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Novel approach to automatically classify rat social behavior using a video tracking system

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HIGHLIGHTS

- Current animal models, if involving social behavior at all, are limited to very short and simple measurements.
- Social behavior is mostly manually annotated, despite available automated observation technology.
- We developed a method that combines velocity of movement with inter-individual distance of rat pairs.
- Our methods result in different behavioral classes that are naturally present in juvenile rats.
- Our approach allows automated and objective measurement of social rat behavior.

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ABSTRACT

Background: In the past, studies in behavioral neuroscience and drug development have relied on simple and quick readout parameters of animal behavior to assess treatment efficacy or to understand underlying brain mechanisms. The predominant use of classical behavioral tests has been repeatedly criticized during the last decades because of their poor reproducibility, poor translational value and the limited explanatory power in functional terms.
New method: We present a new method to monitor social behavior of rats using automated video tracking. The velocity of moving and the distance between two rats were plotted in frequency distributions. In addition, behavior was manually annotated and related to the automatically obtained parameters for a validated interpretation.
Results: Inter-individual distance in combination with velocity of movement provided specific behavioral classes, such as moving with high velocity when "in contact" or "in proximity". Human observations showed that these classes coincide with following (chasing) behavior. In addition, when animals are "in contact", but at low velocity, behaviors such as allogrooming and social investigation were observed. Also, low dose treatment with morphine and short isolation increased the time animals spent in contact or in proximity at high velocity.
Comparison with existing methods: Current methods that involve the investigation of social rat behavior are mostly limited to short and relatively simple manual observations.
Conclusion: A new and automated method for analyzing social behavior in a social interaction test is presented here and shows to be sensitive to drug treatment and housing conditions known to influence social behavior in rats.

1. Introduction

Measuring social rat behavior should be a necessity in studies using animal models for human psychopathologies, such as autism, depression and schizophrenia. However, there is a striking inconsistency between the strong relevance of social behavior and its relative minimal use in animal studies on brain disorders. Apart from methodological issues that will be addressed below, this may

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be due to the complex nature of social behavior. Its highly interactive character requires the continuous perception of the other animal's reaction to its own behavior and thus, continuous adaptive responses. This dynamic behavior is more difficult to recognize, analyze and interpret, than a single behavioral element that is usually manually scored. Traditional social behavioral parameters, such as time spent in playful interaction or inter-individual distance, initially seem easy to quantify and understand. However, an important shortcoming here is that behavior is simplified and degraded to a single readout parameter, thereby, losing the representation of the complex dynamic features of this behavior. Another issue is that there seems to be neither a generally adopted strategy, nor a definition, by which social behavior, especially social play, is measured and analyzed (Pellis and Pellis, 1998).

Most studies measure behavior in a relatively small and novel environment in which animals are placed for a short time period. In addition, in some cases animals are tested during their inactive phase of the diurnal cycle using bright light conditions. These test conditions do not provide an optimal challenging environment for the expression of complex social behavior. Moreover, social behavior is typically scored by a human observer quantifying only frequencies and durations of a few specific social elements. On the other hand, it is possibly to acquire more meaningful behavioral data by using detailed ethograms in combination with multivariate approaches. For example, the application of temporal pattern analysis has proven to be an effective tool to investigate the behavior of rats in an elevated plus maze (e.g. Casarrubea et al., 2015).

Whereas, in general, innovative methodologies in neuroscience are continuously becoming available and are rapidly applied, behavioral science seems hesitant in adopting new advanced hard- and software tools to measure behavior of rodents (Fonio et al., 2012; Spruijt et al., 2014). This is surprising in view of the successful use of technology in behavioral studies introduced in the 80s and 90s (e.g. Sams-Dodd, 1995; Spruijt and Gispen, 1984; Spruijt et al., 1992) and the acknowledgment of numerous clear disadvantages of manually scored behavior. For instance, it is very laborious, time consuming, error prone and subjective.

The category 'social behavior' encompasses play behaviors and other affiliative social behaviors, on which we focus here, as well as agonistic, parental and sexual behaviors. Play behavior has received attention across different species because of its typical form, crucial effect on development (Cooke and Shukla, 2011; Pellis and Pellis, 2009) and because of its possible application as a welfare indicator (Boissy et al., 2007; Held and Špinka, 2011). In addition, studies have focused on the rewarding aspects of play, often in relation to reward sensitivity and abnormal brain function (for reviews see e.g. Siviy and Panksepp, 2011; Trezza et al., 2011). Besides play, affiliative social behaviors comprise allogrooming, crawling over or under each other, huddling or sitting close together and following/approach behaviors. The aim of the current study is to provide an automated method that allows the detection of changes in both affiliative as well as play behaviors.

The development of advanced techniques for automated identification and objective analysis of behavior has been ongoing for a number of years now. Recently, several methods have been described allowing automated monitoring of social dyads in rodents (Giancardo et al., 2013; Kabra et al., 2013; Ohayon et al., 2013; Shemesh et al., 2013; Weissbrod et al., 2013). When sophisticated software tools and systems are used to observe and identify behavior in full detail this automatically leads to a second issue: how to deal with the complex data. Unfortunately, little attention has been paid to the manner in which automatically acquired data, both from individual and multiple animals can be analyzed. The use of (top view) video images of behavior enables detailed analysis of these images. A few studies have tried to adopt and develop advanced statistical methods for analysis of trajectory data from automated video tracking of rats and mice, e.g. (Drai and Golani, 2001; Drai et al., 2000; Kafkafi et al., 2003). The elegant approach of Golani and co-workers includes statistical methods to search for natural categories or so called 'modes' in the data itself. They could demonstrate that locomotor behavior of exploring rats can be divided into distinct categories of movement (Drai et al., 2000), comparable to the use of different gears when for instance driving a car. For example, a rat can still move around while hardly leaving its location, i.e. as if it is using its first "gear". Alternatively, it can make a run from point a to b with a much higher velocity using its second or third "gear". This method of analyzing locomotor behavior by defining different categories of velocities has been successfully applied in a few other studies using rats or mice, however, using individual animals e.g. (Grieb et al., 2014; McGinty et al., 2013). Periods of stationary movements have been called 'lingering' behavior since animals are not necessarily inactive, but can perform behaviors such as rearing and scanning of the environment. Movements with higher velocity are often called 'progression' or 'progressing'. We recently showed that drugs may differently affect lingering or progressing. For instance, morphine can enhance or inhibit progressing depending on the selected dose and time-interval after injection (Spruijt et al., 2014).

