comparison of the duration of
the searching phase for well-fed and hungry leeches.
The searching phase duration is longer for hungry
leeches (Student’s \( t \)-test, *\( P = 4.5E-9 \)). G: comparison of
behavior percentage of well-fed and hungry leeches.
The small circular tank had a volume of 2 liters (lower
diameter 14 cm; upper diameter 18.2 cm), and the large
circular tank had a volume of 6 liters (lower diameter 18
cm; upper diameter 27.7 cm).
RESULTS

Leeches, like many other invertebrate species, exhibit both solitary behavior and also a more social attitude when conspecifics are present (Lang 1974). Social behavior is broadly defined as an interaction between members of the same species that changes their subsequent behavior (Sokolowski 2010). The present study aimed at analyzing the behavior of leeches and in particular the transition from a solitary behavior to a more gregarious, social behavior. We attached two colored beads to the skin of a small number (typically 2 or 3) of leeches, to tag individual leeches and follow their behavior for long periods of time.

Searching behavior of leeches. Three leeches tagged with colored beads were kept in a tank in darkness for at least 2 h. In some experiments, the light source illuminating the tank was switched on and their behavior was observed (Fig. 2A). In these experiments, leeches started to move around, exploring the environment for some minutes (1.3 ± 1.8 min; n = 9). In other experiments leeches were moved into a different tank (Fig. 2B) and their behaviors were similarly monitored; in this case, leeches were active for longer periods (54 ± 13 min; n = 13). To quantify these movements, we computed the leech center of mass and its velocity (see METHODS). Leeches were assumed to search the environment if the velocity of the center of mass was larger than 3 pixels/s. The duration of the searching phase (Fig. 2D, red background) was calculated: we compared how long leeches searched in a known environment (Fig. 2E, same tank) with the duration of searching a new environment (Fig. 2F, different tank). Leeches searched the new tank for 54 ± 13 min and then stopped moving and remained stationary for a time varying from tens of minutes up to 1 or 2 h. The duration of this initial searching phase in 13 leeches had a Gaussian distribution (Jarque-Bera test; P = 0.14) (Fig. 2F). From the center of mass trajectories, we determined the time spent in each location (x, y) of the tank (Fig. 2G): leeches preferentially explored the external edge of the bottom of the tank and its upper rim. When the explored location was determined in polar coordinates (R, φ), no radial preference was observed either during the searching phase (Fig. 2H) or during the following stationary phase (Fig. 2I).

Behavior during searching phase. Using the procedures described in METHODS, we classified the behavior during the searching phase and the following stationary phase (Fig. 3A). During the searching phase (Fig. 3B), leeches exhibited all five stereotyped behaviors (Mazzoni et al. 2005): they alternated swimming (blue), pseudoswimming (dark green), crawling (red), episodes in which they explored the environment with their head (pink), and brief periods in which they were static (light green). During the early searching phase (Fig. 3C), the most common behavior was crawling (44.5 ± 17.3% of the total searching phase), followed by static behavior (30.2 ± 17.9%), swimming (8.9 ± 11.4%), exploration (8.9 ± 8.5%), and pseudoswimming (6.1 ± 7.9%). A different static behavior percentage was observed during the stationary phase: leeches were static for most of the time (89.5 ± 11.4%) but moved their head (5.9 ± 9.7) with a percentage very similar to that observed during the initial searching phase and very rarely exhibited crawling (2.3 ± 2.5%), pseudoswimming (1.8 ± 2.4%), or swimming (0.6 ± 1.4%).

We also investigated whether the size of the tank and the state of the animal affected the searching phase. We compared the statistics of the searching phase of leeches moving in a small and in a large tank, and the same analysis was performed on fed leeches (shipped no more than 2 mo earlier) and on hungry leeches (kept unfed for at least 8 mo). The duration of the searching phase was statistically longer (Student’s t-test; P < 0.05) in the larger tank (48 ± 25.4 min, 5 leeches) than in the smaller tank (21 ± 7.8 min, 7 leeches) (Fig. 3D). The fraction of time spent in swimming, pseudoswimming, crawling, resting, and moving the head was not different when leeches were in a large or small tank (Fig. 3E). As shown in Fig. 3, F and G, the duration of the searching phase was longer for hungry (53.6 ± 13.1 min, 13 leeches) than for fed (11.4 ± 6.4 min, 18 leeches) leeches (Student’s t-test, P < 0.01) and the behavioral percentages during the searching phase were similar for fed and hungry leeches. Therefore, hungry leeches search a new environment for a longer period of time, but the specific behavior adopted during the searching phase is independent of the size of the tank and the state of the animal.

