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Blocking NK1 receptors disrupts the sequential and temporal organization of chain grooming in rats

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Abstract

The basal ganglia are a group of sub-cortical structures believed to play a critical role in action selection and sequencing. The striatum is the largest input structure of the basal ganglia and contains the neuropeptide substance P in abundance. Recent computational work has suggested that substance P could play a critical role in action sequence performance and acquisition, but this has not been tested experimentally before. The aim of the present study was to test how blocking substance P's main NK1-type receptors, affected the sequential and temporal organization of spontaneous behavioral patterns. We did this in rats by focusing on the grooming chain, an innate and highly stereotyped ordered sequence. We performed an open field experiment in which NK1 receptor antagonist L-733,060 was injected intraperitoneally in rats at two doses (2 and 4 mg/kg/ml), in a within-subject counterbalanced design. We used first order transition probabilities, Variable Length Markov Models, entropy metrics and T-pattern analysis to evaluate the effects of L-733,060 on sequential and temporal aspects of spontaneously ordered behavioral sequences. Our results suggest that blocking NK1 receptors made the transitions between the grooming chain elements significantly more variable, the transition structure of the grooming bouts simpler, and it increased the probability of transitioning from active to inactive states. Overall, this suggest that blocking substance P receptors led to a general break down in the fluency of spontaneous behavioral sequences, suggesting that substance P could be playing a key role in the implementation of sequential patterns.

Keywords: Action sequences, chunking, substance P, NK1 receptors, grooming chain, Markov analysis.

1 Introduction

From pressing a lever, to making a cup of tea, learning to perform most behavioral patterns requires being able to execute sequences of actions with some degree of order. Once learned, behavioral patterns tend to group themselves into units that are performed in a fluent and seemingly effortless way. In animals, spontaneous behavioral patterns such as rearing and grooming seem apparently undirected and unordered (Renner, 1990; Lever et al., 2006), however, the innate stream of behavior also tends to group itself into "natural units", among which the most easily identifiable ones are fixed actions patterns (Drummond, 1981). Thus, being able to perform sequences of actions as integrated behavioral units has been suggested to be a fundamental process in motor control (Graybriel, 2000; Jin and Costa, 2015).

Action sequencing is believed to depend on several brain areas (Penhune et al., 2012). Evidence from several experiments have indicated that the basal ganglia -a subcortical network believed to be involved in action selection- are a key component in a variety of sequential behaviors, with a predominant role for its main input nucleus, the striatum (Jog et al., 1999; Ölveczky et al., 2005; Yin, 2010; Smith and Graybiel, 2013; Jin et al., 2014; Tecuapetla et al., 2016; Nakamura et al., 2017). Furthermore, several motor cortical areas, such as primary and secondary motor cortex, have been reported to be fundamental for action sequence learning (Kawai et al., 2015; Rothwell et al., 2015), and possibly for the representation of sequences as behavioral units (Ostlund et al., 2009). Thalamic nuclei are also believed to be relevant, since they have been found to be necessary for the smooth initiation and execution of sequential patterns (Chen et al., 2014; Diaz-Hernandez et al., 2018). However, although the involvement of several brain areas in

sequential behaviors has been demonstrated, the mechanistic substrate of action sequence concatenation is still not fully understood.

A recent computational model has suggested that a neuropeptide abundant in the striatum, called substance P (SP), could be a key neuromodulator of action sequencing (Buxton et al., 2017). Substance P is part of a family of neuropeptides called tachykinins that is present both in the central and peripheral nervous systems. Its effects are mediated primarily through NK1 receptors, a G-protein coupled receptor, but it also binds to NK2 and NK3 receptors in a lesser degree (Rupniak and Kramer, 2002). In the central nervous system, NK1 receptors and SP fibers can be found in the striatum, nucleus accumbens (NAc), amygdala, thalamus and hypothalamus, amongst other areas (Beaujouan et al., 2000). In the basal ganglia, one of the main networks involved in action sequencing, SP fibers can be found in subtantia nigra pars reticulata (SNr), globus pallidus, NAc and striatum, however, cell bodies containing SP are only present in striatum and NAc (Shults et al.,1984; Ribeiro-da-Silva and Hökfelt, 2000), with the striatum showing one of the highest NK1 binding densities, both in humans and several rodent species (Griffante et al., 2006; Haneda et al., 2007; Okumara et al., 2008).