Here, we describe a novel automated method of quantifying social interactions. We take advantage of available sophisticated analysis techniques by applying automated continuous video tracking of pairs of rats. The previously noted methodological and biological shortcomings are addressed by using the proposed method as introduced above, for the distinction of different velocity categories. Yet, we extend this by applying it in a social context and on inter-individual distances to reveal possible different categories of proximity. Our method does not require a priori defined and arbitrarily chosen criteria that define movement or being in proximity, rather, these thresholds are extracted from the frequency distribution data. In addition, we now combine velocity with inter-individual distances which leads to new behavioral classes. These behavioral classes represent for instance moving with high velocity and being in (close) proximity. We hypothesized that such classes composed of velocity of movement and inter-individual distance are sensitive to treatment with morphine and short social isolation as this has been repeatedly shown to increase social behaviors in rats e.g. (Niesink and Van Ree, 1989; Vanderschuren et al., 1995a). In addition, to provide a full validation of our method automatically obtained behavioral classes were also compared with human observer data.

2. Materials and methods

2.1. Animals

A total of 26 male Sprague Dawley (Hsd:SD) rats were weaned at Harlan, the Netherlands, at an age of 3 weeks and housed in sibling pairs. Two individuals from each mother were selected and formed a test pair. Subsequently, the pairs arrived in this configuration at Delta Phenomics research facility (Utrecht, the Netherlands) and were housed under reversed light-dark regime (red light on at 09:00 h, white light on at 21:00 h). Rats were housed in Macrolon IV-S cages with a flat lid (Techniplast, Italy). Each cage contained wood chipped bedding (Abedd® wood chips, LAB & VET Service GmbH, Vienna, Austria), a plastic tube and some tissue material. Food (CRM (E), Special Diets Services, United Kingdom) and tap water were provided ad libitum. The holding room was maintained at $21 \pm 1\,^{\circ}C$, with relative humidity set between 45 and 65%. All animals were habituated on regular basis to human handling before start of the experiments. Experimental testing started when the animals were 5 weeks of age, weighing an average of 91.4 ± 6.5 g. This age was chosen to ensure high levels of social (play)

behavior. It is known that rats have a peak in play activity between 30 and 40 days of age (Panksepp, 1981). The experiments were performed in adherence to the legal requirements of Dutch legislation on laboratory animals (WOD/Dutch 'Experiments on Animals Act') and were reviewed and approved by an Animal Ethics Committee ('Lely-DEC').

2.2. Apparatus and software

All testing took place in an enlarged PhenoTyper® instrumented cage (Noldus Information Technology, the Netherlands) under red light conditions. The animals are provided with this large environment because the expression of social behavior requires space, see for example (Spruijt et al., 2014). The cage consisted of a black floor plate (floor dimensions: 90 × 90 cm), transparent Perspex walls (high: 100 cm) and a roof equipped with infrared emitting LED's (peak range average of 950 nm), on which a PhenoTyper top-unit was placed (Noldus Information Technology) containing an infrared sensitive camera (CCD 1/3″ SONY SUPER HAD CCD black/white) and IR-filter (type Kodak 87C). Digital top view video recordings (25 samples per seconds) were made using a computer placed in an adjacent room. Video recordings were processed afterwards with the video tracking software EthoVision XT 8.0 (Noldus Information Technology) using the detection settings 'static subtraction'. For each sample the software stores the *x*- and *y*-coordinate of the animal's position. See Noldus et al. (2001) for more detailed information on the software. Animals were marked red or black using a permanent marker (Edding, Germany) in order for the software to individually recognize both individuals. In contrast to the black marking, the red marking is not visible in the video because of the infrared light conditions. This way, the software recognizes a marked versus an unmarked animal, while both animals experience the same handling procedure. A similar procedure is used by Sams-Dodd (1995) and Spruijt et al. (1992). Occasional identity swops made by the software were corrected manually after video tracking.

2.3. Drugs

We validated our method by using a low dose of morphine that stimulates social behaviors, see e.g. Niesink and Van Ree (1989), Vanderschuren et al. (1995a). Morphine-HCL (Centrafarm, the Netherlands) at a dose of 1 mg/kg and in a volume of 2.5 ml/kg was dissolved in NaCl (0.9%) and administered subcutaneously in the nape of the neck 30 min prior to testing. Control saline injections consisted of an equivalent volume of NaCl (0.9%) using the same route of administration.

2.4. Social interaction test

Before the social interaction test, animals were habituated to the experimental setup and procedure on two separate days before the start of the first test. This involved marking of the animals, a subcutaneous saline injection in the nape of the neck and habituation to the PhenoTyper cage for 30 min. The effect of repeated testing was considered to be minimized by these 2 days of habituation due to the fact that any initial novelty induced behavior declines after repeated exposure to the test environment (Spruijt et al., 2014). In the social interaction test animals were allowed to freely interact for 30 min with their familiar and similarly treated cage mate. Two social interaction tests were conducted: (1) without any isolation, thus, socially-housed (SOC) and (2) with 48 h of short isolation of all pairs prior to the test (ISO). A repeated mixed cross-over design was used. In the first social SOC interaction test pairs were treated with either morphine ($n = 7$ pairs) or saline ($n = 6$ pairs) 30 min prior to testing. In the second social interaction test (ISO), the same pairs were tested again, hence the repeated design, and, again both animals of a pair received either morphine ($n = 7$ pairs) or saline ($n = 6$ pairs) treatment 30 min prior to the social interaction test.

