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Behavioral mirroring in Wistar rats investigated through temporal pattern analysis

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The study of social interactions lies at the core of several disciplines such as psychiatry, psychology and ethology, just to name a few. In this context, understanding the temporal patterns underlying interactive behaviors is of crucial importance. Here, we employed T-pattern detection and analysis to study social interactions in ten pairs of Wistar rats tested in an Open-Field environment. We found four different categories of interactive behaviors. One of them was of particular interest to us because it consisted of behavioral events that, taken individually, should not underlie an interaction of any kind; however, they were included in T-patterns, which is suggestive of a dyadic temporal coordination in the behavioral expression of two individuals. Within this category, we described for the first time a new subcategory of apparent interaction patterns characterized by events that one of the two rats repeats only if previously produced by the partner (i.e., behavioral mirroring). These findings are discussed in functional terms for rodents and in light of our current understanding of social interactions in humans.

Keywords T-pattern analysis, TPA, Social interaction, Wistar rat, Open-Field

The exploration of the behavioral dynamics underlying social interactions is a key center of gravity around which various disciplines such as Ethology[1], Psychology[2], Psychiatry[3], Ecology[4] and Sports Science[5] orbit. Many studies on social interactions are carried out on laboratory animals, in particular, rodents. Despite numerous differences between laboratory animals and their wild counterparts (for a review see Kondrakiewicz et al.[6]), the use of animal subjects remains the only solution to study (social) phenomena that would be ethically difficult to assess in humans. Laboratory rats and mice have been successfully used in interaction studies because, when challenged with one or more conspecifics, these animals show marked social behavior[7]. Consistently, it has been shown that, under normal housing conditions, these rodents prefer to remain in close contact when they sleep; during normal behavioral observation conditions their interactions result in activities such as allogrooming, partner sniffing, partner approaching[8–11] or play fighting, i.e. a species-typical behavior patterns of agonism spontaneously produced by intact animals[12]. From a translational perspective, rodent models of interactions have a high utility in studies on neuropsychiatric diseases in humans since a number of severe conditions, like autism spectrum disorders, show dysfunctions in social interaction processes[13]. Thus, studying the behavioral dynamics underlying rodents' interactions could lead to a better understanding of the etiology of these diseases and result in the development of new therapeutic approaches.

One of the most interesting aspects of social interactions is their temporal dimension. This aspect has been extensively studied, over the past decades, through the use of T-pattern analysis (TPA)[14–18], a technique employed to reveal statistically significant sequences of events. On the one hand, this approach allows researchers to detect otherwise hidden features of the temporal dimension of social behavior in humans[14–18]; on the other hand, its application in the study of interactions in non-human subjects, and particularly in rodents, has been very limited. To date, the only investigation of interactions among rodents via TPA was conducted in our laboratory[8]. In this previous study, our ethogram (Fig. 1) allowed us to divide the rats' repertoire into interactive behaviors, which we called "inter-subject", and non-interactive behaviors, which we called "intra-subject". During *inter-subject* (Fig. 1A), the rat is in contact with the partner (e.g. leaning on, social grooming, etc.) or an evident approach/withdrawing is produced (e.g. approaching, following etc.). During *intra-subject* (Fig. 1B), the rat

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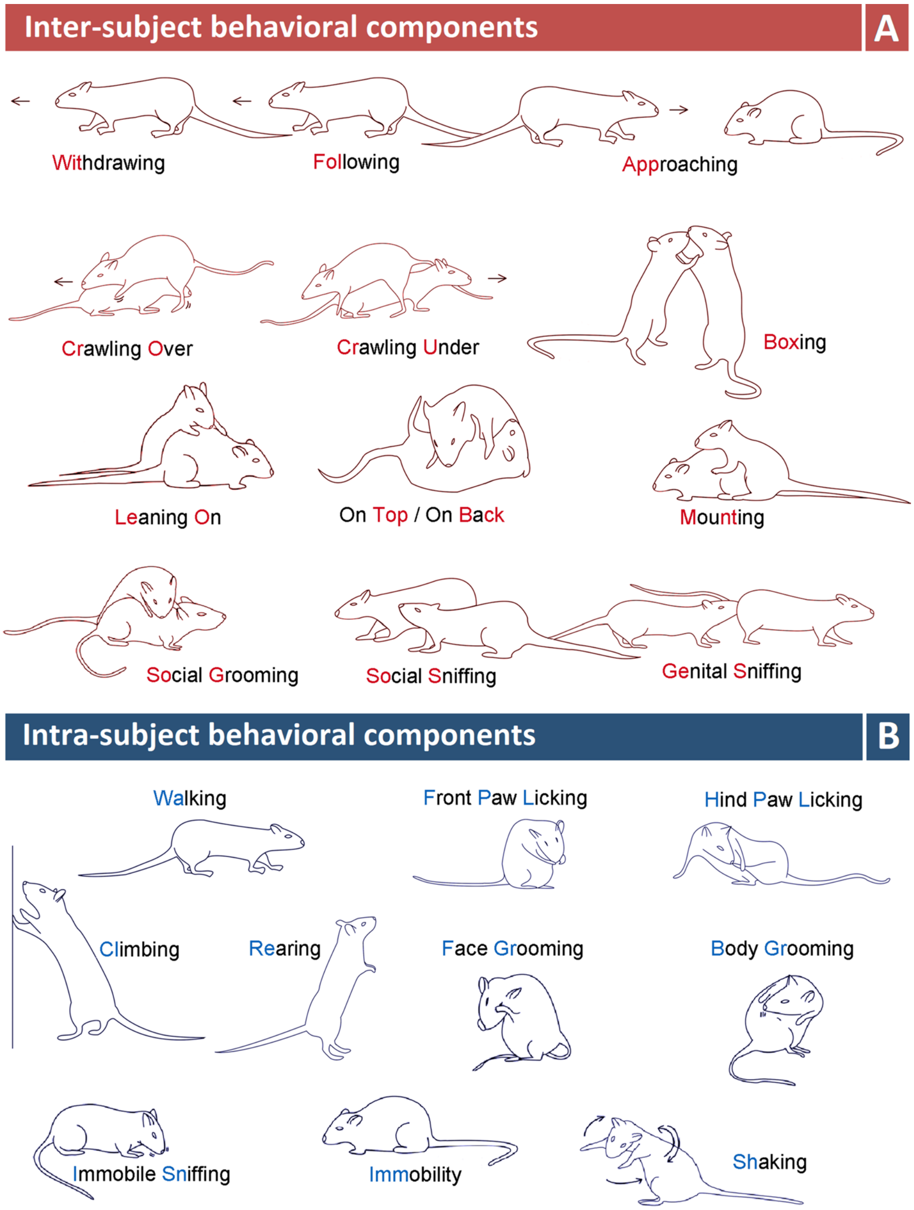


Fig. 1. Ethogram of social interaction in rats. (**A**) Inter-subject (i.e. directed toward the partner) behavioral components: Withdrawing (Wit): a rat walks/runs away from the partner; Following (Fol): a rat follows the partner walking/running away; Approaching (App): a rat walks in the direction of the partner, which is immobile or is already approaching; Crawling over (CrO): a rat walks over the partner; Crawling under (CrU): a rat walks under the partner; Boxing (Box): offensive/aggressive behaviors such as pawing, pouncing, nosing, biting, boxing, kicking, wrestling; Leaning on (LeO): one of the rats leans with its forelimbs on the other rat, which, in turn, maintains all the four paws on the ground; On-top (Top): one of the animals stands over the partner lying with its back on the floor; On-back (Bck): one of the animals lies with its back on the floor with the other animal standing on it; Mounting (Mnt): one of the rats holds the other rat's trunk with its forelimbs; Social grooming (SoG): one of the rats grooms its partner's body, neck or face; Social sniffing (SoS): one of the rats sniffs the partner's face and/or body; Genital sniffing (GeS): one of the rats sniffs the partner's ano-genital area. (**B**) Intra-subject (i.e. not directed toward the partner) behavioral components: Walking (Wa): the rat walks around sniffing the environment; Climbing (Cl): the rat maintains an erect posture leaning against the Plexiglas wall; Rearing (Re): the rat maintains an erect posture without leaning against the wall; Front Paw Licking (FPL): the rat licks or grooms its forepaws; Hind Paw Licking (HPL): the rat licks or grooms its hind paws; Face Grooming (FGr): the rat rubs its face with the forepaws; Body Grooming (BGr): the rat rubs the body combing the fur by fast movement of the incisors; Shaking (Sh): the rat shakes its head and body with rapid semi-circular movements; Immobility/Resting (Imm): the rat maintains a fixed posture and no movements are produced; Immobile Sniffing (ISn): the rat sniffs the environment, standing on the ground. Modified with permission from Casarrubea et al.[8].

is engaged in activities directed toward itself (e.g. paw lickings or grooming) or toward the exploration of its environment (e.g. walking or sniffing). Our working hypotheses postulated the occurrence of three categories of behavioral sequences (Fig. 2): (a) sequences in which the animals would display exclusively inter-subject behaviors, for example, when the two rats are engaged in behaviors involving mutual contact; (b) sequences in which the animals would display both inter-subject and intra-subject behaviors, for example, when one of the two rats approaches the other who is engaged in sniffing the environment; finally, (c) sequences performed by only one of the two rats, with no interaction toward the partner. Surprisingly, a fourth (d) category of sequences emerged containing events produced by both subjects while neither of them was, *apparently*, exhibiting any explicit interactive behavior toward their partner. The detection of this fourth category[8] has opened up intriguing questions about the intrinsic nature of social interactions. If TPA can detect behavioral events performed by (apparently) non-interacting subjects, but that are sequentially associated in a statistically significant manner, should such instances be considered "interactions"? How are these sequences qualitatively structured? What about the events within each behavioral sequence? Do they hold peculiar characteristics? Last but not least, from a translational point of view, does this putative behavioral category have any relevance to our understanding of collective phenomena in humans? Following up on the above-mentioned study[8] and using newly available and improved means of detection and analysis, we aimed to further address these questions and shed a new light on interactional processes.