In the striatum, substance P is known to facilitate the response of neighboring medium spiny neurons (MSNs) (Blomeley and Bracci, 2008; Blomeley et al., 2009) and it interacts with several neurotransmitters known to be important for motor control, such as dopamine and acetylcholine (Anderson et al., 1993; Aosaki and Kawaguchi, 1996; Tremblay et al., 1992; Gauchy et al., 1996; Kraft et al., 2001; Brimblecombe and Cragg, 2015). Thus, not surprisingly, studies in which SP, NK1 agonists or antagonists have been injected, either locally or systemically, have produced numerous and not always consistent results in behavior. In terms of general locomotion, systemic injections of SP have been

reported to increase behavioral output, with increased locomotion, grooming, scratching, and rearing (Katz and Gelbart, 1978; Hall et al., 1987; Van Wimersma Greidanus and Maigret, 1988). Accordingly, systemically blocking SP receptors has been found to inhibit stereotypical behaviors (Duffy et al., 2002). Nevertheless, others have reported that mice injected with NK1 antagonist and mice lacking NK1 receptors display hyperactivity or no effect on locomotion (Kertes et al., 2010; Yan et al., 2011; Porter et al., 2015).

Studies focusing on the role of SP in the serial organization of behavior have been infrequent. To our knowledge, the only studies that have analyzed SP's role in serial action selection have used the 5-choice serial reaction time task, a task that uses random sequences guided by lights. Using this task, it has been found that mice lacking NK1 receptors display a greater percentage of omissions, perseverations, premature responses, and that they take longer times to retrieve the reward (Yan et al., 2009; Yan et al., 2011; Weir et al., 2013; Porter et al., 2015). Overall, these results suggest that mice lacking NK1 receptors display disrupted action selection in a sequential unordered task. Although interesting, the structure of the task (i.e. random sequences with guiding stimuli) probably means that the mice were not able to learn the sequences as behavioral units.

The aim of the present study was to research the role of substance P in the serial organization of innate and spontaneous behavioral patterns, which provide a behavioral model to study action sequencing in a relatively isolated preparation, since cofounding cognitive mechanisms, such as learning and memory, are minimal (Kalueff et al., 2007). To do this, we performed an open field experiment in which rats were injected with saline and NK1 receptor antagonist L-733,060 at two doses, and we analyzed the effects on sequential and temporal structure of spontaneous activity patterns, focusing on the grooming bouts. In particular, we analyzed the effects of blocking SP receptors on the innate grooming chain, a

naturally highly ordered sequence, whose sequential pattern is known to depend on the striatum (Berridge and Wishaw, 1992; Cromwell and Berrdige, 1996; Kalueff et al., 2007). We found that blocking substance P led to more variable transitions between the behaviors of the highly stereotypical grooming chain, and made the overall transition and temporal structure of the grooming bouts simpler.

2 Materials and methods

2.1 Subjects

Male Lister Hooded rats (400-500 g, N = 12), approximately 16-week-old, were purchased from Charles River (Kent, UK). Rats were housed in pairs and maintained in a 12-h light/dark cycle with free access to food and water at all times. All procedures were performed under the Scientific (Animal Procedures) Act 1986 and in accordance with the ethical guidelines of The University of Sheffield.

2.2 Drugs

NK1 receptor antagonist L-733,060 (Tocris Bioscience, Abingdon, UK) was dissolved in sterile saline and administered intraperitoneally (i.p.) in a volume of 1 ml/kg 15 min prior to each experimental session. L-733,060 was administered at two doses, 2 and 4 mg/kg, based on previous experiments who used doses ranging from 1 mg to 10 mg (Duffy et al., 2002; Wicke et al., 2007; Yan et al., 2011).

2.3 Open field test

Each rat was individually placed in a transparent open field box (30 × 30 × 30 cm) as shown in Fig. 1A. Mirrors were positioned on the top and the sides of the box, and a

light box was kept between 800 and 1000 lux, providing even illumination from below. Animals could freely move within the recording chamber, and all grooming behaviors were spontaneous, they were not triggered with water since this has been reported to cause more disorganized grooming (Kyzar et al., 2011). Animals were initially allowed to habituate to the test box for 1 h for three consecutive days. On the fourth day, half of the rats received an injection of L-733,060 and half received an injection of saline solution. On the fifth day, the rats that on the previous day had been injected with L-733,060 were injected with an equivalent volume of saline solution, and the rats that had received the saline injection were now injected with L-733,060, in a counterbalanced within-subject design (Fig. 1B). To see any dose dependent effects, half of the rats received L-733,060 in a low dose (2 mg/kg, N = 6) and half of the rats in a high dose (4 mg/kg, N = 6). Behaviors were recorded on the fourth and fifth day for 1 h using two cameras, one located in front of the box and the other one in the back of the box. White noise was presented in all sessions to mask external noises.