2.5. Manual scoring

Ten social interactions tests, were randomly selected across treatment groups and manually scored by one observer blind to the treatment. Software (The Observer XT10, Noldus Information Technology) was used to score behavior afterwards from captured video files using ½ playback speed of the video to precisely code the behavior. See Table 1 for the used ethogram. Continuous focal animal sampling was used in such a way that only one animal of the pair was followed. However, when for instance following behavior displayed by the focal animal is scored, this animal can either be the one that is following or is followed (thus being actor or receiver, respectively). Individual animals of a similarly treated pair displayed. Passive or active behaviors in the same way. As a consequence, all social behaviors were listed as the behavior of a pair and information on the role of actor or receiver was not distinguished in data analysis.

2.6. Data analysis

2.6.1. Determining arrests and movements

After video tracking, the raw data containing the *x*- and *y*-coordinates of each animal in the pair was exported from EthoVision to MatLab® R2012b (The MathWorks, United States). Data was further analyzed with help of custom made MatLab scripts. To remove any noise, raw track data was smoothed using a robust Locally Weighted Scatter Plot Smoothing (LOWESS) filter with a 1-s time window. This connects points that are representative of the animal's trajectory and finds the most optimal fit, see Hen et al. (2004) for a more extensive description. After smoothing other variables were calculated, such as velocity and distance between animals. Based on the statistical method introduced by Golani and coworkers (as described in Drai et al., 2000) the velocity with which a pair of animals moved was profiled.

Raw data was filtered with a repeated running median using a one-dimensional median filter with window size 13, 11, 9, 9 respectively. Data was collected with 25 Hz, producing a series of 25 *x*- and *y*-coordinates per second. This relative high sampling rate required a repeated running median approach. Basically, this means that a moving window of a few samples (13, 11, 9 and 9) is moved over the data. In this moving window the median of the consecutive samples is determined.

Tracking data was divided into movement bouts by detecting the 'segments of arrest', i.e. moments were the animal really has come to a stop without any clear visible movement of the body. Hereby, movements bouts are defined as the path between two arrests and subsequently, the maximal velocity that is reached by the animal during a movement bout can be determined. Because the tracking software always detects minor displacement of the center point, even when animals are at arrest, a threshold for arrests had to be determined. To find the optimal threshold, clear visible moments of arrests of one animal in the video were manually scored by an observer using ½ playback speed. In addition, it was scored when the animal was moving but without clear forward movement, i.e. more than two steps in the same direction, and it was scored when there was clear forward movement. Based on the distribution of arrests, a tolerance for arrest was set at 0.07 cm between 2 samples lasting at least 4 samples (0.16 s). The threshold was verified by visual inspection of graphs of track visualization with velocity that were integrated with results of manually scored: (1) arrests, (2) movements when staying in place and (3) forward movements using this threshold.

Table 1
Ethogram. All behaviors were scored as states. Since the behavioral elements were scored from the view of one of the animals (focal animal) of a pair, behavioral elements from the category 'social' are scored either as receiver or as actor.

Behavioral category	Behavioral element	Description
Social	In proximity	Being within one tail length from the partner without actively seeking or engaging in social interaction.
	Following	Following the partner within a tail length distance.
	Social sniffing	Exploration of the partner's body by sniffing (except the anogenital region).
	Anogenital inspection	Exploration of the partner's anogenital region and tail.
	Allogrooming	Chewing and licking the fur of the partner.
	Crawling over/under	Climbing over or crawling under the partner.
	Nape attack	Snout or oral contact is directed to the neck region of the partner can be accompanied with biting and pulling fur in that region.
	Pinning/supine	The animal is standing over the partner, often using its front paws, to hold the other down (pinning), while partner is lying on its back (supine position).
	Biting/pulling	Pulling or biting the fur of the partner at any part of the body, except the neck region. Often accompanied by the receiver reacting defensively.
	Boxing/kicking	Both animals rear and box at each other with their front paws or kick with hind paws at each other defensively.
	Defense (push off/away)	Pushing away the partner, but not moving away. Includes reposition of the body during interaction. Can also be followed by an avoiding response ('avoid') if the animal pushes away and then moves away.
	Approach	Moving toward the partner. The animal moves in a straight line.
	Avoid	Moving away from the partner with at least one body length. Often the opposite of an approach, the animal moves in a straight line away.
Non-social	Other	All individual behaviors performed when not in proximity of the partner, e.g. rearing, self-grooming, sitting.
	Mobile exploration	All individual movements through the cage.

2.6.2. Frequency distributions

Then, frequency distributions (histogram) of the maximal velocities of each movement bout were made. This was done for both social interaction tests (ISO and SOC) and per treatment. On these distributions the best Gaussian curves that represent the data were fitted with an expectation maximization (EM) method. In short, it was first determined if different components, in this case Gaussian curves, could be recognized within the population. Thereafter, proportion, mean and standard deviation were estimated of each Gaussian curve with the EM algorithm. For more details on this method see for example Drai et al., 2000.

After visual inspection of all frequency distributions plots, two Gaussian curves were plotted on the frequency distributions plots (see also Fig. 1, left panel). This was verified by assuring that the mean values of the curves were at least two standard deviations apart. A positive outcome of this verification step confirmed that the two curves are indeed representing the data. Subsequently, the intersections of the curves created with the EM method were used to determine a threshold/cutoff value for the different modes or categories in which an animal moves.