Methods

Subjects

We sampled the behavior of twenty S.P.F. male rats Wistar strain (Harlan Laboratories, Italy), 55–60 days old and weighing 220–250 g. Animals were housed in a room maintained at a constant temperature of 23 ± 1 °C, under a 12-h light/dark cycle (lights on 07:00 a.m.; lights off at 07:00 p.m.). Food (standard laboratory pellets, Mucedola, Italy) and water were freely accessible. We sampled nine pairs of animals already used in our previous study[8], to which we added a new pair of rats. In this study, our data stem from the video files of these ten pairs of rats as described in the following sections. We did not use any data from the previous study[8].

Experimental apparatus

The Open-Field (OF) apparatus consisted of a square 50 × 50 cm arena surrounded by three white opaque walls, a front transparent wall, and a white opaque Plexiglas floor. Illumination of the OF, during all the observation sessions, was 300 lx, i.e. high light conditions[19]. The behavior of all subjects was recorded by using a digital camera (Toshiba P10, Tokyo, Japan) and video files stored in a personal computer for future analyses.

Procedure

Before testing, each rat was individually housed for 5 days[8,20]. On the testing day, two rats were separately transferred, inside their own home cages, from the housing room to the testing room and allowed to acclimate for 30 min, in different zones of the room and far from the OF apparatus. This procedure reduced any transfer effects and minimized any visual and/or olfactory cues originating from the other home cage and/or from the testing arena. Then, both animals were moved from their own home cages to the OF and placed in two opposite corners of the arena. The behavior of the animals was continuously video-recorded for 15 min. Temperature in the testing room was maintained equal to the temperature in the housing room (23 ± 1 °C). During observation sessions, each rat was paired with a rat from a different home-cage. All rats were experimentally naïve. At the end of the experiment the animals were euthanized with an overdose of urethane injected intraperitoneally.

Ethogram, behavioral observations, and event log files

We developed an ethogram (Fig. 1) adapted from our previous study[8] and from other studies[9–11,21–23]. All video files, previously recorded during the experimental sessions, were scored by a highly trained observer by using the software tool "The Observer XT 14" (Noldus Information Technology, The Netherlands. https://www.noldus.com/). The propaedeutic assessment of intra-rater reliability was evaluated on three video files, randomly taken from the experimental recordings, and each file was scored twice by the same observer and at two different points in time. The video-scoring process produced an event log file (ELF) containing a sequence of events performed by the animal and their corresponding time points. ELFs represent the starting point of all the analyses performed in the present study and described in the following sections.

Quantitative analysis

Percent distributions of each item of the ethogram were calculated to provide an immediate view of the impact of each component in the context of the comprehensive behavioral repertoire. Mean durations and mean occurrences of inter- and intra-subject components were calculated to assess how much time was spent in each of the two categories and their overall frequencies. Finally, time course of mean occurrences and mean durations of inter- and intra-subject components were evaluated to investigate any temporal changes in the behavioral sequences. These occurrences and durations were compared on the basis of the following five 3-min windows: 1–3, 4–6, 7–9, 10–12 and 13–15 min.

T-pattern analysis

We utilized T-pattern analysis (TPA) to elucidate the temporal structure of behavior. This procedure produces structures known as "*T-patterns"* (TPs), i.e. sequences of events characterized by statistically significant temporal constraints. TPA can be performed by means of Theme 6 software tool (PatternVision Ltd, Reykjavik, Iceland.

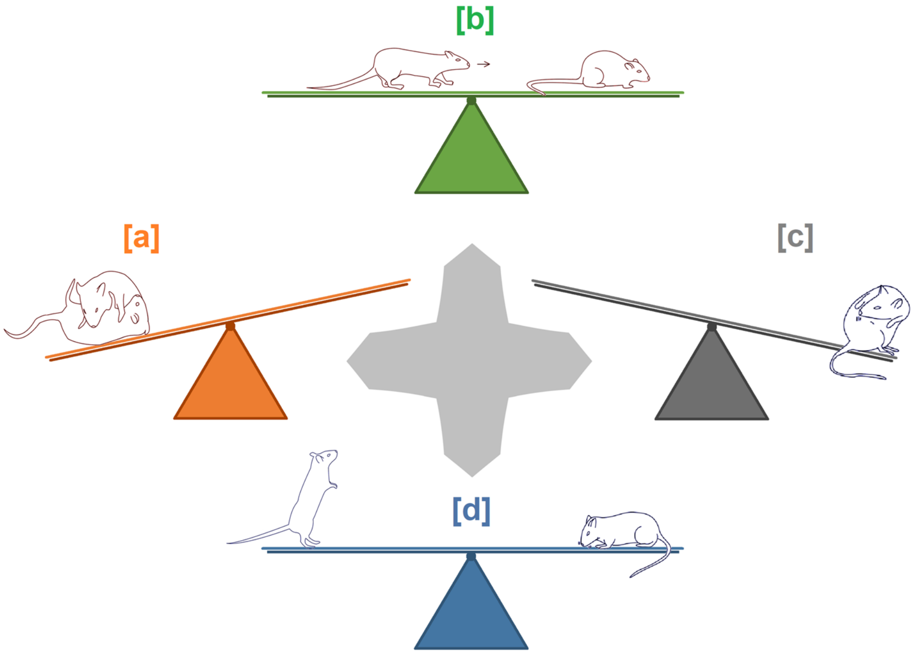


Fig. 2. Categories of T-patterns in social interaction. [a] TPs in which the animals display exclusively inter-subject behaviors; [b] TPs in which the animals display both inter-subject and intra-subject behaviors; [c] TPs made by only one of the two rats, in which no interaction toward the partner is present; [d] TPs produced by both subjects while neither of them is, apparently, exhibiting any explicit interactive behavior toward the partner.

https://patternvision.com/). In brief, Theme's detection algorithm delves into the interconnections among events within behavioral data, considering their sequencing, timing, and occurrence frequency[14,15]. For example, within an observation timeframe denoted as T0-Tx (Fig. 3), which encompasses various hypothetical events (as exemplified by the fifty letters along the axis in Fig. 3), the detection algorithm systematically compares the distribution of each pair of events, seeking temporal windows wherein an event, such as "a", is statistically more likely to be followed by another event, such as "b", compared to chance expectations. In such instances, where the event "a" is frequently followed by "b" within a defined temporal window, they constitute a two-event TP, denoted by the sequence (a b). Then, employing a hierarchical bottom-up detection approach, all primary level TPs, such as (a b), are evaluated as prospective constituents for higher-order patterns like ((a b) c), ((a b) (c d))…, and so forth. Through this iterative process, the detection algorithm identifies increasingly complex TPs, spanning across multiple levels. The search process terminates once no further TPs can be identified. Thus, TPA is a very useful tool to describe sequences of events such as those performed by interacting subjects. More exhaustive descriptions of concepts, theories and procedures pertaining to TPA can be found in previous papers[14–18,24–26].

Statistics

To evaluate any changes during the time-course of occurrences and durations, and to compare the mean occurrences and mean length of detected TPs, we used ANOVAs followed by the Newman–Keuls post-hoc test for multiple comparisons. To assess any significant differences in the mean occurrences and mean durations between inter- and intra-subject components, we used Student's *t*-test. For each pattern detected via TPA, the time interval among events implies the existence of a statistical significance. Nonetheless, the large number of such potential relationships, in data containing numerous occurrences of events, raises the question of whether the TPs detected

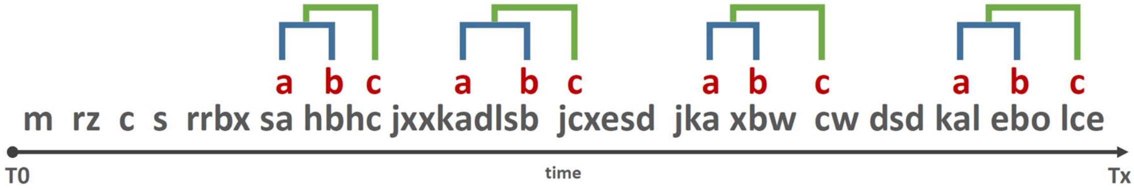


Fig. 3. Example of T-pattern. X-axis: observation period (T0–Tx) consisting of 50 hypothetical behavioral events (bold letters near time axis). The ((a b) c) T-pattern, occurring 4 times, becomes evident when all the remaining events are left out. See text for details.

were different by chance. Theme software deals with this issue by performing repeated randomizations and analyses of the original data. In all analyses, $P < 0.05$ was considered statistically significant.