Effect of 5-HT and DA. Leech behavior is affected by specific neuromodulators such as 5-HT and DA: a high blood concentration of 5-HT promotes swimming in intact leeches and in isolated nerve cord preparations (Willard 1981). Similarly, DA induces fictive crawling in isolated nerve cord preparations or in single isolated ganglia (Puhl and Mesce 2008). To test the effects of these modulators on intact leeches, we determined the percentage of time that leeches injected with different amounts of 5-HT (Fig. 4, A and B) and DA (Fig. 4, D and E) spent either pseudoswimming or crawling during the initial search of a new environment. Leeches injected with 300 μl of a saline solution—with no neuromodulator added—spent a small amount of time pseudoswimming (see Fig. 4A), whereas uninjected leeches rarely exhibited pseudoswimming (Fig. 3).

Uninjected leeches pseudoswam for ~6% of the searching phase, suggesting a low level of 5-HT in the blood (<50 nM) (Willard 1981): a subsequent saline injection is expected to lower further the endogenous 5-HT concentration in the blood (Fig. 4A, saline control value). Injection of 5-HT concentrations higher than 100 μM promotes pseudoswimming: leeches injected with 150–200 μM 5-HT spent almost half of their searching time pseudoswimming (Fig. 4A). The modal duration of pseudoswimming episodes is also increased (Fig. 4, B and C). Under the same conditions leeches hardly swam: the time spent swimming was 0.5 ± 0.3%. 5-HT is known to control the swimming behavior through two different mechanisms: Retzius cells exert a neurohormonal action whereas cells 21 and 61 act synaptically (Kristan and Nusbaum 1982), but the mechanisms underlying the generation of two similar behaviors such as swimming and pseudoswimming are still unknown because previous studies have been carried out on isolated nerve cord preparations, in which these two behaviors cannot be distinguished.

Uninjected leeches crawl for ~45% of the searching phase, but leeches injected with saline solution crawl for only 9.9 ± 3.0% of the searching phase. These observations suggest that a significant level of DA is present in the blood and that the injection of 300 μl of saline solution may significantly lower the endogenous DA concentration in the blood. This is supported by experiments in which we measured the percentage of...
either 5-HT or DA was injected at a high concentration (500 μM) elicit the highest percentage of pseudoswimming. B: comparison of duration of uninterrupted bouts of pseudoswimming in leeches injected with 150–200 μM 5-HT (black line) and in noninjected leeches (gray line). C: cumulative distribution plot (same data as B): 5-HT-injected leeches show longer pseudoswimming bouts (Kolmogorov-Smirnov test, P < 0.01). D: percentage of crawling after the injection of varying DA concentrations (circle) and comparison with saline-injected (square) and noninjected (triangle) controls. DA concentrations of 650–700 μM elicit the highest percentage of crawling. E: comparison of duration of uninterrupted bouts of crawling in leeches injected with 650–700 μM DA (black line) and in noninjected leeches (gray line). A statistical test between the cumulative distributions did not show a significant difference (Kolmogorov-Smirnov test). Reported amounts of 5-HT and of DA were dissolved in 300 μl of leech saline and were injected in leeches as described in METHODS. F: percentage of crawling after the injection of increasing volumes of saline. Higher volumes have the effect of reducing the crawling percentages (Student’s t-test; *P < 0.01), suggesting an increasing dilution of DA in the blood.