Additionally, a similar analysis was done on the inter-individual distance between animals. The difference here is that distance between animals was calculated for each sample in the tracking data. Subsequently, all the inter-individual distances per sample were plotted in a frequency distributions. After visual inspection of all frequency distributions plots, three Gaussian curves were plotted on the frequency distributions plots (see also Fig. 1, right panel). The intersections of the curves created with the EM method were used to determine the different modes or categories of inter-individual distance.

2.6.3. Behavioral classes and statistical analysis

The identified modes or categories in velocity and inter-individual distances were combined into behavioral classes. After that, data were statistically tested using R statistical software (version 2.15.2). The pair of animals was considered as the statistical unit (thus samples size is the number of pairs) for all behaviors. Normal distribution and outlier checks were performed and after that all analyses was done using non-parametric testing. To compare the effect of morphine with saline treatment and the effect of 48 h short isolation, an unpaired Wilcoxon rank sum test (also known as the Mann–Whitney *U* test) was executed over the full length of the social interaction test: 30 min.

3. Results

3.1. Frequency distributions

Visual inspection of the inter-individual distance and the velocity frequency distributions per pair revealed no major differences between pairs of the same group and treatment. Therefore, inter-individual distance and velocity profiles were created across test condition (ISO or SOC) and across treatment (MOR or SAL). Fitting the Gaussian expectation maximization yielded two curves that divide the movements of the animals in two separate categories or modes: (1) "low velocities" and (2) "high velocities". For inter-individual distance, the application of the Gaussian method resulted in three curves and thus, three different categories or modes of sociality: (1) "in contact" (2) "in proximity" and (3) "not in proximity".

3.2. Manual scoring versus automatic parameters

The percentage that each manually scored behavior occurs relative to the nine different classes of automated behavioral classes is depicted in Table 2. The three distinct velocity categories are indicated with 'low' (i.e. both animals are moving with low velocity), 'high' (both are moving with low velocity) and 'low + high' (one rat is moving with high velocity, while the other is moving with low velocity). In addition, the behavior of the rat pair can fall into the "in contact", "in proximity" and not in proximity" while moving with a certain speed. The shading in Table 2 highlights a high level of coincidence of the manually scored behavior (first item in each row) and the automated behavioral classes (first item of each column).

For example, it shows that all manually scored social behaviors were only occurring in the "in contact" and "in proximity" category but not in the "not in proximity". Furthermore, some behaviors were almost exclusively represented in one specific behavioral distance/velocity class. For instance allogrooming, nearly only occurred when being "in contact at low velocity". Also, all behaviors

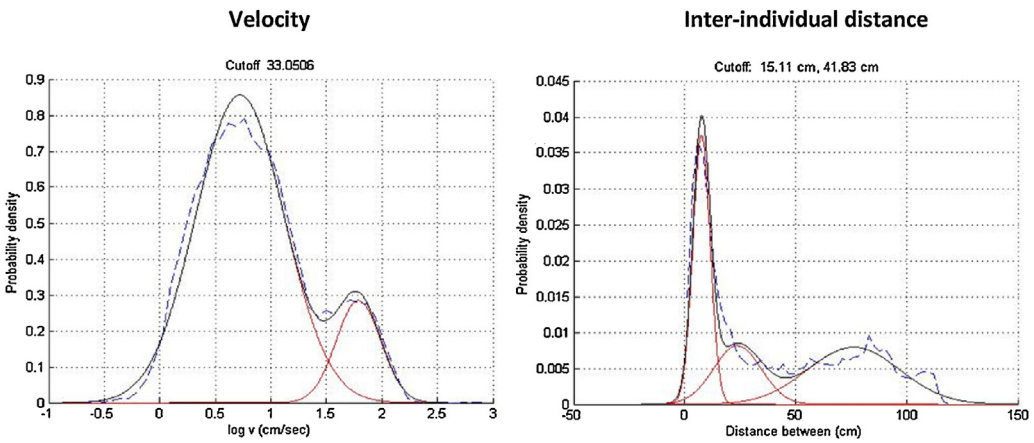

Fig. 1. Two examples of the data analysis process to identify different modes or categories of velocity and social behavior (proximity) using frequency distributions of velocity (left) and distance between the two rats (right). The blue dashed line represents the empirical data, the red lines are Gaussian curves that are the result of the Gaussian expectation maximization. The cut-off values above the graphs are the intersection points of the red lines which determines the thresholds by which different modes or categories of velocity and proximity are defined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Comparison of the manually scored behaviors in relation to the automatically obtained behavioral parameters. The percentage that each manually scored behavior occurs relative to the nine different classes of automated behavioral classes is depicted. The three distinct velocity categories are indicated with 'low' (i.e. both animals are moving with low velocity), 'high' (both are moving with low velocity) and 'low + high' (one rat is moving with high velocity, while the other is moving with low velocity). The shading highlights a high level of coincidence of the manually scored behavior (first item in each row) and the automated behavioral classes (first item of each column).

	Contact			In proximity			Not in proximity		
Category	low	high	low + high	low	high	low + high	low	high	low + high
Allogrooming	93.8	1.4	4.3	0.4	0.1	0.1	0.0	0.0	0.0
Anogenital inspection	37.9	17.0	26.6	4.1	9.5	4.8	0.0	0.1	0.0
Social sniffing	38.3	11.4	21.1	9.0	9.0	10.7	0.0	0.3	0.2
In proximity	27.8	3.0	8.3	37.5	7.2	15.0	0.2	0.5	0.5
Approaching	0.5	3.6	4.7	2.6	19.2	19.8	1.1	27.7	20.9
Avoid (moving away)	2.2	18.3	8.3	1.7	40.0	10.5	0.2	13.5	5.4
Nape attacking	24.8	38.0	24.5	0.5	9.4	2.1	0.0	0.6	0.1
Pinning/supine	65.0	16.2	18.1	0.1	0.4	0.3	0.0	0.0	0.0
Biting/pulling	33.1	34.2	23.4	2.3	5.3	1.7	0.0	0.0	0.0
Boxing/kicking	25.0	32.0	24.6	1.3	11.4	4.2	0.0	1.2	0.2
Crawling over/under	42.2	28.3	26.3	0.6	1.5	1.1	0.0	0.0	0.0
Defense	11.7	46.1	22.6	0.6	15.0	3.3	0.0	0.5	0.2
Following	1.0	26.5	4.4	0.5	56.7	3.4	0.0	7.3	0.1
Other	0.1	0.2	0.3	6.5	1.7	5.0	50.5	6.6	29.2
Mobile exploration	0.0	0.7	0.5	1.2	7.6	7.9	9.5	27.2	45.4