2.4 Behavioral video-analysis

The software Observer XT 11 (https://www.noldus.com/observer-xt) was used to classify the behaviors registered in the videos into seven standard open field behavioral categories: moving, still, sniffing, rearing, grooming, grooming chain and scanning. All grooming episodes were further classified into: elliptical strokes, unilateral strokes, bilateral strokes, body licking and paw licking (Berridge and Wishaw, 1992; Kalueff et al., 2007). More detailed descriptions of each behavioral category are shown in Table 1 for general behaviors and in Table 2 for grooming behaviors. The criterion to identify the initiation of a grooming chain was the execution of its first phase, the very fast and tight elliptical strokes,

which tend to occur at such speed only when the grooming chain is being executed (Kalueff et al., 2007). We also recorded the interruptions within the grooming chain as any behavior not belonging to the four stereotypical phases, including momentarily stopping for 400 ms or longer. Behaviors were classified as mutually exclusive categories, so that two behaviors could not occur at the same time. A second observer blind to the treatment classified a randomly selected sample of 40% of the grooming chains (50 out of a total of 125 registered), which were used to calculate Cohen's Kappa coefficient to assess the agreement between the two observers' classifications. We found an agreement of 96% between the two observers and a Cohen's Kappa coefficient of 0.95, thus we considered the observations to be reliable.

Table 1. Ethological classification of general behaviors.

Behavior	**Description**
Rearing	Standing on back paws with the body in a vertical position leaning or not towards any wall.
Sniffing	Bumping nose repeatedly against the ground, walls or corners of the test box.
Scanning	Large head orienting movements, usually accompanied by sniffing the air.
Moving	Moving from one place to another, and big changes in posture after long periods of inactivity.
Still	Inactivity and momentarily stopping between two actions.
Grooming	Any grooming behavior, such as paw licking, unilateral strokes, etc. not-including the grooming chain.
Grooming chain	Determined by the initiation of very fast elliptical strokes usually followed by unilateral strokes, bilateral strokes and body licking.

Table 2. Ethological classification of grooming behaviors.

Behavior	**Description**
Paw licking	Licking frontal paws.
Elliptical strokes	Very fast, small strokes close to the nose.

Unilateral strokes	Very small, small or medium unilateral paw strokes along the mystacial vibrissae.
Bilateral strokes	Large symmetrical or semi-symmetrical bilateral strokes, usually extending over the ears. Paw strokes were allowed to start with small time and amplitude differences.
Body licking	Bout of licking over the lateral and ventral torso, sometimes including the genitals.

2.5 Statistical analysis

Mixed effects ANOVAs were conducted to compare the effect of the within variable treatment (L-733,060 vs saline) and the between variable dose (low vs high) on the frequency, time, probability, and duration of the behaviors. Bonferroni corrected post hoc tests were performed when an interaction was found to be significant. Statistical significance was established as $p < 0.05$. Data are shown as mean and SEM. All statistical analyses were performed using the R studio software environment.

2.6 Transition analysis

To obtain a general measurement of locomotion, first order transition probabilities between activity and inactivity were calculated. To do this, the behavioral category "still" was considered as inactivity, whereas all other general behaviors, moving, sniffing, rearing, etc., were considered as activity. To characterize effects on the highly fixed grooming chain, first order transition probabilities between its four phases were calculated. Given that when L-733,060 was injected rats performed significantly fewer grooming chains, the transition probabilities were calculated by pooling the chains from all the rats in each group, in an attempt to avoid spurious inflation of the probabilities.

Then, to quantify how fixed or random these first order transition probabilities were, a transition entropy metric was calculated. Thus, let p_{ij} be the probability that behavior i is

followed by behavior *j*, and *n* the total number of unique behaviors, then, the transition entropy is calculated as follows:

$$H_i = \sum_{i=1}^{n} - p_{ij} log_2(p_{ij})$$

When $p_{ij} = 0$, then $- p_{ij} log_2(p_{ij}) = 0$. The value of the entropy was normalized by the largest possible entropy (H_{max}), which occurs when all behaviors have the same transition probability (i.e. when $p_{ij} = 1/n$). Thus, an entropy of zero, $H_i = 0$, indicates that a transition is completely fixed, that is, behavior *i* will always be followed by the same behavior *j*. Whereas $H_i = 1$ indicates that the transition is completely random, meaning that after behavior *i* all other behaviors are equally likely to occur (Miller et al., 2010).