5-HT concentrations (circle) and comparison with saline-injected (square) and noninjected (triangle) controls. 5-HT concentrations of 150–200 μM elicit the highest percentage of pseudoswimming. B: comparison of duration of uninterrupted bouts of pseudoswimming in leeches injected with 150–200 μM 5-HT (black line) and in noninjected leeches (gray line). C: cumulative distribution plot (same data as B): 5-HT-injected leeches show longer pseudoswimming bouts (Kolmogorov-Smirnov test, P < 0.01). D: percentage of crawling after the injection of varying DA concentrations (circle) and comparison with saline-injected (square) and noninjected (triangle) controls. DA concentrations of 650–700 μM elicit the highest percentage of crawling. E: comparison of duration of uninterrupted bouts of crawling in leeches injected with 650–700 μM DA (black line) and in noninjected leeches (gray line). A statistical test between the cumulative distributions did not show a significant difference (Kolmogorov-Smirnov test). Reported amounts of 5-HT and of DA were dissolved in 300 μl of leech saline and were injected in leeches as described in METHODS. F: percentage of crawling after the injection of increasing volumes of saline. Higher volumes have the effect of reducing the crawling percentages (Student’s t-test; *P < 0.01), suggesting an increasing dilution of DA in the blood.

crawling after the injection of increasing saline volumes (Fig. 4F). Higher volumes reduced the crawling percentages (Student’s t-test, P < 0.01), suggesting an increasing dilution of DA in the blood. When DA was added to the injected saline, over a broad range of DA concentrations, leeches exhibited the usual amount of crawling behavior and the modal duration of crawling episodes was unchanged (Fig. 4, D and E). When either 5-HT or DA was injected at a high concentration (>500 μM), neither pseudoswimming nor crawling was observed. The resulting dose-response curves relating the amount of injected neuromodulator and the percentage of pseudoswimming and crawling were “bell shaped.” This kind of shape could originate from two distinct mechanisms: first, receptor desensitization occurring at higher agonist concentrations (Lopez-Garcia and King 1996; Wilkie et al. 1996) and second, the existence of two different types of 5-HT receptors, as in leech mechanosensory P cells (Drapeau and Sanchez-Armass 1988; Henderson 1983) and S cells (Burrell et al. 2001). In leech P cells, application of low levels of 5-HT onto the soma produces an increase in Cl⁻ conductance and a consequent hyperpolarization, possibly mediated by one type of 5-HT₁ receptor (Ali et al. 1998), whereas a higher level of 5-HT induces depolarization resulting from the activation of one type of 5-HT₂ receptor causing an increase of a nonspecific cationic conductance (Sanchez-Armass et al. 1991; Catarsi and Drapeau 1997). Ionic mechanisms for 5-HT-mediated inhibition and excitation are unknown in the S cell but could be explained by different 5-HT receptors and receptor desensitization (Burrell et al. 2001).

The body concentration of 5-HT clearly influences the percentage and the duration of pseudoswimming episodes (compare black and gray lines in Fig. 4, B and C). The change in the modal duration suggests that other modulatory effects could be present in different regions: for example, it is known that the head brain contributes not only to swimming initiation but also to the quality (duration and rhythmicity) of these episodes (Crisp and Mesce 2006).

The percentage of crawling was clearly influenced by the body concentration of DA, but the duration was not: crawling episodes had similar duration in the noninjected and injected leeches (compare black and gray lines in Fig. 4E). Similar results were observed in isolated nerve cord preparations with DA (Puhl and Mesce 2008).

Social interactions among conspecifics: experiments with three leeches. Having characterized searching behaviors in solitary leeches in a new environment, we asked whether their behavior was influenced by the presence of other conspecifics. To test this possibility, we analyzed the distribution density of swimming initiation and correlated behaviors in other leeches, so that their behavior would become correlated. This possibility was tested by analyzing whether pairs of leeches adopted a similar behavior in the same time windows (Fig. 6). Deviation from statistical independence would indicate social interactions.

We first investigated social interactions among leeches in groups of three. For each leech we determined its center of...
mass (see METHODS) and computed the distribution density (pdd) of pairwise distances (Dleeches) among all pairs of leeches in the observation tank. In Fig. 5A (gray bars), the pdd of Dleeches was expressed in relation to the normalized radius of the recording arena (equal to 1), so that two diametrically opposite leeches are at a distance of 2 normalized units. The pdd was calculated from data collected during the initial searching phase (i.e., the first 100 min). The pdd obtained from the same experiments, after shuffling the data points, had a similar probability density (Fig. 5A, blue curve), suggesting independent behaviors of the three leeches.