Results

Quantitative analyses

Total percent distributions of intra- and inter-subject components (Fig. 4A) and percent distributions of each component of the behavioral repertoire (Fig. 4B) showed, respectively, a clear-cut prevalence of intra-subject components (Fig. 4A) and the contribution of each activity in the comprehensive behavioral repertoire (Fig. 4B). To evaluate the involvement of each component within intra- and inter-subject activities, we calculated their percent distributions separately (Fig. 4C,D). Among intra-subject components (Fig. 4C), Immobile-Sniffing (ISn) was, by far, the most represented with a percent score of 36.81%, followed by Walking (Wa) 16.69%, Climbing (Cl) 12.64% and Immobility (Imm) 9.46%. The remaining intra-subject components fell below 10%. Among inter-subject components (Fig. 4D), Social-Sniffing (SoS) was the most represented, with 33.93%, followed by Genital-Sniffing (GeS) 13.23%, Leaning-On (LeO) 12.96% and Withdrawing (Wit) 12.77%. The remaining inter-subject components fell below 10%.

The overall activity of the rats was illustrated by means of time courses of mean occurrences and mean durations of inter-subject and intra-subject components (Fig. 5). Regarding mean occurrences (Fig. 5A), the ANOVA showed a significant change in the time course of intra-subject components with a score of $F(4,49) = 3.36$, $p < 0.05$; Newman–Keuls post-hoc test for multiple comparisons revealed a significant ($p < 0.05$) reduction of mean occurrences during the 10–12 and 13–15 ranges compared to range 1–3. We found no significant changes in the time course of inter-subject components (ANOVA of $F(4,49) = 0.68$, $p = 0.612$). We found a significant difference between intra- and inter-subject mean occurrences (Student's *t*-test, $t(18) = 3.076$, $p < 0.01$; Fig. 5A, upper-right inset). Regarding mean durations (Fig. 5B), no significant changes were detected neither for time course of intra-subject components nor inter-subject ones ($F(4,49) = 2.19$, $p = 0.085$, and $F(4,49) = 0.15$, $p = 0.964$, respectively). We found a highly significant difference between intra- and inter-subject mean durations (Student's *t*-test, $t(18) = 13.926$, $p < 0.0001$; Fig. 5B, upper-right inset).

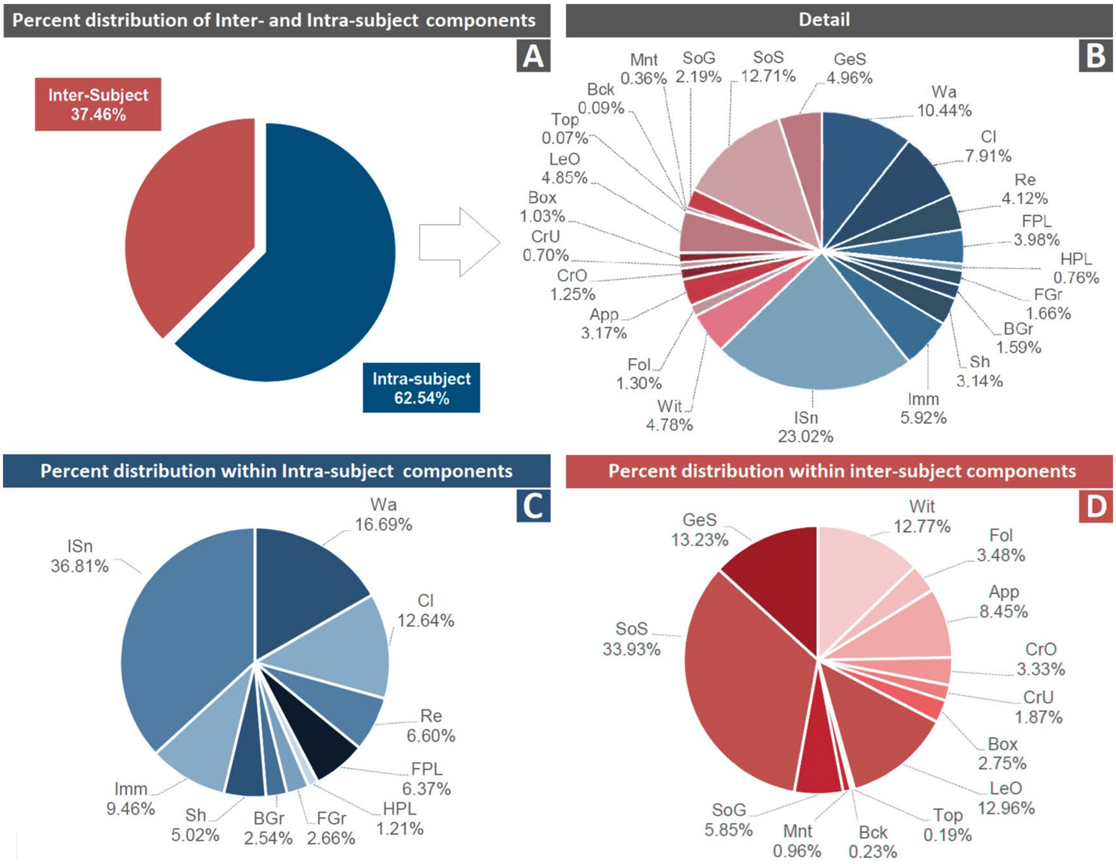


Fig. 4. Percent distributions. (**A**) Overall percent of inter and intra-subject components; (**B**) percent of each inter- and intra-subject component; (**C**) percent distribution within intra-subject components; (**D**) percent distribution within inter-subject components. Data obtained from the analysis of 20 subjects. For abbreviations see Fig. 1.

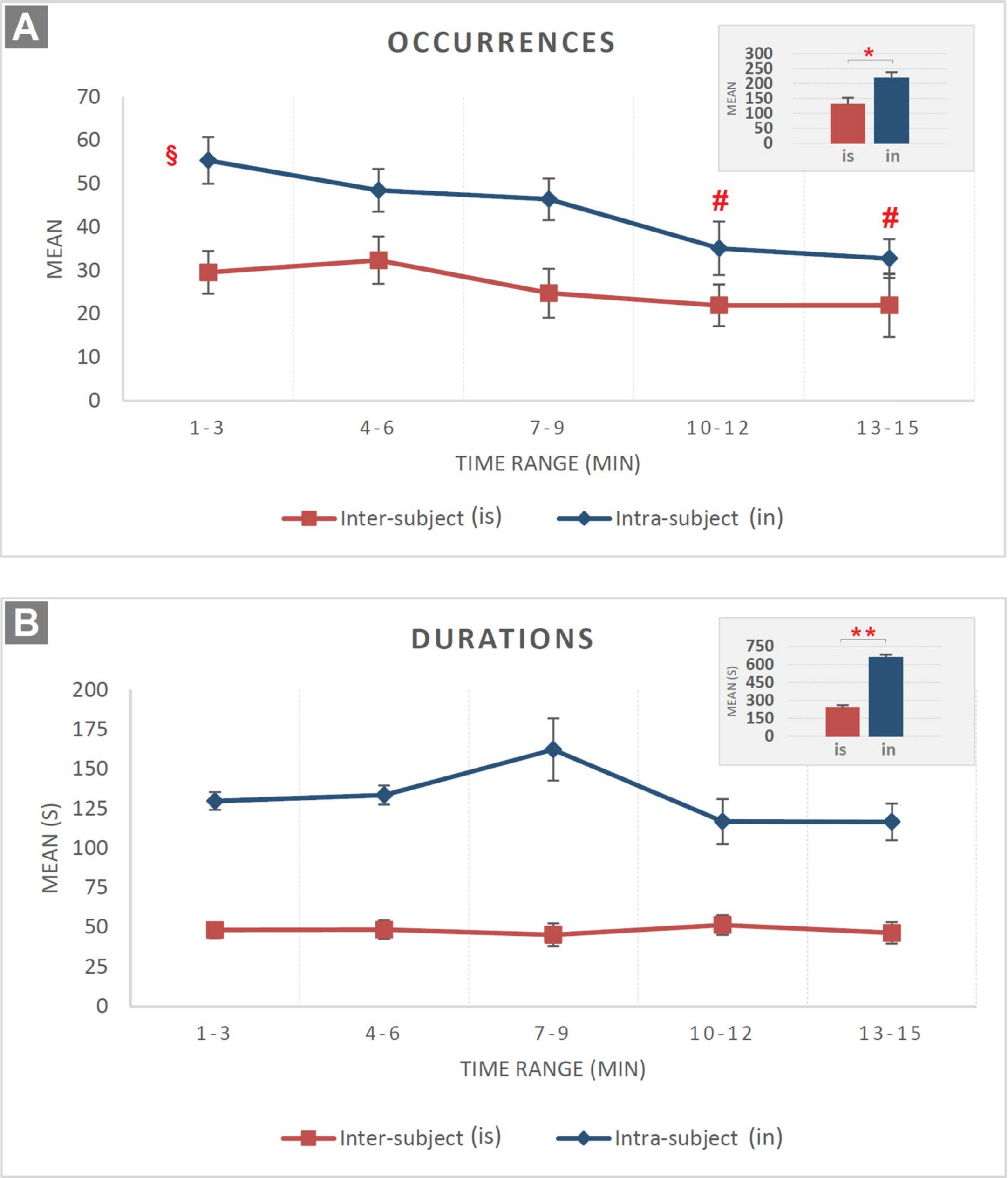


Fig. 5. Time course of the overall activity within 3 min ranges. (**A**) Time course of the mean occurrences of inter-subject (filled red square) and intra-subject (filled blue diamond) components; inset in the upper-right: overall mean occurrences of inter-subject and intra-subject components. (**B**) Time course of the mean durations (s) of inter-subject (filled red square) and intra-subject (filled blue diamond) components; inset in the upper-right: overall mean durations (s) of inter-subject and intra-subject components. S = seconds, MIN = minutes, is = inter-subject components, in = intra-subject components, *$p < 0.01$ (Student's *t*-test), **$p < 0.0001$ (Student's *t*-test), §significant $p < 0.05$ change in the time course (ANOVA), #significant $p < 0.05$ difference in comparison with range 1–3 (Newman–Keuls post-hoc test for multiple comparisons). Data obtained from the analysis of 20 subjects.