related to play were mainly found in the "in contact" category while individual behaviors (other and mobile exploration) were almost only seen in the "not in proximity" category. Also, following (chasing) behavior is performed at high velocity being both "in contact" and "in proximity".

3.3. Effects of short isolation and compound validation

3.3.1. Total distance moved

An overall effect of morphine on the total distance moved per animal pair during the 30 min social interaction test was detected (Fig. 2). Morphine significantly increased the total distance moved as compared to saline ($U = 37$, $p = 0.022$) in the social test condition. In the short isolation test condition, morphine also increased the total distance moved as compared to saline, however, this was only a near significant trend ($U = 35$, $p = 0.051$). Furthermore, an effect of short isolation (48 h) on total distance moved per pair was also observed in the saline ($U = 36$, $p = 0.002$) and morphine ($U = 47$, $p = 0.002$) treated groups.

3.3.2. Movement with low or high velocities

Both high velocity (Fig. 3) and low velocity movements (data not shown) were affected by drug treatment when given after a short isolation. In the social interaction test after short isolation, morphine significantly increased the time spent moving with high velocity as compared to saline ($U = 38$, $p = 0.014$). Also, morphine treated animals spent significantly more time moving with high velocity when tested after short isolation ($U = 43$, $p = 0.017$).

3.3.3. Inter-individual distance combined with velocity of movement

The classification of the velocity modes combined with that of the inter-individual distance resulted in nine different behavioral classes that represent sociability. These nine classifications

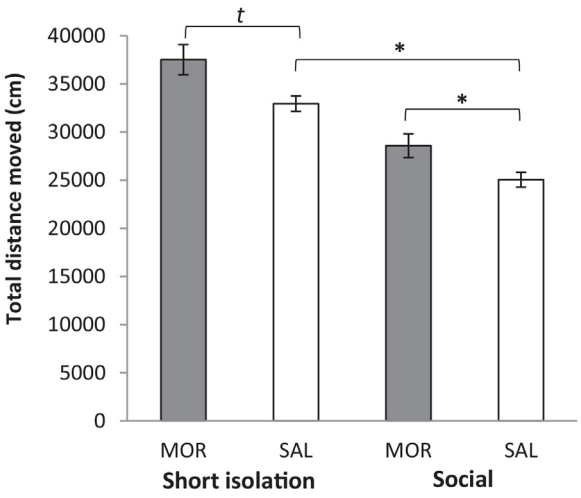

Fig. 2. Average (±SEM) total distance moved in centimeters during the 30 min-social interaction tests for the two treatment groups after short isolation housing (48 h) and social housing. Morphine (MOR, $n = 7$) 1 mg/kg and saline control (SAL, $n = 6$). Asterisks indicate significant differences; $^*p < 0.05$ and t indicates a trend $p < 0.1$.

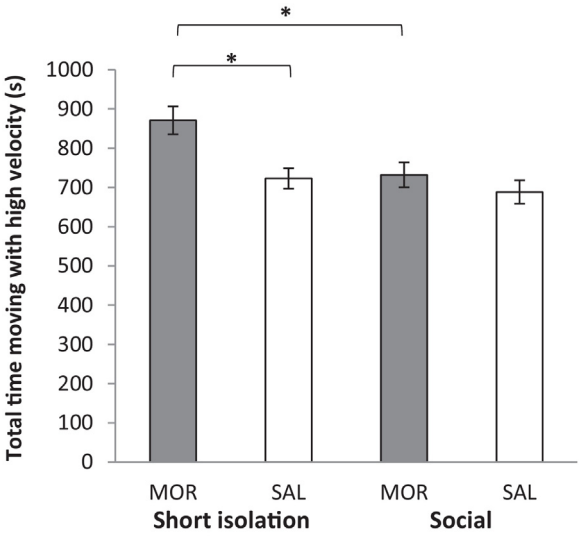

Fig. 3. Average (±SEM) total time moving with high velocity during the 30 min-social interaction tests for the two treatment groups after short isolation housing (48 h) and social housing. Morphine (MOR, $n = 7$) 1 mg/kg and saline control (SAL, $n = 6$). Asterisks indicate significant differences; $^*p < 0.05$.

are defined by a specific combination of a pair that can either be "in contact", "in proximity" or "not in proximity" (the "social modes") and move with high velocity or low velocity, or one is moving with high velocity and the other is moving with low velocity ('the velocity modes'). In the latter mode, both animals moving at different velocity, there were no differences seen between the treatments in both conditions (social and short isolation).