To verify that the three leeches searched the tank in an independent fashion, we simulated leech trajectories with built-in reciprocal independence. From these simulated trajectories we determined the correspondence between the experimental and simulated trajectories is shown in Fig. 5D. A comparison between the experimental and simulated trajectories is shown in Fig. 5D. From these simulated trajectories we determined the corresponding pdd (red curve in Fig. 5A). The pdd of experimentally observed pairwise distances Dleeches is well fitted by those obtained from independence hypotheses with the data-shuffling technique ($R^2 = 0.963$) or the stochastic point process model ($R^2 = 0.922$).

To test whether the presence of other leeches influenced the behavior of each leech, we compared the behavior percentage of each leech (Fig. 6A) while one was crawling (dark gray bars) or was static (light gray bars). The percentage of a given behavior was very similar in control conditions (black bars) and in groups of three leeches while one was crawling (dark gray bars) or was static (light gray bars).

A more accurate analysis relies on the fact that the time structure of leech behavior is well described as a first-order Markov process with transitions among five different behavioral categories (Garcia-Perez et al. 2005): swimming, pseudoswimming, crawling, static, and exploratory (Fig. 6B). To determine whether behaviors were performed independently, we implemented a conditional test for independence of two Markov chains as described in detail in METHODS. In a temporal window of 20 min we performed a conditional significance test for an independent Markov process (Fig. 6C). The final result is a $5 \times 5$ matrix with entries showing the conditional significance level for all possible behavior couples and the fraction $5B$. The angular density $f_\theta(\theta)$ was assumed to be uniform, producing the red circle in Fig. 5C. From these quantities we were able to obtain simulations of the leech trajectories satisfying the experimentally observed radial and angular distribution densities (Fig. 5, B and C). A comparison between the experimental and simulated trajectories is shown in Fig. 5D.

Fig. 5. Independent motion in 3 leeches. A: probability density (pdd) of pairwise distances ($D_{\text{leeches}}$) from 5 experiments with 3 leeches (gray bars), pdd obtained from the same experiments but with shuffled data (blue trace), and pdd from simulated data using the independent model (red trace). The similarity between the 3 pdds indicates independent motion in groups of 3 leeches. B and C: the radial and angular densities of the location of solitary leeches obtained from 15 experiments (blue data in A and B); the radial density was fitted with the sum of a log-normal and a normal distribution (see Eq. 1), providing the red line in B along with 0.95 confidence bounds (dotted red lines); the angular density was assumed to be uniform and is shown as a red line in C. By using these theoretical densities we have simulated independent spatial point processes describing leeches moving independently from each other. D: comparison between 3 experimental trajectories and 3 simulated trajectories.
of samples for which statistical independence is observed with a $P$ value $< 0.01$ ($N = 12$ couples of leeches).

Fig. 6. A: comparison of behavior percentage as in Fig. 3C (black bars) and when 1 other leech was crawling (dark gray bars) or was static (light gray bars). These behavior percentages do not depend on whether another leech is static or is crawling, suggesting that the behavior of the 3 leeches in reciprocally independent. B: model of the dynamics of the leech behavior as a 5-state homogeneous Markov chain (not all transitions are shown) C: statistical analysis of the independence of leech behavior. The behavioral sequences of pairs of leeches were divided in windows of 20 min, and each couple of windowed sequences was tested for the null hypothesis that the 2 sequences form a couple of independent Markov chains. D: $5 \times 5$ matrix with entries reporting the fraction of samples for which statistical independence is observed with a $P$ value $> 0.1$ ($N = 12$ couples of leeches).

Emergence of social interactions in leeches: effect of number of individuals and of 5-HT. The three leeches observed were in a tank with a volume of 6 liters, corresponding to a density of 0.5 leeches/dm$^3$. Because the absence of social interactions (Figs. 5 and 6) could result from the low leech density, we increased the number of leeches in the observation tank. Our CCD camera was not able to reliably distinguish the color of more than six beads, so we adopted a different experimental approach. We developed an algorithm to follow in real time the contour of individual leeches and to keep track of the number of leeches that had aggregated in a group. Also, a red bead was placed onto the skin of up to 10 leeches in the midbody. The distance between leeches was measured as the distance between the red beads. A shallow tank (8 cm high) was used, to ensure that leeches belonging to the same group were not at different depths, yet allowing the performance of all possible behaviors.