T-patterns' detection and analysis

The number of different TPs detected, based on their length, is shown in Fig. 6. Overall, 1054 different TPs were organized as follows: 121 different TPs had a length (L) of 2 events (= L2, i.e. 121 different TPs contained 2 events in sequence), 198 were = L3, 249 = L4, 209 = L5, 131 = L6, 67 = L7, 37 = L8, 22 = L9, 4 = L10, 6 = L11, 2 = L12, 3 = L13, 4 = L14, 1 = L15. Comparison of TPs detected in random generated data showed a negligible amount of TPs detected when compared to the real data: using the "rotation" randomization procedure, on the basis of 5 random runs, a mean of 7.7 TPs were detected containing 2 events and no TPs of higher order containing 3 or more events (Fig. 6, empty bar); using the "shuffling" procedure, on the basis of 5 random runs, a mean of 0.6

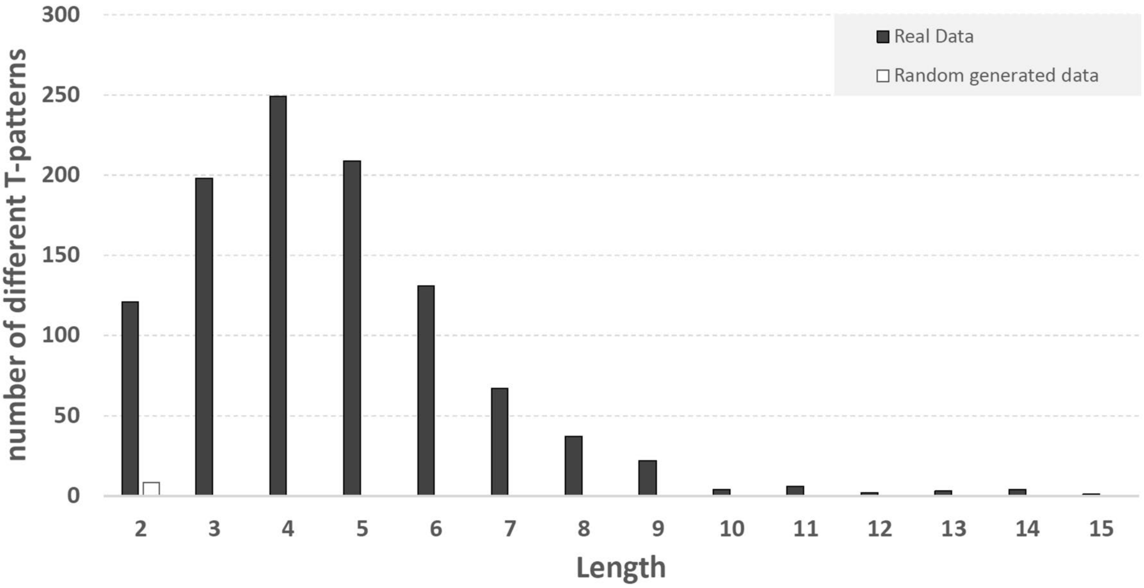


Fig. 6. T-patterns' length distribution. Dark bars = T-patterns detected in real data; empty bars = T-patterns detected in random generated data. X-axis = T-pattern length, i.e., number of events in T-pattern's structure; Y-axis = number of different T-patterns detected. Data obtained from the analysis of 20 subjects.

TPs was detected containing 2 events and no TPs of higher order containing 3 or more events (result not shown). Complete results of the detection process are presented in the supplementary online material.

On the basis of the events in TPs' sequences, four main categories of TPs were identified (see supplementary online material):

- Category [a]—consists of only inter-subject events and is characterized by a sequence in which both subjects, or only one of them, produce exclusively behavioral events toward the partner.
- Category [b]—by far the most represented, consists of both inter-subject and intra-subject events and is characterized by a sequence in which both animals, or only one of them, produce events directed toward the partner as well as not directed toward the partner.
- Category [c]—consists only of intra-subject events and shows a sequence in which only one of the two rats produces behavioral events not directed toward the partner
- Category [d]—consists only of intra-subject events and shows a sequence in which *both subjects produce behavioral events not directed toward the partner.*

Mean occurrences and mean lengths of TPs in the four categories, detailed in the supplementary online material, are graphically illustrated in Fig. 7. Regarding mean occurrences (Fig. 7A), ANOVA showed highly significant changes with a score of $F(3,1053) = 141.98$, $p < 0.0001$; Newman–Keuls post-hoc test for multiple comparisons showed significant ($p < 0.05$) differences among all the categories. Regarding mean length (Fig. 7B), ANOVA showed highly significant changes with a score of $F(3,1053) = 80.11$, $p < 0.0001$; Newman–Keuls post-hoc test for multiple comparisons showed significant ($p < 0.05$) differences only between category [b] and all the remaining ones.

Percent distributions of the occurrences of the TPs belonging to the above-mentioned four categories, detailed in the supplementary online material, are graphically illustrated in Fig. 8: category [a] = 9.89%, category [b] = 58.71%, category [c] = 20.19%, category [d] = 11.21%.

Percent composition of TPs, on the basis of events in structure, is illustrated in Fig. 9.

- Category [a]—consists of 74 different TPs containing, proceeding clockwise, Wit = 16.08%, App = 20.60%, CrO = 2.01%, LeO = 12.56%, SoS = 35.68% and GeS = 13.07% (Fig. 9A).
- Category [b]—consists of 840 different TPs encompassing Wa = 13.66%, Cl = 5.81%, Re = 0.68%, Sh = 0.05%, Imm = 0.66%, ISn = 38.05%, Wit = 3.17%, App = 15.76%, CrO = 0.12%, LeO = 3.72%, SoS = 16.42% and GeS = 1.90% (Fig. 9B).
- Category [c]—consists of 73 different TPs containing Wa = 28.22%, Cl = 23.75%, Re = 8.91%, FPL = 2.97%, FGr = 4.95%, BGr = 1.49%, Sh = 0.50%, Imm = 2.48% and ISn = 26.73% (Fig. 9C).
- Category [d]—consists of 67 different TPs. This category is structured almost exclusively on the basis of the events of environmental exploration which, taken together, account for more than 99.5% of all the events in TPs' structure within this category; in detail, Wa = 30.99%, Cl = 28.17%, Re = 6.57% and ISn = 33.80%; the remaining small percent value is represented by Sh = 0.47% (Fig. 9D). Terminal strings of category [d] are presented in Table 1. Within this category, a sub-set of 23 TPs consists of sequences in which at least one

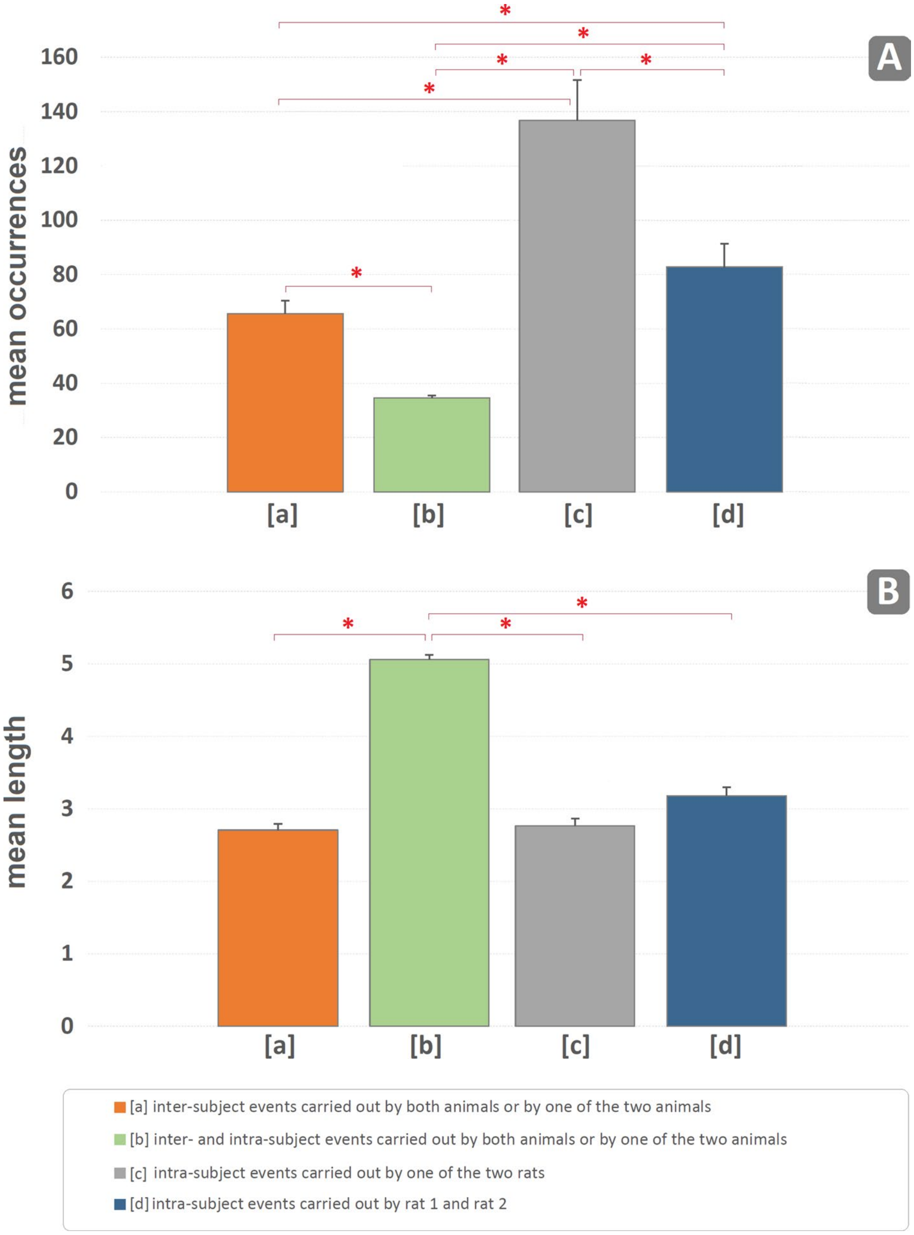


Fig. 7. Mean occurrences and mean length of T-patterns. (**A**) Mean occurrences (± SE) of T-patterns belonging to each category (a–d); (**B**) mean durations (± SE) of T-patterns belonging to each category (a–d). *Significant differences between categories (ANOVA followed by Newman–Keuls post-hoc test for multiple comparisons). Data obtained from the analysis of 20 subjects.