Although first order transitions are very informative, higher order relationships between behaviors are most likely common. However, fitting a full Markov model of all the higher order relationships between behaviors would be computationally challenging since they grow exponentially. Further, it is not clear what the explanatory power of such complex descriptions would be in mechanistic terms, given limited internal representational capacity of animals. Thus, to parsimoniously model higher order relations between the behaviors, we fitted Variable Length Markov Models (VLMM) using the R package VLMC (Machler and Buhlmann, 2004). In this type of Markov model, the current behavior is allowed to depend on a variable number of previous behaviors; that is, not all higher order transitions are present in the model, only those that significantly add information to predict the following behavior, giving a sparser and more flexible way of modelling behavioral time series (Machler and Buhlmann, 2004). To calculate these models, the data from all the rats of each group were concatenated together, obtaining a single VLMM per

group as has been done previously (Maubourguet et al., 2008). To constrain the construction process of the model, behavioral sequences were only included in the VLMM if they appeared a minimum of 18 times for general behaviors (3 per rat), and 12 for grooming behaviors (2 per rat) and the significance level was established at $p < 0.05$ (Maubourguet et al., 2008). The resulting VLMMs are shown as Prediction Suffix Trees (PST). These are tree-structures where the branches show the higher order sequences that significantly predict the next behavior. Fig. 2 shows an example of a PST, the root is placed on the right, and the behavioral dependencies on the left, where each column of nodes indicates a step in the past. These tree structures are built of Markov chains, our example in Fig. 2 has three first order chains, and two second order chains. For the case of the second order chains, it means that the probability of the next behavior is different if sequence A-B was performed than if sequence C-B was previously performed. Associated with each node of a PST there is a probability vector of the next behavior, which can be used to generate or predict specific sequences.

To make an overall comparison of two VLMM models, A and B, we used a probabilistic divergence measurement, which compares how similarly two models predict the occurrence of a sequence (Gabadinho and Ritschard, 2016). Let x_i, i = 1,…,n, be the *ith* sequence of length *m* generated by model A, and $P_A(x_i)$ and $P_B(x_i)$ be the predicted probabilities for the *ith* sequence by model A and B, respectively. Then, the probabilistic divergence between model A and B is calculated as:

$$D = \frac{1}{n}\sum_{i=1}^{n}\frac{1}{m}\left(log\frac{P_A(x_i)}{P_B(x_i)}\right)$$

To compute this divergence, we generated *n* = 5,000 sequences of length *m* = 10 with model A, and then, we used models A and B to predict these sequences. Therefore, if both

models make very similar predictions about the sequences, then $|D| \approx 0$. On the other hand, the bigger the value of $|D|$, the more different the two model's predictions are. It should be noted that this measurement is not symmetric, thus the distance between model A and B is not necessarily the same as the distance between models B and A (Gabadinho and Ritschard, 2016). Thus, it is possible that model B poorly estimates the sequences produced by model A, leading to a large divergence, but that model A accurately predicts the sequences generated by model B, leading to a small divergence.

2.7 Temporal analysis

Given that Markov analyses only take into consideration the serial order, but not the time when the behaviors were executed, we further explored the data by carrying out T-pattern analysis (Magnusson, 2000). T-pattern analysis returns the number and length of the significant temporal patterns found. We used these two measurements as indicators of size and complexity of behavioral repertoires as has been done previously (de Haas et al., 2010; Casarrubea et al., 2019). We performed the T-pattern analysis with the following parameters: we used fast intervals, a lump factor of 0.9, a significance level of $p < 0.001$, and minimum occurrences of 3 patterns per rat. The significance level was set very strict to discard spurious patterns and the rest of the parameters were set as suggested by Magnusson (2000) when exploring a data set. The temporal patterns found were further classified into short (2-3 behaviors), medium (4-5 behaviors) and long (6 or more behaviors). T-pattern analysis was performed using the software Theme (https://patternvision.com/products/theme/).