The distribution density of pairwise distances ($D_{\text{leeches}}$) among leeches was markedly different in the case of 3 (example in Fig. 7A) and 10 (example Fig. 7B) leeches. With 10 leeches in the observation tank, many times leeches congregated in a group (13 of 15 experiments), so that the distribution density of pairwise distances ($D_{\text{leeches}}$) had a peak close to 0 (Fig. 7B); such a peak was not present when only 3 leeches were observed (Fig. 7A, peak value at a normalized distance of 1.8). When the three leeches had all been injected with 150–200 $\mu$M 5-HT, the distribution density of pairwise distances ($D_{\text{leeches}}$) had a large peak corresponding to a normalized distance of 0.7 (Fig. 7C, average distribution, $N = 5$), which was different from the independent behavior observed among three noninjected leeches (Fig. 7A). The injection of 650–700 $\mu$M DA produced similar but less marked changes (peak at a normalized distance of 1.40) from the independent behavior (Fig. 7D, average distribution, $N = 5$).

The median pairwise normalized distance ($D_{\text{leeches}}$) when 3 and 10 leeches were in the observation tank was 1.29 and 1.09, respectively. Therefore, the median value of $D_{\text{leeches}}$ is lower when 10 leeches are present in the tank (Mann-Whitney $U$-test; $P < 0.01$). In leeches moving independently in the tank, the...
The percentage of swimming is increased. Ten leeches explored the tank (A) and came to rest either in a scattered configuration (B) or in a gregarious configuration (C). All 10 leeches could also swim (D) in a collective manner. Beads are also shown in gray. 

Fig. 8. Emergence of collective behavior when the number of conspecifics is increased. Ten leeches explored the tank (A) and came to rest either in a scattered configuration (B) or in a gregarious configuration (C). All 10 leeches could also swim (D) in a collective manner. Beads are also shown in gray. E: comparison of the experimental percentage of collective swimming in k leeches (gray line) and the binomial distribution where the probability of swimming is 0.10 (black line). The lines are different according to the 2-sample Kolmogorov-Smirnov test—P < 0.01. F: comparison of the estimated probability that at least 5 leeches swim contemporarily and the theoretical value (horizontal line) that would be expected in the case of independent motion where the probability of swimming is 0.10.

The median value of D_leeches does not depend on the number of conspecifics; therefore social interactions emerge when the leech density in the tank is increased. To determine whether the neuromodulators affected social interactions, we measured interleech distances after injecting leeches with 5-HT or DA. The median pairwise normalized distance D_leeches when three leeches were injected with 5-HT was 0.89. The same quantity when leeches were injected with DA was 1.01. Therefore, 5-HT (Mann-Whitney U-test; P < 0.01) and to a lower extent DA (Mann-Whitney U-test; P < 0.01) promote the emergence of social interactions also in groups of three leeches. As shown in Figs. 5–7, the behavior of three leeches in the same tank was not significantly affected by the presence of conspecifics.

In the presence of 10 leeches in the observation tank, the emergence of collective behavior was observed (Fig. 7B). Indeed, leeches could explore the environment (Fig. 8A) or be stationary (Fig. 8B) but could also exhibit a gregarious behavior (Fig. 8C). We also observed episodes in which all 10 leeches swim at the same time (Fig. 8D).

The probability f_k that exactly only k leeches swim follows a binomial distribution. As shown in Fig. 8E, the binomial distribution (black line) is in agreement with the experimentally observed percentages (gray line) only for values of k < 5 (2-sample Kolmogorov-Smirnov test, P < 0.01) and are different for k > 5. Indeed, groups of equal to or less than five leeches behave almost independently, and social interactions characterized by the occurrence of collective behaviors occur with more than five conspecifics. In six of the nine experiments analyzed (Fig. 8F), the percentage that at least five leeches swim simultaneously was above the theoretical value (where the percentage of swimming is ~10%) expected in the case of independent behavior (Fig. 8F, horizontal black line). The statistical analysis shown in Fig. 8E was performed on a single experiment.