of the occurring events is mirrored, i.e. it occurs after the occurrence of the same event performed by the partner. These events are marked with asterisks (tab. 1, "M" column) and illustrated in Fig. 10 as well.

Discussion

We used TPA to analyze the temporal structure of social interactions in 10 pairs of male Wistar rats. Using a comprehensive ethogram, both in terms of interactive (i.e., inter-subject) and non-interactive (i.e., intra-subject) behaviors, we expanded and deepened several aspects of previous observations[8]. We also discovered a new behavioral category of interaction, that was indicative of dyadic temporal coordination in the behavioral expression and behavioral mirroring between two individuals. We also described some peculiar aspects, related to the temporal trends of the observed phenomena.

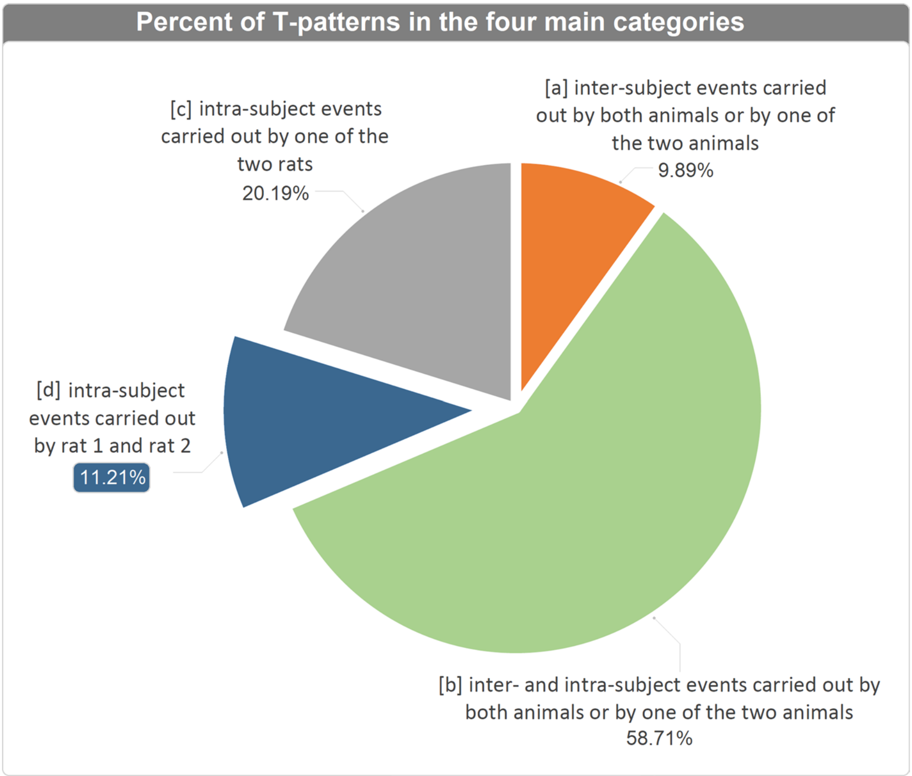


Fig. 8. Percent distribution of T-patterns. Percent of T-patterns in the four main categories (a–d). See text for details. Data obtained from the analysis of 20 subjects.

Quantitative analyses

Percent distributions highlighted the contribution of each category and component in the comprehensive behavioral repertoire of interactions (Fig. 4A). Non-interactive behaviors were prevalent, and among them, those pertaining to environmental exploration (Wa, Cl, Re, and ISn). In this context, components involving physical contact between the two animals (CrO, CrU, Box, LeO, Top, BcK, Mnt and SoG) slightly exceeded 10% (Fig. 4B). A careful look into each of the two categories allowed us to better appreciate these aspects, emphasizing the preponderance of exploration behaviors as far as the intra-subject category was concerned; regarding the inter-subject category, the two sniffing behaviors (SoS and GeS) were prevalent and approximated 50%. By adding the components involving walking (Wit, Fol and App), we noticed that even within the inter-subject category the partner-contact components were, in fact, a minority. Taken together, these data show that two rats placed in interaction orient their behavior predominantly toward exploration of the environment, rather than toward interaction; in addition, within the interaction category, physical contact with the partner represented the smallest percentage of the entire behavioral repertoire.

Considering that all subjects were tested under environmental settings conducive to high anxiety (i.e., intense lightning and unfamiliar environment, see studies by File and Seth[20] and Irvine et al.[19]), we argue that this outcome may be the result of stressful conditions. A look at temporal trends (Fig. 5) provides further insights for discussion. Regarding occurrences (Fig. 5A), post-hoc test for multiple comparisons showed that time has a marked effect only on the occurrences belonging to the intra-subject category. Part of this result may be explained by the fact that, during the first half of the observations, the animals' exploratory activity was more represented, whereas a physiological decrease occurred during the later time ranges; this phenomenon was not present in the occurrences within the inter-subject category. Regarding durations (Fig. 5B), ANOVA showed no significant variations for both categories. It is noteworthy that, for both occurrences and durations, the inter-subject category maintained an almost "flat" trend. In line with our argument that the environmental settings may induce high levels of anxiety in the sampled subjects (see File and Seth[20]; Irvine and Cheeta[19]), it is likely that while the exploration responses stabilized over time, the relationship with the partner (which was unknown, because each subject was tested with a partner from another home-cage, see section concerning Procedure) did not, possibly leading to sustained arousal during inter-subject responses.

T-pattern analysis

TPA allowed us to detect four categories of TPs, each with its own peculiar characteristics (Fig. 2): [a] = TPs containing only partner-directed events, produced by one or both animals; [b] TPs containing both partner-directed and non-partner-directed events, produced by one or both animals; [c] TPs containing only non-partner-directed events, produced by only one of the animals; [d] TPs containing only non-partner-directed events, produced