3 Results

3.1 Effects of L-733,060 on time, duration, and frequency of behaviors

We first addressed whether L-733,060 had affected basic properties of behavior such as its frequency, duration, distribution, and total time active/inactive. Injecting L-733,060 significantly increased the total time rats remained inactive, with a significant main treatment ($F(1,11) = 14.5$, $p = 0.002$) and dose effect ($F(1,10) = 7.3$, $p = 0.02$; Fig. 3A). This increase in inactivity was related to a significant treatment effect ($F(1,11) = 9.2$, $p = 0.01$; Fig. 3B) on the mean duration of the inactive episodes, which went from an average of 11 s in the control condition to 17 s when L-733,060 was injected. This increase in inactivity led to a significant decrease in the proportion of time rats spent doing other behaviors, with a significant treatment effect on rearing ($F(1,11) = 6.7$, $p = 0.03$), grooming ($F(1,11) = 9.0$, $p = 0.01$), sniffing ($F(1,11) = 12.9$, $p = 0.004$), and chain grooming ($F(1,11) = 7.9$, $p = 0.02$). Since there was no significant dose effect on proportions of time, data from both doses were pooled together and are shown in Fig. 3C. Despite spending less time doing these behaviors, the duration of each individual behavior, that is, rearing, sniffing, etc., was not significantly affected by the injection of L-733,060, except for remaining still (Fig. 3B). Furthermore, all rats from the high and low dose groups displayed similar levels of activity when saline was injected, as shown by the lack of a significant dose effect on the frequency of any of the open field behaviors (Fig S1 Supplementary Material). This suggests that there were no systematic a priori differences in the number of general behaviors performed by the rats of the high and low dose group.

In the case of grooming behaviors, the number of grooming chains performed significantly decreased when L-733,060 was injected with a significant main treatment effect ($F(1,11) = 12.3$, $p = 0.005$; Fig. 3D). However, the mean duration of the overall

grooming bouts ($F(1,11) = 1.7$, $p = 0.22$, n. s.) or of the complete grooming chains ($F(1,11) = 1.2$, $p = 0.3$, n. s.; Fig. 3E) were not significantly affected by L-733,060. When analyzing each of the behaviors of the grooming chain, we found that only the duration of the bilateral strokes slightly increased when L-733,060 was injected ($F(1,11) = 5.9$, $p = 0.03$), but the duration of all other grooming chain behaviors, that is, elliptical strokes ($F(1,11) = 0.97$, $p = 0.34$, n. s.), unilateral strokes ($F(1,11) = 2.26$, $p = 0.16$, n. s.) and body licking ($F(1,11) = 1.53$, $p = 0.24$, n. s.), were not significantly affected. In summary, blocking NK1 receptors with L-733,060 significantly decreased the time rats spent rearing, sniffing, grooming, and chain-grooming, but without affecting the duration of the grooming bouts or of most general behaviors.

3.2 Effects of L-733,060 on first and higher order transitions between general behaviors

In the most general way, the behavior of an animal can be divided into active and inactive states. We first analyzed whether the alternation between these two basic states had been affected. We found that L-733,060 significantly increased the transition probabilities from active to inactive states, with a significant main treatment effect ($F(1,11) = 17.4$, $p = 0.002$; Fig. 4), and although the treatment×dose interaction did not reach significance ($F(1,10) = 3.34$, $p = 0.09$, n. s.), there seems to be a dose dependent trend.

To analyze whether higher order transitions, that is sequences involving two or more behaviors, had been affected by the NK1 antagonist L-733,060, we computed the Variable Length Markov Models of each group. The diagrams in Fig. 4 display the significant first, second, third and fourth order sequences found in the general behaviors of the rats when saline and L-733,060 were injected at the low dose (Fig. 5A and B) and at the high dose (Fig. 5C and D). When L-733,060 was injected at a low dose (Fig. 5B), there was no effect

on the amount of higher order sequences found, with 23 and 25 sequences for the saline and L-733,060 groups, respectively. On the other hand, when L-733,060 was injected at the high dose (Fig. 5D) there was a reduction in the higher order sequences found, with no significant third or fourth order relationships present in the behavior of the rats. To assess whether the transition of open field behavioral sequences became more or less variable after the L-733,060 injection, we calculated the transition entropy of the first order sequences present in the VLMMs, such as sniffing-rearing, scanning-moving, grooming-chain grooming, etc. We did not find any significant treatment effect on the entropies of these sequences (Fig. S2 Supplementary Material) which were quite high even in the saline condition, suggesting that L-733,060 has no significant effect on sequences that are already naturally variable.