As the behavior of leeches injected with 5-HT (Fig. 7) deviates from statistical independence for groups of only three conspecifics, we investigated whether we could observe collective behavior in groups of five injected leeches. Indeed, five leeches injected with 200 μM 5-HT (volume 300 μl) initially explored the tank (Fig. 9A) and could come to rest in a solitary manner, but more often aggregated (Fig. 9C) and more notably showed collective swimming episodes (Fig. 9D). The observed collective behavior occurred with a percentage significantly higher (Fig. 9E, gray line) than that expected from independent behavior (Fig. 9E, black line) (2-sample Kolmogorov-Smirnov test, P < 0.01; statistical analysis performed on a single experiment). 5-HT injection increases the percentage of spon-
taneous pseudoswimming, but also of swimming, if a mechanical stimulation is applied on the tail (data not shown), as reported also by Willard (1981). In the presence of several conspecifics there are more mechanical perturbations in the water, leading to mechanical stimulations possibly inducing the leeches to swim.

We analyzed the emergence of social interactions induced by 5-HT in a series of other experiments. In experiments with several leeches (such as 10), we waited for the formation of a large group formed by at least five conspecifics (Fig. 9F). We then introduced into the observation tank a leech injected with 5-HT (300 μl of 200 μM 5-HT) that had a colored bead attached to the skin on its back. Therefore, the motion of this leech could be followed and we could verify whether this leech was attracted by the large group of conspecifics. The injected leech explored the tank for ~10–20 min (see light gray line indicating its trajectory in Fig. 9F) and then invariably (N = 7) joined the group of conspecifics not injected with 5-HT (dark gray circle in Fig. 9F). In other experiments (N = 3), the injected leech was introduced into a tank where a black large object was positioned, roughly mimicking a large group of leeches or a black stone. Under these circumstances, the injected leech explored the tank and the object but neither rested in its proximity nor took shelter underneath it (Fig. 9G).

In all seven analyzed experiments, the percentage that at least four leeches swim simultaneously was above the theoretical value (where the percentage of swimming is ~10%) expected in the case of independent behavior (Fig. 9H, horizontal black line). Moreover, the percentage of time spent in a group of at least four conspecifics by a leech injected with 300 μl of saline (N = 5) was significantly lower than the time spent by a leech injected with 5-HT (N = 7) (Fig. 9I; Student’s t-test, P < 0.01). The same could be observed in experiments with a fake group (N = 3). These results suggest that leeches injected with 5-HT are attracted by their conspecifics and have a clear tendency to establish social interactions.

To test whether the collective swimming behavior is the mere result of the presence of an object moving in the water or is truly a social behavior, we introduced a goldfish into the tank (Fig. 10A). The goldfish swam most of the time, producing an almost continuous periodic water movement leading to mechanical stimulations of the leech bodies (Fig. 10A).

Our real-time algorithm allowed precise tracking of the fish motion (as its color is red) and of the leeches (as they are black), as shown in Fig. 10B. By studying the movies we observed several episodes in which the fish apparently chased a leech (Fig. 10B). This observation is also confirmed by the fact that the scalar product (Fig. 10C) between the velocity vector v of the goldfish and the average velocity vector of all leeches <v> shows more positive than negative peaks (Fig. 10D). The scalar product v · <v> is positive when the two vectors have the same direction, implying that the goldfish and some leeches are swimming in the same direction. When the goldfish is present in the tank, its continuous swimming produces mechanical waves, certainly reaching the leech body, and the movement is likely to produce a mechanical stimulation to their tail, known to initiate swimming. Nevertheless, we observed a decrease in the swimming percentage, and very rarely more than three leeches swim simultaneously (Fig. 10E). This analysis suggests that leeches are able to sense the presence of a nonconspecific organism in the neighborhood, which could be a predator. As a consequence, leeches could adopt a quieter behavior, in order to minimize the risk of being predated.