Morphine compared to saline, significantly increased moving with "high velocity when in contact" ($U = 42$, $p = 0.001$) after the short isolation condition, (Fig. 4A) but not after the social condition. In the interaction test after social housing morphine significantly increased moving "with high velocity when in proximity" compared to saline ($U = 41$, $p = 0.002$) but not in the isolation condition (Fig. 4B). When animals were moving "with high velocity when *not* in proximity" no effects of morphine or short isolation were observed (Fig. 4C). In addition, the effects of short isolation housing were observed in both morphine and saline treated animals. For morphine treated animals, short isolation significantly enhanced the time moving with "high velocity in contact" (Fig. 4A, $U = 49$, $p < 0.005$) and "high velocity in proximity" (Fig. 4B, $U = 44$, $p = 0.011$) compared to social housing. Saline treated animals also spent significantly more time in moving with "high velocity in proximity" in the social interaction test after short isolation as compared to social housing (Fig. 4B, $U = 36$, $p = 0.002$).

4. Discussion

Our method revealed that juvenile rats show two distinct modes or categories of movement: movements with low or high velocity. This is in line with observations of others in mice (e.g. Kafkafi et al., 2003) and in rats (Drai et al., 2001). In addition, by applying the same method – now for the first time – on inter-individual distance we detected three distinct modes or categories of inter-individual distances: "in contact", "in proximity" and "not in proximity". Interestingly, the combination of velocity with inter-individual distance yielded distinct behavioral classes which are sensitive to pharmacological and environmental treatment and which are consistently in line with human hand scored behavioral data.

In the "in proximity category", hand scored behaviors mainly observed are: in proximity (without being actively engaging in social interaction), approach or avoidance and following (socially active). While, in the "in contact" category (very close: one whisker-length away) most observed behaviors are the play behaviors, social investigation or social grooming. This suggests that our automated method is capable of distinguishing real physical contact behaviors from social behaviors that do not necessarily involve physical contact (touch) such as following behavior (chasing) or approach/avoidance. In addition, by combining inter-individual distance with velocity of movement the automated behavioral classes also identifies following behavior which takes place at high velocity. Moreover, it characterizes behaviors such as allogrooming, social sniffing and play contact behaviors that are mostly performed when animals are both moving with a lower velocity. The automated behavioral classes are in line with manual scoring at a compatible level as two human observers, which usually achieve 80% reliability. In the present study this is sufficient to detect the effects of morphine treatment and environmental manipulation, i.e. isolation.

Short isolation from peers before the test is a well-known manipulation to increase social behavior of rats (e.g. Niesink and Van Ree, 1982). The here defined (automated) behavioral classes demonstrate a clear effect of short isolation on social behavior. Both morphine and saline treated animals move more with high velocity when "in contact" or "in proximity" in the social interaction test after short isolation. In addition, our study shows that morphine intensifies social behavior by increasing moving with high velocity, while simultaneously decreasing the inter-individual distance from "in proximity" to "in contact". Probably, morphine strengthens the intrinsic motivation to engage in social behavior with the two aforementioned effects as a consequence. This is in line with previous studies showing that morphine increases levels of social (play) behavior (Niesink and Van Ree, 1989; Vanderschuren et al., 1995b) after a short period of individual housing before treatment. The effects of morphine are most clearly seen in the high velocity category which is comparable with the earlier finding that morphine increases moving with high velocity in individual animals (Spruijt et al., 2014).

The automatically determined "in proximity" matches relatively poor with the manually scored "in proximity". In proximity is defined in the manual soring as: "Being within one tail length from the partner without actively seeking or engaging in social interaction". A human observer is biased toward scoring changes in behavior in contrast to the system, which scores at a fixed frequency

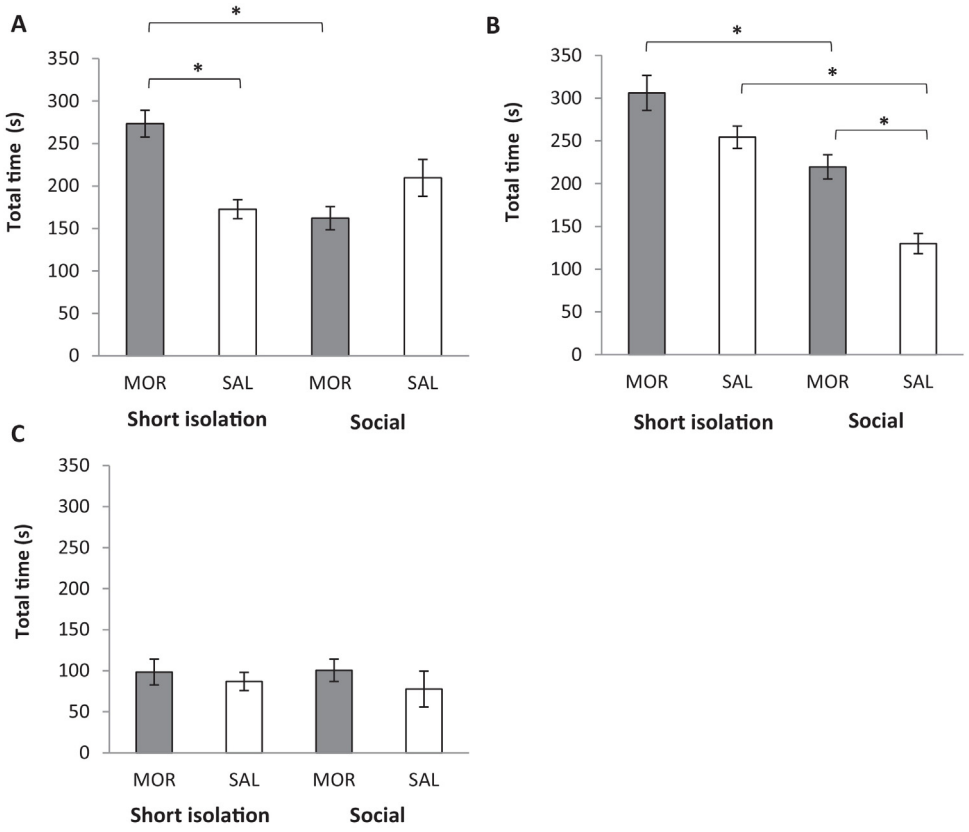

Fig. 4. Average (±SEM) time in each of the distance classes (**A**, "in contact"; **B**, "in proximity"; **C**, "not in proximity") and moving with "high velocity" during the 30 min social interaction tests for the two treatment groups after short isolation housing (48 h), left panel and social housing, right panel. Morphine ($n = 7$) 1 mg/kg, saline control ($n = 6$). Asterisks indicate significant differences; $^{*}p < 0.05$.