To quantify the overall difference between the VLMMs, the probabilistic divergence between them was calculated at each dose. Furthermore, we calculated the divergence of the found models with: 1) a model in which behaviors were simulated to be completely independent from each other (i.e. a zero-order model); and 2) a model whose behaviors were simulated to depend only on one previous behavior (i.e. a first-order model). Results are shown in Table 3, where the values displayed in the cells are the divergences between the models in the corresponding row (in bold) and column (in italics). The models in the rows (in bold) were the ones used to produce the sequences. Thus, a divergence larger than zero indicates that the models in the columns (in italics) were not able to accurately predict the sequences generated by the models in the rows. It is important to note that because the divergence is not a symmetrical measurement, the divergence between L-733,060 and saline models does not need to be the same as the divergence between saline and L-733,060 models.

Table 3. Probabilistic divergence between general behavior VLMMs. The values in the cells show the probabilistic divergence between the models in the corresponding rows and columns. The top rows show low dose results, and the bottom rows the high dose results.

Low dose	*saline VLMM*	*L-733,060 LMM*	*0^{th} order model*	*1^{st} order model*
saline VLMM	—	0.07	0.46	0.07
L-733,060 VLMM	0.03	—	0.45	0.06
High dose				
saline VLMM	—	0.08	0.45	0.06
L-733,060 VLMM	0.05	—	0.47	0.06

Overall, it seems that the predictions made by the VLMMs fitted for saline and L-733,060 were very similar, as indicated by the relatively small divergences found between them, which ranged from 0.03 to 0.08, at both doses. Furthermore, the largest divergences for the saline and L-733,060 VLMMs were with the zero-order model, suggesting that general behaviors are not independent from each other, even when L-733,060 was injected. On the other hand, both saline and L-733,060 VLMMs had small divergences with the first order model, which suggests that a first order relationship explains a lot of the transitions between general behaviors. This make sense given that exploration patterns are more variable sequences, so although there exist some higher order sequences, a lot can be explained by first order transitions. In summary, blocking NK1 receptors does not seem to have had a strong effect on the general behaviors' transition structure, besides removing some third and fourth order relationships at the high dose group as shown in Fig. 5D.

3.3 Effects of L-733,060 on first and higher order transitions between grooming behaviors

To analyze whether the highly fixed grooming chain had been affected by L-733,060 we first looked at the first order transition probabilities between the four stereotypical phases of the grooming chain shown in Fig. 6, with low dose results on the top diagrams (Fig. 6A

and B), and high dose results on the bottom ones (Fig. 6C and D). Red arrows indicate the transition probabilities that changed by 0.10 or more when L-733,060 was injected. These diagrams suggest that the first order transition probabilities between the four stereotypical phases of the grooming chain became more variable when NK1 receptors were blocked, with the largest changes in the middle portion of the chains, that is, from unilateral to bilateral strokes, and from bilateral strokes to body licking. Behaviorally, when rats were injected with L-733,060 they tended to skip middle elements more frequently, and they momentarily stopped before reaching the last element of the sequence more frequently than under control conditions. Transition entropies were significantly larger when L-733,060 was injected ($F(1,5) = 7.9$, $p = 0.036$, Fig. 6E), suggesting that the transitions within the grooming chain were significantly more variable. These results from the grouped data have similar trends when seen at the individual level, shown in Fig. S3 Supplementary Materials, were we calculated the transition probabilities of each rat' grooming chain, and then plotted the average ± SEM to see the mean and spread of the effect in each transition and dose. We found a significant Treatment×Transition interaction ($F(4,88) = 2.74$, $p = 0.03$), indicating that the NK1 antagonist L-733,060 had a larger effect on specific transitions inside the grooming chain, in particular in the middle transitions, as already suggested by the group diagrams in Fig 6.

We also wanted to explore if the transition structure of the overall grooming bouts had been affected by blocking NK1 receptors. To do this, we fitted VLMMs to find the higher order structure of the grooming bouts in each group. Fig. 7 shows the significant first, second and third order sequences found when saline and L-733,060 were injected at the low dose (Fig. 7A and B) and at the high dose (Fig. 7C and D). These diagrams suggest that blocking NK1 receptors at both doses produced an important reduction in the number of

second and third order sequences found in the grooming bouts. The effects were stronger in the high dose group, in which the sequences found were mainly of first order degree (Fig. 7D). Interestingly, the third order transition of the grooming chain that links the four phases together, that is, *P*(*Body licking* | *Elliptical strokes – Unilateral strokes – Bilateral strokes*), was not present in the diagram of the high dose drug group (Fig. 7D), indicating that this higher order transition was less fixed and frequent when L-733,060 was injected in a high dose.