DISCUSSION

In the present report we present the first analysis of the occurrence of social interactions in medicinal leeches. Social interactions consist in group decision making (Sokolowski 2010), characterized by a mutual attraction leading to aggregation of conspecifics and an imitative behavior causing episodes in which conspecifics adopt the same behavior. This study provides three major results: first, when the leech density is low (<3 leeches in 6 liters), leech behavior is unaffected by the presence of other conspecifics; second, when the leech density is increased (>8–10 leeches in 3 liters), leeches no longer move in a statistically independent way but are attracted to each other and exhibit episodes of collective behavior; third, the leech density at which social interactions emerge is lowered.
Leech ethology. Leech ethology has been extensively studied, and several excellent books describe natural leech behavior in great detail (Muller et al. 1981; Sawyer 1986a, 1986b). In their daily lives, leeches perceive and are affected by a broad range of stimuli; those with the highest biological relevance are light, odorants, water movements, touch, and temperature.

Leeches are negative phototactic, which means that leeches crawl and move away from light sources, seeking protection under a stone or other shelter, where they then remain. Different leech species show different degrees of phototaxis, in response to the dynamics of environmental light, leading to the regulation of their reproductive cycle during the year (photoperiodism), inactivity during the daytime and increased activity at night (diurnal rhythmicity), and alerting posture following sudden changes in light intensity (shadow reflex). The diurnal rhythm can be reversed by reversing the lighting regime, and it is abolished under constant dark or light conditions (Elliott 1973). Leeches are very sensitive to touch, and in Hirudo four behaviors—local bending, whole body and local shortening, crawling, and swimming—can be elicited by tactile stimulation of the leech. At a threshold level of mechanosensory stimulation, the predominant response elicited depends on the location of the stimulus; stimulating the anterior end produces shortening, stimulating the posterior end produces crawling or swimming, and stimulating midbody sites produces local bending (Kristan et al. 1982). In addition to localized tactile stimulation, leeches also show a generalized response to touch, as they are often found under rocks, leaf axils of aquatic vegetation, or other tight-fitting places that offer maximum physical contact with the substrate (positive thigmotaxis) (Lang 1974; Sawyer 1986a). Therefore, it is likely that positive thigmotaxis and negative phototaxis contribute to the deviation of the statistical independence here observed: the former could favor the physical contact between conspecifics, while the latter can be achieved by mutual covering. Nevertheless, we speculate that conspecific recognition may occur as well, as suggested by the data shown in Fig. 9.

Searching behavior, deviation from independence, and emergence of social interactions. When placed in a new environment, leeches begin to explore it, with particular interest for its boundaries. This “searching behavior” lasts for an amount of time dependent on the size of the tank (Figs. 2 and 3). This initial searching phase was not described in a previous study (Mazzoni et al. 2005) because in that study data collection and behavioral characterization began 30–60 min after positioning leeches in the new environment. Therefore this initial phase was not observed. Our new Bayesian classifier of pixel color made it possible to track six or more beads in real time so that we could follow the position and the behavior of three leeches placed into an unexplored water tank, and follow the emergence of social interactions.

When a small number of leeches are in the same environment they hardly notice one another, and their motion and behavior are independent with a high statistical significance (Figs. 5 and 6). When a higher number of leeches are in the same tank (Fig. 7), they spend more time closer to one another and assemble in groups (peak at short distances in Fig. 7B). This attraction could be due in part to positive thigmotaxis and negative phototaxis, the former leading the leeches to stay in contact and the latter leading the leeches to mutually cover each other. However, the deviation from independence observed in small groups of leeches injected with 5-HT cannot be explained by positive thigmotaxis and negative phototaxis.

In some cases, we have observed 10 leeches swimming simultaneously. Because solitary leeches swim at a low probability ($p_{sw} = 0.12$, see Fig. 3C), seeing 10 independently acting leeches swimming simultaneously is extremely unlikely and it is a clear indication of the emergence of social interactions among leeches. Therefore leeches, like other lower invertebrates, exhibit a collective behavior, in which conspecifics adopt the same behavior. Leeches are also able to sense the presence of a nonconspecific and are able to collectively modify their behavior (Fig. 10) accordingly. Locusts can switch from a solitary to a gregarious behavior in different conditions such as dispersal and migration (Anstey et al. 2009; Buhl et al. 2006). Neurobiological mechanisms underlying social interactions in animals are largely unknown, but several reports indicate that well-known neuromodulators such as 5-HT can play a major role. Indeed, the internal level of 5-HT, a conserved mediator of neuronal plasticity, mediates the shift toward a gregarious behavioral pattern in locusts (Anstey et al. 2009). Thus our results (Figs. 7–9) suggest the emergence of social interactions among leeches when their density is increased and/or the level of endogenous 5-HT is elevated.