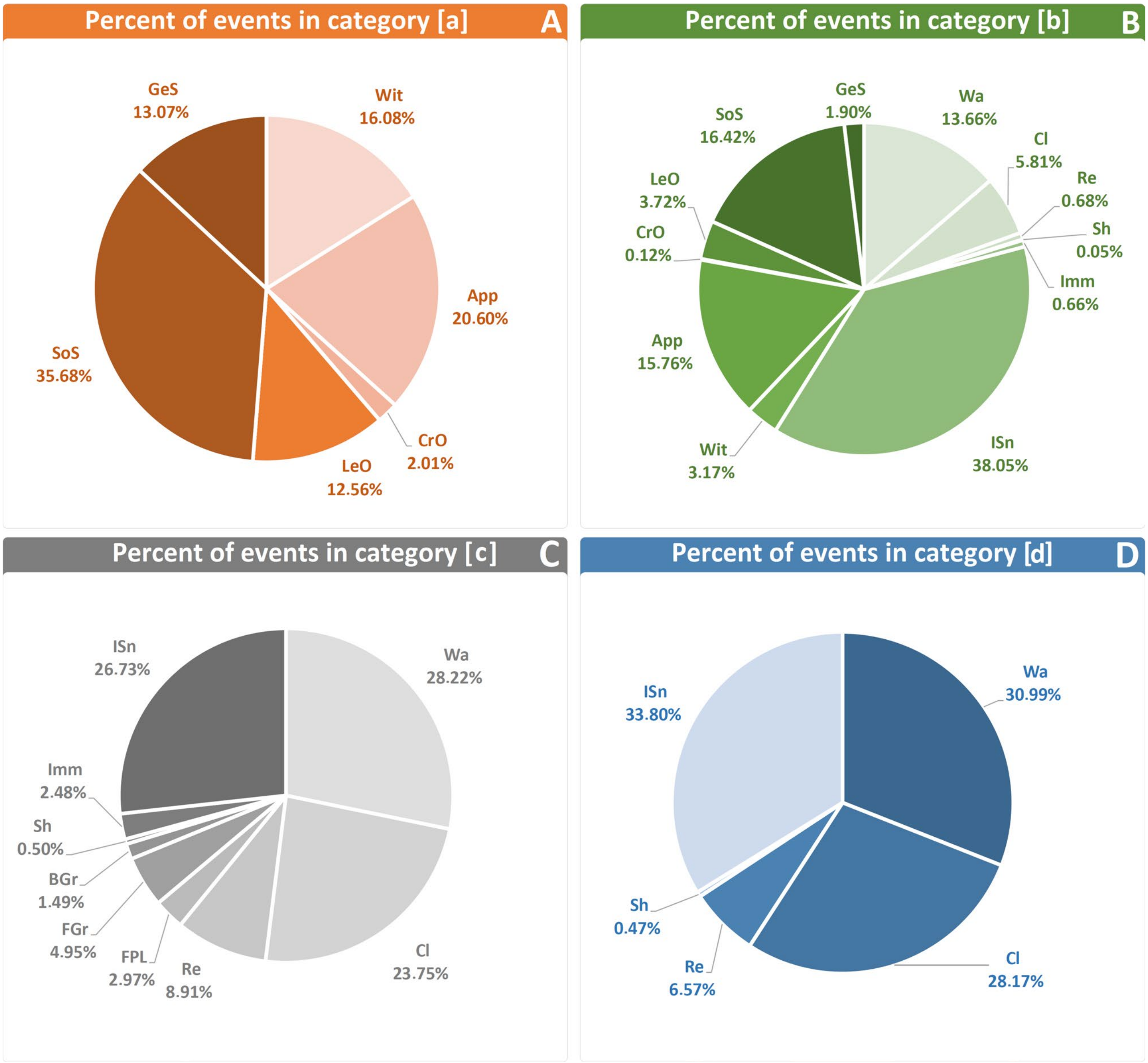


Fig. 9. Percent distribution of events in T-patterns for each category. (**A**) Percent of events in the structure of T-patterns belonging to the category [a], i.e. T-patterns encompassing only inter-subject events produced by one or both the animals; (**B**) percent of events in the structure of T-patterns belonging to the category [b], i.e. T-patterns encompassing inter and intra-subject events produced by one or both the animals; (**C**) percent of events in the structure of T-patterns belonging to the category [c], i.e. T-patterns encompassing only intra-subject events produced by one of the two animals; (**D**) percent of events in the structure of T-patterns belonging to the category [d], i.e. T-patterns encompassing only intra-subject events produced by both the animals. See text for details. For abbreviations, see Fig. 1. Data obtained from the analysis of 20 subjects.

by *both* animals. Among these categories, the one that triggered specific interest and represented the primum movens of the present study, was [d], i.e., TPs produced by *both* subjects but composed only of (apparently) non-interactive behaviors. While these results are consistent with our previous work[8], they greatly expand its boundaries. In fact, we have highlighted that this category [d] encompasses a new subcategory of TPs characterized by not only apparently non-interactive events between the two animals but also by *events that one of the two subjects repeats after the partner has produced them.*

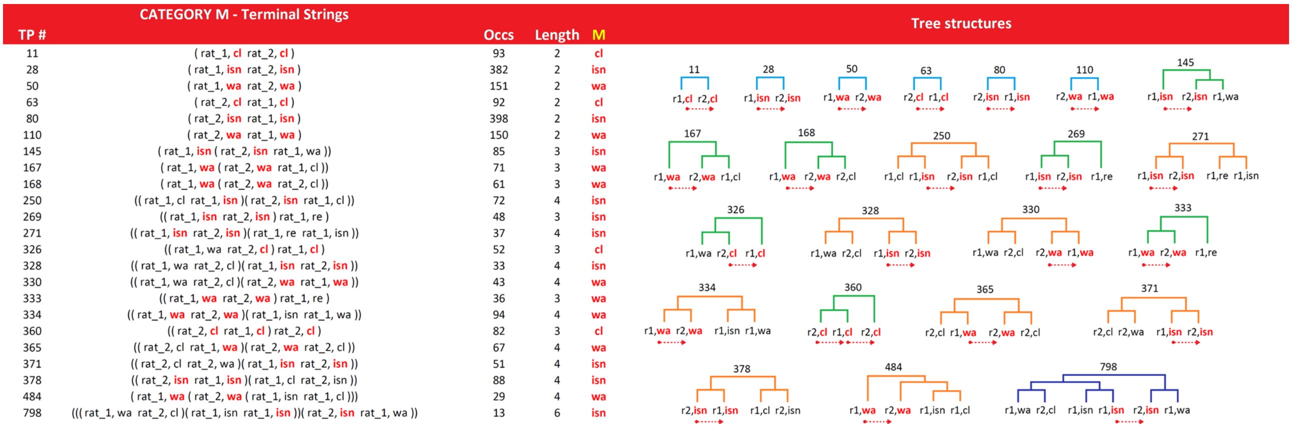
- Category [a]: This category encompasses sequences in which both subjects, e.g. TP#6 (rat_1,app rat_2,sos), or only one of them e.g. TP#1 (rat_1,app rat_1,ges), produce(s) exclusively behavioral events toward the partner. Two more complex examples could be represented by TP#748 ((rat_2,sos (rat_2,wit rat_2,app)) (rat_2,leo rat_2,sos)) or TP#764 (((rat_1,app rat_1,sos) rat_2,sos) rat_1,leo). In all the above examples, and in all TPs belonging to this category, there are significant relationships exclusively between inter-subject

TP #	M	CATEGORY [d]—Terminal Strings	Occs	Length
11	*	(rat_1, **cl** rat_2, **cl**)	93	2
12		(rat_1, cl rat_2, isn)	128	2
28	*	(rat_1, **isn** rat_2, **isn**)	382	2
30		(rat_1, isn rat_2, wa)	183	2
34		(rat_1, re rat_2, cl)	26	2
36		(rat_1, sh rat_2, isn)	55	2
48		(rat_1, wa rat_2, cl)	92	2
49		(rat_1, wa rat_2, isn)	221	2
50	*	(rat_1, **wa** rat_2, **wa**)	151	2
63	*	(rat_2, **cl** rat_1, **cl**)	92	2
64		(rat_2, cl rat_1, isn)	152	2
65		(rat_2, cl rat_1, wa)	121	2
79		(rat_2, isn rat_1, cl)	146	2
80	*	(rat_2, **isn** rat_1, **isn**)	398	2
81		(rat_2, isn rat_1, re)	62	2
82		(rat_2, isn rat_1, wa)	191	2
107		(rat_2, wa rat_1, cl)	103	2
108		(rat_2, wa rat_1, isn)	178	2
109		(rat_2, wa rat_1, re)	57	2
110	*	(rat_2, **wa** rat_1, **wa**)	150	2
145	*	(rat_1, **isn** (rat_2, **isn** rat_1, wa))	85	3
165		(rat_1, wa (rat_2, cl rat_1, wa))	67	3
166		(rat_1, wa (rat_2, isn rat_1, cl))	49	3
167	*	(rat_1, **wa** (rat_2, **wa** rat_1, cl))	71	3
168	*	(rat_1, **wa** (rat_2, **wa** rat_2, cl))	61	3
181		(rat_2, cl (rat_1, isn rat_1, cl))	51	3
182		(rat_2, cl (rat_1, isn rat_2, wa))	88	3
183		(rat_2, cl (rat_1, wa rat_2, cl))	62	3
188		(rat_2, cl (rat_2, wa rat_1, cl))	62	3
192		(rat_2, isn (rat_1, re rat_1, isn))	39	3
206		(rat_2, re (rat_1, isn rat_2, wa))	45	3
222		(rat_2, wa (rat_1, isn rat_1, cl))	48	3
250	*	((rat_1, cl rat_1, **isn**)(rat_2, **isn** rat_1, cl))	72	4
252		((rat_1, cl rat_2, isn) rat_1, wa)	96	3
269	*	((rat_1, **isn** rat_2, **isn**) rat_1, re)	48	3
271	*	((rat_1, **isn** rat_2, **isn**)(rat_1, re rat_1, isn))	37	4
282		((rat_1, isn rat_2, wa) rat_1, re)	46	3
285		((rat_1, isn rat_2, wa)(rat_2, cl rat_1, isn))	77	4
317		((rat_1, wa rat_1, isn) rat_2, re)	28	3
324		((rat_1, wa rat_1, isn)(rat_2, re rat_2, isn))	27	4
326	*	((rat_1, wa rat_2, **cl**) rat_1, **cl**)	52	3
327		((rat_1, wa rat_2, cl)(rat_1, isn rat_1, isn))	19	4
328	*	((rat_1, wa rat_2, cl)(rat_1, **isn** rat_2, **isn**))	33	4
329		((rat_1, wa rat_2, cl)(rat_2, isn rat_1, wa))	60	4
330	*	((rat_1, wa rat_2, cl)(rat_2, **wa** rat_1, **wa**))	43	4
333	*	((rat_1, **wa** rat_2, **wa**) rat_1, re)	36	3
334	*	((rat_1, **wa** rat_2, **wa**)(rat_1, isn rat_1, wa))	94	4
360	*	((rat_2, **cl** rat_1, **cl**) rat_2, **cl**)	82	3
361		((rat_2, cl rat_1, isn)(rat_1, wa rat_2, cl))	53	4
363		((rat_2, cl rat_1, isn)(rat_2, wa rat_2, cl))	64	4
364		((rat_2, cl rat_1, wa) rat_2, cl)	110	3
365	*	((rat_2, cl rat_1, **wa**)(rat_2, **wa** rat_2, cl))	67	4
371	*	((rat_2, cl rat_2, wa)(rat_1, **isn** rat_2, **isn**))	51	4
378	*	((rat_2, **isn** rat_1, **isn**)(rat_1, cl rat_2, isn))	88	4
387		((rat_2, isn rat_1, re)(rat_1, isn rat_1, isn))	42	4
398		((rat_2, isn rat_2, cl)(rat_1, wa rat_2, isn))	85	4