(every sample) the behavior again. Thus, our current automated observation tool determines per sample in which category the sample belongs which could have resulted in a slight overestimation of frequencies as compared to human scoring. In addition, human observers tend to interpret behavior after a behavior or behavioral sequence has occurred and, therefore, short events are tend to be neglected by human observers. This way of observing may escape human awareness. On the other hand, also non intended proximity behaviors may have occurred which is not recognized by the human observer as in proximity, for example when the animal is highly active and runs around it can cross the other individual's proximity area. When humans do not see the intention to be in proximity they also do not score it, whereas, when objectively measured they are in proximity. The factor intention as easily and unaware used by humans to identify behaviors is not part of automatic system and may, thus result, into differences.

In the social interaction test animals perform a mixture of social behavior and otherwise motivated behaviors. The present method provides an objective tool to focus on the efficacy of a treatment on the "real" social episodes in the test. Those episodes are characterized by a decrease in the inter-individual distance which occurs in the "in contact" or "in proximity" distance categories. When trial duration increases to hours and even days, our method could extract the specific episodes of behavior in which social interaction takes place. This is important when possible effects of a treatment are expected to preferentially occur in the social domain. Hypothetically, when animals which show aberrant social behavior are treated with an antidepressant agent or anxiolytics this could restore baseline social behavior. In our test setup, which has the benefits of a home-cage approach, this could easily be measured by automatically selecting the social episodes and subject those episodes to further analysis.

The use of automated (social) behavioral parameters based on coordinates of the animals is not new. For example, Sams-Dodd (1995) used an automated parameter for social interaction in rats. In his study, a fixed threshold was used: animals are in close proximity when their center of gravity points are within 20 centimeters from each other. It was mentioned that (p. 161): "*selection of a criterion value of 20 cm is based on systematic variation of this parameter from 0 to 50 cm*" and "*the value of 20 cm resulted in the least variation in the data*". It is exactly this variation that we now use to obtain the different categories of proximity by using the frequency distributions of inter-individual distances. An important benefit of our approach is that the threshold is not assessed artificially (arbitrarily) by limiting variation but based on the animal's own behavior. When the size of animals changes (due to age or gender) or different setups are used, the arbitrarily chosen cutoff values have to be assessed again, whereas in our approach this is deduced from the actual data. What is regarded as high velocity or being in proximity is defined by the variation in the occurrence of different velocities or inter-individual distances and not by an arbitrarily chosen value.

In the future, nose and tail point recognition or even 3 dimensional image building of the animals, see for example (Matsumoto et al., 2013), could have an added value, because, it might be possible then to capture the orientation of animals toward each other. However, the current tracking of body contour is still a challenge for the software when the animals are close together. Here, the overlapping pixels cause merging of body contours and that makes it difficult for algorithms to recognize the individual rats and, thus,

also to correctly identify the nose or head area. Still, in future, ideally, tracking software should be able to recognize full body contour and movement of individuals even when they are close together. Many behavioral elements have such specific characteristics such as the pinning behavior and nape attacks, that these characteristics might be useful in "learning" the software to recognize these behaviors. A similar approach was recently applied in mice, where 2 relatively simple behaviors 'walk and follow' could be recognized by the system (Kabra et al., 2013). Most computers systems are not a hundred percent error free. It is especially difficult for a computer system relying on only top view camera images to identify different animals properly when they are close together or overlap partially. In our setup, using commercially available tracking software, we needed to manually correct identity swops before our analysis could take place. Also, other software systems have dealt with these issues. For example, some provide tracking supervision in which the tracks can be corrected frame-by-frame when two blobs of animals are overlapping in such a way the software does not recognize two individuals (De Chaumont et al., 2012).

Due to all the recently developed new systems it seems that long term monitoring of laboratory rodents in a group should be readily adopted in future research. Application of such methodology though, requires a vast understanding of the basic principles behind the software and the subsequent statistical methods to analyze the data. There is still a huge gap between the extraction of biological relevant information from complex data sets derived from long-term group housed animals and the current state of the techniques (Branson, 2014). Regardless of the system used, our approach of data analysis could potentially also be applied on data output from other systems, as long as it allows an accurate identification, determination of the velocity of movements and the inter-individual distance. In the future, we plan to integrate the behavior with ultrasonic vocalizations of the rat pairs which will further increase the sensitivity of our system and leads to a better understanding of the social behavioral profile of rats. The ultimate goal is to continuously monitor group housed rats in a home-cage environment to study their behavior in a relatively fast, automated and objective way without interference of human handling.

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References

Boissy A, Manteuffel G, Jensen MB, Moe RO, Spruijt B, Keeling LJ, Winckler C, Forkman B, Dimitrov I, Langbein J, Bakken M, Veissier I, Aubert A. Assessment of positive emotions in animals to improve their welfare. Physiol. Behav 2007;92:375–97.

Branson K. Distinguishing seemingly indistinguishable animals with computer vision. Nat. Methods 2014;11:721–2.

Casarrubea M, Jonsson GK, Faulisi F, Sorbera F, Di Giovanni G, Benigno A, Crescimanno G, Magnusson MS. T-pattern analysis for the study of temporal structure of animal and human behavior: a comprehensive review. J. Neurosci. Methods 2015;239:34–46.

Cooke BM, Shukla D. Double Helix: reciprocity between juvenile play and brain development. Dev. Cogn. Neurosci 2011;1:459–70.