Effect of 5-HT and DA. In the leech, each segmental ganglion contains a network of serotoninergic neurons (De-Miguel and Trueta 2005; Lent and Frazer 1977) that acts on different targets to modulate the swimming motor program (Friesen 1989; Kristan and Nusbaum 1982; Ort et al. 1974); these cells are a pair of large Retzius cells, one pair of dorsolateral (DL) and ventrolateral (VL) interneurons, and two pairs of medial interneurons (cells E and M) (Lent et al. 1991). The neuropil of the supraesophageal ganglion is densely filled with serotoninergic arbor, and a number of 5-HT-immunoreactive fibers were found in a region previously identified as a neurohemal release site (Crisp et al. 2002; Webb 1980); these 5-HT fibers contained varicosities and represent possible sites of neurohemal secretion. The subsesophageal ganglion is a fusion of four neuromeres; each of these contains a pair of large Retzius cells and two pairs of serotoninergic interneurons (cells E and M); in addition, the large serotonin effector cells (LL) are contained in neurorme 1 (Crisp and Mesce, 2006; Lent et al. 1991). It has also been shown that fine 5-HT-immunopositive fibers are associated to the lateral blood vessels close to the dorsoventral and the longitudinal body wall muscles (Leake et al. 1985). Moreover, there is also evidence that Retzius cell stimulation, which causes mucus secretion from the skin, can be mimicked by exogenous 5-HT injected on the dorsal side (Lent 1973). These observations support the notion that 5-HT can act peripherally and hormonally, by circulating in the blood (Willard 1981).

Dopaminergic neurons have a distribution different from serotoninergic neurons: two pairs of cells are present in the supraesophageal ganglion and seven pairs in the subesophageal ganglion and in the tail brain, while each segmental ganglion receives dopaminergic inputs from a peripheral cell located in the anterior root ganglion (Crisp et al. 2002). Thus the combined action of neurons able to secrete DA and 5-HT and those with the corresponding receptors affects and modulates several aspects of leech behavior. Indeed, the biological mechanisms...
of behavioral selection of the leech have been gradually uncovered during the last decade. The decision to swim is distributed across populations of swim-activating and swim-inactivating neurons descending from the subesophageal ganglion of the head brain (Crisp and Mesce 2006). The modulatory effect of 5-HT on the generation of the swimming motor program has been quantified with isolated nerve cord preparations (Willard 1981). The choice between swimming and crawling in leeches is also mediated by DA, which represses swimming and evokes crawling (Friesen and Kristan 2007; Puhl and Mesce 2008). In our work we extend these findings, by demonstrating that in intact animals—and not only in isolated nerve cords—5-HT made the swimming motor program more likely, leading to an increased percentage of pseudoswimming episodes (Fig. 4). Leeches injected with 5-HT were also more prone to swim after tail stimulation (data not shown), and although the swimming probability did not change significantly (Figs. 8 and 9) leeches were more prone to swim collectively. Similarly, DA increased the percentage of crawling episodes, but no collective behavior has been observed. These two amines are released locally into the leech ganglia by peripheral and central neurons and may be released into the blood by a putative neurochemical complex in the subesophageal ganglion of the head brain (Crisp et al. 2002; Webb 1980), or by the ganglia themselves (Glover and Lent 1991). In this work we observed that 5-HT and—to a lesser extent—DA affect social leech behavior, and indeed these two neuromodulators have a role in determining how leeches alter their behavior in the presence of their conspecifics: injection of these neuromodulators decreases the pairwise average distance between conspecifics, promoting the emergence of social interactions. These observations are very similar to what has already been observed in other simple nervous systems such as those of C. elegans, Drosophila, and other invertebrates (Sokolowski 2010). The effect of 5-HT, here described, is similar to that observed in desert locusts Schistocerca gregaria (Anstey et al. 2009) becoming gregarious after injection of 5-HT. The neurobiological mechanisms generating and modulating these social interactions in the leech are not yet known; therefore such studies are appropriate subjects for the newly developing field of social neuroscience (Adolphs 2010).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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