Continued

TP #	M	CATEGORY [d]—Terminal Strings	Occs	Length
451		((rat_2, wa rat_2, isn) rat_1, cl)	92	3
461		(rat_1, cl (rat_1, wa (rat_2, isn rat_1, cl)))	39	4
466		(rat_1, isn (rat_1, wa (rat_2, isn rat_1, cl)))	34	4
484	*	(rat_1, **wa** (rat_2, **wa** (rat_1, isn rat_1, cl)))	29	4
494		(rat_1, wa ((rat_1, cl rat_2, isn) rat_1, wa))	70	4
536		(rat_2, wa (rat_2, cl (rat_1, isn rat_2, wa)))	50	4
548		((rat_1, cl rat_1, isn)(rat_1, wa (rat_2, isn rat_1, cl)))	33	5
600		((rat_2, cl rat_1, isn)((rat_1, wa rat_1, isn) rat_2, re))	17	5
792		(((rat_1, wa rat_1, isn) rat_2, re)(rat_2, isn rat_1, wa))	22	5
798	*	(((rat_1, wa rat_2, cl)(rat_1, isn rat_1, **isn**))(rat_2, **isn** rat_1, wa))	13	6
845		(rat_1, cl (rat_1, isn (rat_1, wa (rat_2, isn rat_1, cl))))	27	5

Table 1. Terminal strings. Textual representations of T-patterns belonging to the category [d]. Parentheses indicate the structure of the given pattern on the basis of the hierarchical bottom-up detection process. Events are presented in the order in which they occur: the first event is on the left of each string, the last on the right. Code on the left of each string (TP# column) indicates the corresponding T-pattern present in the additional online material; asterisks (M column) indicate the presence of a mirrored event in the structure of the corresponding T-pattern; numbers on the right of each string indicate occurrences (Occs) and length respectively. For abbreviations see Fig. 1. Data obtained from the analysis of 20 subjects.



CATEGORY M - Terminal Strings

TP #		Occs	Length	M
11	(rat_1, cl rat_2, cl)	93	2	cl
28	(rat_1, isn rat_2, isn)	382	2	isn
50	(rat_1, wa rat_2, wa)	151	2	wa
63	(rat_2, cl rat_1, cl)	92	2	cl
80	(rat_2, isn rat_1, isn)	398	2	isn
110	(rat_2, wa rat_1, wa)	150	2	wa
145	(rat_1, isn (rat_2, isn rat_1, wa))	85	3	isn
167	(rat_1, wa (rat_2, wa rat_1, cl))	71	3	wa
168	(rat_1, wa (rat_2, wa rat_2, cl))	61	3	wa
250	((rat_1, cl rat_1, isn)(rat_2, isn rat_1, cl))	72	4	isn
269	((rat_1, isn rat_2, isn) rat_1, re)	48	3	isn
271	((rat_1, isn rat_2, isn)(rat_1, re rat_1, isn))	37	4	isn
326	((rat_1, wa rat_2, cl) rat_1, cl)	52	3	cl
328	((rat_1, wa rat_2, cl)(rat_1, isn rat_2, isn))	33	4	isn
330	((rat_1, wa rat_2, cl)(rat_2, wa rat_1, wa))	43	4	wa
333	((rat_1, wa rat_2, wa) rat_1, re)	36	3	wa
334	((rat_1, wa rat_2, wa)(rat_1, isn rat_1, wa))	94	4	wa
360	((rat_2, cl rat_1, cl) rat_2, cl)	82	3	cl
365	((rat_2, cl rat_1, wa)(rat_2, wa rat_2, cl))	67	4	wa
371	((rat_2, cl rat_2, wa)(rat_1, isn rat_2, isn))	51	4	isn
378	((rat_2, isn rat_1, isn)(rat_1, cl rat_2, isn))	88	4	isn
484	(rat_1, wa (rat_2, wa (rat_1, isn rat_1, cl)))	29	4	wa
798	(((rat_1, wa rat_2, cl)(rat_1, isn rat_1, isn))(rat_2, isn rat_1, wa))	13	6	isn

Fig. 10. Terminal strings and tree structures. T-patterns belonging to the [d]'s sub-category M, i.e. T-patterns containing intra-subject events produced by both subjects where a mirrored event (M column) is present. Terminal strings (left) present T-patterns in textual format with parentheses indicating the structure of the given pattern on the basis of the bottom-up detection process. Tree structures (right) graphically illustrate T-patterns by showing events and how they are connected. In both representations events are presented in the order in which they occur: the first event in pattern is on the left, the last on the right. Code on the left of each string (TP# column) indicates the corresponding T-pattern present in the additional online material; TP# codes are also reported on the top of each tree structure; numbers on the right of each string indicate occurrences (Occs) and length of the given T-pattern. In tree representations "rat_1" and "rat_2" are abbreviated respectively with "r1" and "r2". Mirrored event, for each tree, is highlighted by a dashed arrow. For abbreviations see Fig. 1. Data obtained from the analysis of 20 subjects.

events. In detail, these TPs mostly comprise only components in which the two animals are in mutual contact with each other (Fig. 9A). This category of TPs, which collectively accounts for 9.89% of the total number of TPs found (Fig. 8) demonstrates that, although a modality of interaction involves contacts between the two subjects, these sequences do represent a small amount of all the occurrences of the sequences detected. Thus, it is evident that the largest extent (i.e., more than 90%) of social interaction in rats orbits around sequences of different architecture.

- Category [b]: This category consists of sequences in which both the animals, e.g. TP#5 (rat_1, app rat_2, isn), or only one of them, e.g. TP#2 (rat_1, app rat_1, isn), produce(s) events directed toward the partner as well as themselves. This category, which overall accounts for 58.71% of the total TPs found (Fig. 8), is comprised of sequences consisting, within the same sequence, of both inter-subject and intra-subject events (Fig. 9B). Thus, these are complex sequences in which both interactive and non-interactive events are present. It is, by far, the most represented category; it shows that a large part of the interactive behaviors of the two subjects (in fact, almost 60%) is based on complex sequences in which the animal interacts with its partner but also directs its activities toward itself and/or the environment.

- Category [c]: This type of TP consists of a sequence in which only one of the two rats produces behavioral events directed toward itself, e.g. TP#13 (rat_1, fgr rat_1, bgr) or TP#70 (rat_2, cl rat_2, wa). This category, overall, encompasses 20.19% of the TPs found (Fig. 8) and in its nature, it is diametrically opposed to category [a]. In fact, while category [a] was represented by pure sequences of interaction between the two animals, category [c] includes pure sequences of non-interaction in which the subject turns its attention exclusively toward itself (e.g. face grooming, body grooming, etc.) or explores the environment (e.g. climbing, walking etc.). Structurally speaking, the events characteristic of category [c] are almost exclusively exploration or grooming (Fig. 9C).
- Category [d]: *interaction or not?*—Among the four categories, [d] is certainly the one worthy of particular attention. It covers 11.21% of the total TPs detected (Fig. 8). It encompasses sequences in which each of the subjects would appear, based on the percentage data apparently in 100 percent of the cases (Fig. 9D), to be engaged in non-interactive events. Being within a TP, however, it is implied that each of the events in the sequence is bound to the others by constraints that would suggest the absence of randomness. This type of TP consists of a sequence in which both the subjects produce behavioral events not directed toward the partner, e.g. TP#11 (rat_1, cl rat_2, cl) or TP#28 (rat_1, isn rat_2, isn). It is important to underline here that if one of the two animals performs a specific event as a result of what the other does, it is not by chance, due to the very definition of TP itself. Implicitly, then, one must admit that even in this case an interaction is present, albeit not manifest to the naked eye. This begs an intriguing question: *what* is really an interaction?—Generally speaking, "interaction", between subjects of the same species, could be defined as the response that an individual produces in presence of a conspecific. In behavioral studies involving rodents, social interaction tests have been defined as experimental approaches able to provide accurate evaluations of social responses that the subject produces when facing the partner[27]. It is widely known that when pairs of laboratory rats are placed in a novel environment such as an Open-Field[7] or an hole-board[28], they engage reciprocal interactions, i.e. a behavioral repertoire characterized by a number of different activities such as allogrooming, following or approaching the partner, walking over, crawling under etc. (Fig. 1), while aggressive behaviors such as boxing or biting are rarely observed[2]. This kind of interaction is often considered "active" to distinguish it from passive contacts occurring when the two conspecifics are in contact without an active participation, like when the animals are immobile or sniff the environment and their body are in contact[28]. There are many studies involving the social interaction test; a discussion of the numerous definitions of social interactions or the paradigms employed to investigate them is beyond the scope of this paper (for a comprehensive review, see File and Seth[20]). All these studies, however, are characterized by the assessment of the response the subject exhibits when in the presence of the partner. For example, imagine one of the two rats approaching the conspecific (i.e. Approaching, Fig. 1) who in turn moves away (i.e. Withdrawing, Fig. 1); again, imagine the same situation in which the partner remains immobile and the subject who was approaching, once in contact, undertakes sniffing of its body (i.e. Social Sniffing, Fig. 1) or its genital region (i.e. Genital Sniffing, Fig. 1). These examples and the countless similar examples we could give are all characterized by the empirical nature of the evaluation, which is rigidly observer-dependent, involving either overt causality (e.g., one subject approaches the other who moves away) or, more simply, the observation of the two subjects in mutual contact (e.g., allogrooming, partner sniffing etc.). Yet, TPA shows that there is a strong link between the behavioral events produced by two subjects engaged in *apparently* non-partner-facing activities. Italicizing the word "*apparently*" is not trivial: if one of the two subjects produces non-partner-facing behavior and the conspecific, *within the strict statistically significant time constraints sanctioned by TPA*, responds with specific activities, is it correct to say that there is no interaction at all? Or, conversely, could we say that such a response, although not intuitively appreciable to the naked eye, must necessarily be linked to the conspecific's behavior by causal constraints and should also be considered an interactive behavior? In this case, category [d] is highly suggestive of a dyadic temporal coordination in the behavioral expression of two individuals. An example involving humans may be clarifying at this point. Imagine two subjects approaching each other and shaking their hands as a greeting: the interaction between the two is evident. Now imagine two subjects at some distance from each other; the first one makes a gesture of greeting with his head, the second one responds with a similar gesture: is there anyone willing to argue that this is not an interaction? Obviously, when speaking of humans, it is easy to pick up causal links (which are rather simple in the two examples above, but might also be much more articulated and/or less appreciable). For obvious reasons, the discussion becomes more complex when the interaction being studied occurs between non-human subjects and, in particular, rodents. The existence of interaction patterns where there seems to be none, i.e. those TPs belonging to the category [d], seriously questions the very concept of interaction between conspecifics as hitherto accepted. We propose that, in the social behavior of two rats, it is necessary to speak of interaction not only when there is a manifest causality to the observer but also when such a causality, by its own nature, is more elusive and/or difficult to appreciate except by appropriate means of investigation such as TPA. The evidence that category [d] also includes numerous TPs in which a repeated behavior from the conspecific, i.e. TPs belonging to the sub-category [m], poses an additional topic of discussion.
- Sub-category [m]: *do rats ape?*—This question overtly recalls a lengthy discussion by Heyes and Byrne[29–32] concerning rodents' ability to replicate behaviors after observing a conspecific. On this subject, an elegant study demonstrated that a rat placed in an observation room at the center of a radial maze becomes more proficient at exploring the environment after observing exploratory activities performed by conspecifics[33]. Other studies have shown that rats[34] or mice[35] perform specific motor tasks (e.g., reaching a food pellet) more efficiently after observing a conspecific perform the same task, with success rates increasing with training[35]. Interestingly, the authors drew stimulating parallels with the activity of mirror neurons and their physiological role. Although further discussion of these concepts is beyond the scope of the present research, some