To quantify the difference between the models shown in Fig. 7, we calculated the probabilistic divergence between the saline and L-733,060 VLMMs, and their divergence with the zero-order model and the first-order model. Results are shown in Table 4. The divergence between saline (in bold) and L733,060 VLMMs (in italics) were 0.22 and 0.12 for the low and high dose, respectively. This means that the models obtained from the L-733,060 data were not able to accurately predict the sequences generated by the saline VLMMs, particularly in the low dose group. If we look at the inverse distance, we can see that the saline VLMMs (in italics) were better at predicting sequences generated by the L-733,060 VLMMs (in bold), as suggested by the smaller divergences, 0.07 and 0.08, for the low and high dose, respectively. Thus, the saline VLMMs were good at prediction the sequences generated from the L-733,060 data, but not the other way around.

Table 4. Probabilistic divergence between grooming VLMMs. The cells show the probabilistic divergence between the models in the corresponding row and column. The top rows show low dose results, and the bottom rows the high dose results.

Low dose	*saline VLMM*	*L-733,060 LMM*	*0^{th} order model*	*1^{st} order model*
saline VLMM	—	0.22	0.56	0.24
L-733,060 VLMM	0.07	—	0.49	0.11
High dose				
saline VLMM	—	0.12	0.45	0.11

L-733,060 VLMM	0.08	—	0.44	0.04

Furthermore, the largest divergence of both saline and L-733,060 VLMMs was again with the zero-order model, which indicates that assuming independence between the grooming behaviors is not a good predictor of the sequences produced by the rats. Finally, at both doses, the L733,060 VLMMs were closer to the first order model than the saline VLMMs, thus a model assuming only first order relationship between grooming behaviors, predicted the sequences produced by the L-733,060 models better than those generated by the saline models. Overall, these divergences suggest that injecting L-733,060 made the transition structure of the grooming bouts simpler, making the overall structure more similar to a first-order model.

3.4 Effects of L-733,060 on temporal patterning

Another important dimension of behavioral patterns is their temporal organization. To analyze whether blocking NK1 receptors had disrupted the sequential timing of behaviors we ran T-pattern analysis. In the general behavior sequences, that is those including moving, rearing, sniffing, etc., most temporal patterns found were formed of 2 or 3 behaviors, and we did not find any significant treatment ($F(1,10) = 2.1$, $p = 0.18$, n. s.) or treatment×dose interaction ($F(1,10) = 0.06$, $p = 0.8$, n. s.). On the other hand, grooming temporal patterns showed a significant main treatment effect ($F(1,10) = 9.1$, $p = 0.01$), and a marginally significant treatment×dose interaction ($F(1,10) = 4.9$, $p = 0.05$; Fig. 8), suggesting that L-733,060 reduced the number of temporal patterns found. Multiple comparisons with Bonferroni correction revealed that there was only a significant difference in the number of temporal patterns between saline and L-733,060 in the high

dose group (Fig. 8B). These results are consistent with the results obtained from fitting VLMMs, which indicated that grooming sequences were reduced more strongly in the high dose group, suggesting that blocking NK1 receptors with L-733,060 had an important effect both on the transition and temporal structure of the grooming behaviors.

4 Discussion

Although a lot of information has been gathered in the last few years about the neural bases of action sequencing, the underlying mechanism is still not fully understood (Jin and Costa, 2015; Nakamura et al., 2017; Díaz-Hernández et al., 2018; Dhawhale et al., 2019). In the present study, using a simple open field preparation, we sought to investigate the role of substance P, a neuropeptide that has been recently implicated in action sequences through a computational model of the basal ganglia (Buxton et al., 2017). Although many previous studies had analyzed the role of substance P in open field behaviors (Duffy et al., 2002; Kertes et al., 2010; Yan et al., 2011; Porter et al., 2015), to our knowledge, SP's role in action sequencing had not been tested experimentally before.

Characterizing spontaneous patterns of animals is not an easy task. Behavior is fluid and segmenting the behavioral continuum into meaningful units is a complicated task (Drummond, 1981). In the present study, by using Markov and T-pattern analyses, we were able to capture the effects of blocking NK1 receptors on the first and higher order sequential and temporal organization of the spontaneous and innate behavioral patterns of rats. Overall, our results suggest that blocking SP led to the transitions between behaviors being more variable and simpler, we interpret this informally as a decrease in *fluency* of behavioral patterning. This was particularly pronounced in grooming bouts, whose

behavioral patterns are known to be rich in structure (Berridge et al., 1987; Berridge, 1990; Kalueff et al., 2007).