De Chaumont F, Coura RD, Serreau P, Cressant A, Chabout J, Granon S, Olivo-Marin J. Computerized video analysis of social interactions in mice. Nat. Methods 2012;9:410–7.

Drai D, Benjamini Y, Golani I. Statistical discrimination of natural modes of motion in rat exploratory behavior. J. Neurosci. Methods 2000;96:119–31.

Drai D, Golani I. SEE: a tool for the visualization and analysis of rodent exploratory behavior. Neurosci. Biobehav. Rev 2001;25:409–26.

Drai D, Kafkafi N, Benjamini Y, Elmer G, Golani I. Rats and mice share common ethologically relevant parameters of exploratory behavior. Behav. Brain Res 2001;125:133–40.

Fonio E, Golani I, Benjamini Y. Measuring behavior of animal models: faults and remedies. Nat. Methods 2012;9:1167–70.

Giancardo L, Sona D, Huang H, Sannino S, Managò F, Scheggia D, Papaleo F, Murino V. Automatic visual tracking and social behaviour analysis with multiple mice. PLOS ONE 2013;8(9):e74557.

Grieb B, Engler G, Sharott A, Von Nicolai C, Streichert T, Papageorgiou I, Schulte A, Westphal M, Lamszus K, Engel AK, Moll CKE, Hamel W. High-frequency stimulation of the subthalamic nucleus counteracts cortical expression of major histocompatibility complex genes in a rat model of Parkinson's disease. PLOS ONE 2014;9.

Hen T, Anat S, Kafkafi N, Golani I, Yoav B. The dynamics of spatial behavior: how can robust smoothing techniques help? J. Neurosci. Methods 2004;133:161–72.

Held SDE, Špinka M. Animal play and animal welfare. Anim. Behav 2011;81:891–9.

Kabra M, Robie AA, Rivera-Alba M, Branson S, Branson K. JAABA: interactive machine learning for automatic annotation of animal behavior. Nat. Methods 2013;10:64–7.

Kafkafi N, Mayo CL, Elmer GI, Lipkind D, Benjamini Y, Golani I. SEE locomotor behavior test discriminates C57BL/6J and DBA/2J mouse inbred strains across laboratories and protocol conditions. Behav. Neurosci 2003;117:464–77.

Matsumoto J, Urakawa S, Takamura Y, Malcher-Lopes R, Hori E, Tomaz C, Ono T, Nishijo H. A 3D-video-based computerized analysis of social and sexual interactions in rats. PLOS ONE 2013;8:e78460.

McGinty V, Lardeux S, Taha S, Kim J, Nicola S. Invigoration of reward seeking by cue and proximity encoding in the nucleus accumbens. Neuron 2013;78:910–22.

Niesink RJM, Van Ree JM. Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 1989;28:411–8.

Niesink RJM, Van Ree JM. Short-term isolation increases social interactions of male rats: a parametric analysis. Physiol. Behav 1982;29:819–25.

Noldus LPJJ, Spink AJ, Tegelenbosch RAJ. EthoVision: a versatile video tracking system for automation of behavioral experiments. Behav. Res. Methods Instrum. Comput 2001;33:398–414.

Ohayon S, Avni O, Taylor AL, Perona P, Roian Egnor SE. Automated multi-day tracking of marked mice for the analysis of social behaviour. J. Neurosci. Methods 2013;219:10–9.

Panksepp J. The ontogeny of play in rats. Dev. Psychobiol 1981;14:327–32.

Pellis SM, Pellis VC. Play fighting of rats in comparative perspective: a schema for neurobehavioral analyses. Neurosci. Biobehav. Rev 1998;23:87–101.

Pellis SM, Pellis VC. The Playful Brain: Venturing to the Limits of Neuroscience. Oxford: One World; 2009.

Sams-Dodd F. Automation of the social interaction test by a video-tracking system: behavioural effects of repeated phencyclidine treatment. J. Neurosci. Methods 1995;59:157–67.

Shemesh Y, Sztainberg Y, Forkosh O, Shlapobersky T, Chen A, Schneidman E. High-order social interactions in groups of mice. eLIFE 2013;2.

Siviy SM, Panksepp J. In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci. Biobehav. Rev 2011;35:1821–30.

Spruijt BM, Gispen WH. Behavioral sequences as an easily quantifiable parameter in experimental studies. Physiol. Behav 1984;32:707–10.

Spruijt BM, Hol T, Rousseau J. Approach, avoidance, and contact behavior of individually recognized animals automatically quantified with an imaging technique. Physiol. Behav 1992;51:747–52.

Spruijt BM, Peters SM, de Heer RC, Pothuizen HHJ, van der Harst JE. Reproducibility and relevance of future behavioral sciences should benefit from a cross fertilization of past recommendations and today's technology: "Back to the future". J. Neurosci. Methods 2014;234:2–12.

Trezza V, Campolongo P, Vanderschuren LJMJ. Evaluating the rewarding nature of social interactions in laboratory animals. Dev. Cogn. Neurosci 2011;1:444–58.

Vanderschuren LJMJ, Niesink RJM, Spruijt BM, Van Ree JM. Effects of morphine on different aspects of social play in juvenile rats. Psychopharmacology (Berl.) 1995a;117:225–31.

Vanderschuren LJMJ, Spruijt BM, Hol T, Niesink RJM, Van Ree JM. Sequential analysis of social play behavior in juvenile rats: effects of morphine. Behav. Brain Res 1995b;72:89–95.

Weissbrod A, Shapiro A, Vasserman G, Edry L, Dayan M, Yitzhaky A, Hertzberg L, Feinerman O, Kimchi T. Automated long-term tracking and social behavioural phenotyping of animal colonies within a semi-natural environment. Nat. Commun 2013;4.