points of tangency with our findings may arise. Indeed, our observations showed a rat repeating something done by a conspecific. However, in contrast to the aforementioned studies, in which the subject observed a conspecific performing a specific task to receive a reward and had to replicate that novel and rather complex behavior, our animals did not receive any rewards as a result of their activities; both animals were subjected to the same housing conditions and interacted in the same new environment. Importantly, none of the animals we tested had to learn to perform behavioral patterns they were unfamiliar with (e.g. performing a specific task to obtain food). Everything we observed and described was already part of the animal's normal behavioral repertoire such as Walking, Climbing, Rearing, Immobile-Sniffing etc. So, the rats we observed were not involved in an "imitative learning" paradigm i.e., the observation of a conspecific doing something new and the subsequent reproduction of that activity[36]. Our subcategory [m] of putative social interactions might be analogous to some forms of behavioral mirroring and physiological concordance in humans that are associated with specific emotional states[37–41].

A stress-mediated response?

As Timmerman[42] underscored, when rats encounter another member of their species, their initial response often involves a combination of orienting themselves towards the unfamiliar conspecific and engaging in exploratory behaviors. This inclination towards exploration is typically observed right at the outset of their interaction, serving as a crucial precursor to subsequent behavioral sequences. However, it's worth noting that such orientation and exploratory behaviors may also reoccur at various points throughout their interaction, indicating ongoing interest or uncertainty[42]. What stands out in all forms of attentive behavior in rats is their consistent tendency to turn their heads towards their conspecifics, indicating a heightened state of awareness and focus. Yet, when observations are conducted within confined spaces, accurately scoring these attentive behaviors presents a distinct challenge. The close proximity of the animals often leads to frequent, unplanned encounters, blurring the delineation between intentional orientation and incidental contact[42]. Consequently, researchers must navigate these complexities to accurately interpret the nuances of rat behavior within such constrained observational settings. Classically, the social interaction test can be used under four different environmental conditions[20]: (1) low light + familiar arena → generating the lowest level of anxiety; (2) high light + familiar arena → generating moderate levels of anxiety; (3) low light + unfamiliar arena → generating moderate levels of anxiety; finally, (4) high light + unfamiliar arena → generating the highest level of anxiety. Pairs of rats we used have been tested in an unfamiliar arena under high light conditions of 300 lux[19], i.e. a well-lit environment and a light threshold sufficient to elicit an anxiogenic condition[43]. Therefore, based on the four conditions above-mentioned, the social interactions described in the present study generate a clear-cut high level of anxiety in the tested rodents. That said, such a stressful situation (e.g., being in an unfamiliar, well-lit environment, in presence of an unknown subject) may release in each rat, viewing its conspecific in the same situation, a behavioral response sustained by the emotional state matching between the two subjects and manifested through a repetition of the conspecific's behaviors; in this context, the behavioral mirroring performed by the subjects could represent a valuable aid in coping with the emotional impact triggered by the stressful situation/environment. From this point of view, the entire category [d] to which the sub-category [m] belongs, could also be an expression of a more general mirroring activity, not of a single behavior, but of a whole class of behaviors of the same type which are, almost exclusively, those of generalized exploration (Fig. 9D). These particularly stressful situations manifested during the social behavior of rats were the main subject of a study, now considered classic, conducted by Grant[44]. The author noted that during encounters, either one or both animals would break off and move away for a distance. They would then return to the encounter after a brief pause. This pause was accompanied by various behaviors, all indicating a conflict between approaching and avoiding. i.e. a condition heavily dependent on subjects' anxiety level. Various TPs in supplementary online material could resemble behavioral sequences described by Grant in his pathway diagrams[44]. However, it is important to consider that in Grant's study the observations were carried out with an entirely different approach where one "intruder" rat was placed into the home cage of another rat. Subsequently, after 15 min of observation, the intruder was returned to its own cage. Importantly, no analysis of the temporal structure of behavior was conducted.

Translational perspectives

Bearing in mind that great caution is required in drawing parallels between results obtained in animal models and potential applications to humans, the focus naturally shifts to the translational implications of our approach. The prospect of employing a technique, such as TPA, specifically designed to analyze real-time patterns in intra- and inter-individual behavior[14,18] holds considerable interest and potential utility. The utility lies just in its intrinsic salient features, namely, the ability to unveil relationships among events over time. Such relationships are often very difficult or impossible to assess without proper means of observation and analysis. By capturing an interaction between two subjects, where there seems to be none, our study design may shed new light into pathological conditions of neuropsychiatric order such as, autism spectrum disorders and schizophrenia spectrum disorders, in which one of the most deleterious aspects is precisely the impairment of social interaction. This applies both to TPs that reveal an interaction between two subjects that would not appear to interact at first glance and to the presence of mirroring behaviors, namely the subcategory we have described. In this way, TPA not only offers crucial insights into understanding behavior but may also suggest potential therapeutic approaches or targeted interventions to effectively address disorders or issues related to social interactions. With these concepts and ideas in mind, TPA has been used to study autism spectrum disorder[45,46] and schizophrenia spectrum disorder[47,48], i.e. prevalent conditions characterized by disruptions in social interaction[13]. TPA has been also successfully applied to study other severe conditions marked by impaired social interactions, including attention-deficit hyperactivity disorder[49], self-injurious behavior[50,51], repeated suicide risk assessment[52], or pervasive developmental disorder[53].

The compelling results obtained underscore the validity of TPA in studying conditions characterized by alterations in social interactions in humans.

Limitations of the study and avenues for future research

In addition to its potential impact, our study offers some guidelines for future research. First, the lighting level in the testing room could provide an opportunity to explore how different light conditions might affect subjects' behavior; test/re-test procedures could offer additional information concerning interaction processes; the sex of the animals used in the study is another variable worth considering, as it opens up avenues to investigate any sex differences in behavioral and physiological responses. Furthermore, the familiarity, or lack thereof, with the partner is another intriguing aspect that could influence the dynamics observed, possibly leading to new findings about the nature of sociality. Examining these variables could strengthen the robustness of the present findings and open the door to novel and stimulating areas of discussion about the mechanisms and evolution of social life.

Conclusion

The present study highlights numerous features of the interactive behavior of male rats during the social interaction test. Beyond a detailed description of various purely qualitative aspects such as the percent distribution of the different components of the behavioral repertoire, the mean, duration and time course of interactive and non-interactive behaviors, a TPA was also conducted to assess the behavioral structure in terms of temporal sequences. This approach revealed peculiar behavioral structures characterized by events that, individually assessed, would in no way imply interaction. Within this category of interaction, the finding of mirrored events between the two animals raises stimulating questions about the meaning of these events. We hope that this newly revealed type of interaction will serve as a launching pad for further research on the topic.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.C.: Conceptualization, data curation, methodology, writing original draft, preparation of all figures and tables; J.B.L., N.G., S.A. and G.C.: Writing, review & editing. All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

Ethical statement

All procedures on animals described in this study were approved and carried out following University of Palermo ethical guidelines and in conformity with Italian and International Laws and Policies (EU Directive, 2010/63/EU for animal experiments, ARRIVE guidelines, and the Basel declaration including the 3R concept). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Additional information

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