The patterned SP connections that striatal MSNs make amongst each other are a potential candidate to regulate the implementation of sequences of actions (Buxton et al., 2017). Most motor control models assume that groups of neurons represent individual actions, and that connections between them might regulate action sequence performance (Penhune et al., 2012; Matheson and Sakata, 2015; Murray and Escola, 2017, Buxton et al., 2017). For example, in the birdsong literature, the transition probabilities and the speed with which syllables are produced are believed to indicate the strength with which the underlying groups of neurons are connected (Matheson and Sakata, 2015). Our results suggest that SP could be playing a key role in mediating these connections and thus the transitions between actions. We found that blocking substance P's main receptors made the transitions inside the highly stereotypical grooming chain significantly more variable and the overall transition structure of the rats' grooming bouts became simpler, with higher order transitions being the most affected. Finally, although blocking substance P increased the probability of transitioning from active to inactive states, suggesting a general effect on the fluency with which behavioral sequences were performed, most of the analysis performed on transition probabilities and transition entropies of general open field behavioral sequences, which are naturally more flexible sequences, showed smaller or non-significant effects. This suggest that the effects of L-733,060 were mostly restricted to more stereotypical sequences, such as the grooming chain, rather than more variables ones.

Sequences not only have a specific order, but they also need to be performed with precise timing. Our results suggest that blocking NK1 receptors not only had an effect on the transition structure, but it also disrupted the timing of the behaviors. After

administration of the NK1 antagonist, we found that rats displayed fewer temporal patterns in the grooming bouts, suggesting that behaviors were performed less consistently in time. This was also evident in the grooming chain, in which the transitions inside the grooming chain not only became more variable after the NK1 antagonist injection, but rats also tended to stop for longer periods before finishing the sequence. The timing and transition probabilities of a sequence are believed to be inversely related, that is, large values of transition probabilities (i.e. more fixed) tend to be accompanied by small gaps between behaviors, whereas lower values of transition probabilities (i.e. more variable), are associated to longer gaps (Matheson and Sakata, 2015). This is in line with our finding that the increase in transition variability induced by the NK1 antagonist was accompanied by disrupted timing as well. This suggests that the very particular firing patterns known to be present when the grooming chain and other less fixed behavioral sequences are executed (Aldridge et al., 1993; Aldridge and Berridge, 1998), were most likely disturbed, leading to sequential and temporal alterations of the behavioral patterns.

Given that SP is known to interact with several neurotransmitters, it is also possible that the effects we observed were due to indirect effects, in particular, due to the interaction of SP with dopamine. Electrophysiologically, SP has been found to increase dopamine release in the striatum, in particular in the striosomes, which directly innervate substantia nigra pars compacta (SNc) (Fujiyama et al., 2011; Brimblecombe and Cragg, 2015). Interestingly, the disruptions in sequencing and timing that we observed are similar to those found in rats with SNc lesions, which show a similar simplification of behavioral sequences (Casarrubea et al., 2019), and disruptions in the grooming chain serial organization (Berridge, 1989; Pelosi et al., 2015). Thus, SP could be acting directly by linking and

facilitating the striatal activity responsible for the serial order, but also indirectly by modulating dopamine release in the striatum.

Given that our NK1 antagonist treatment was systemic, we cannot be certain that the results we observed were due to effects on the striatum alone. However, a lot of evidence has indicated that the implementation of serial order of the grooming chain is a function specific of the striatum, given that disrupting striatal activity leads to problems in sequence performance, whereas disrupting other areas of the brain known to have a role in motor control, such as motor cortex, has no effect on the serial organization of innate sequential patterns (Berridge and Fentress, 1987; Berridge and Wishaw, 1992; Cromwell and Berridge, 1996). Furthermore, the striatum is amongst the brain areas with highest concentrations of NK1 receptors, alongside the amygdala and some thalamic nuclei (Shults et al., 1984; Beaujouan et al., 2000; Duffy et a., 2002). Of course, given the widespread distribution of SP and its receptors, it is most likely that the effects of injecting their antagonists were distributed. For example, SP in the spinal cord has been reported to facilitate glutamatergic reticulospinal inputs (Parker et al., 1998), thus, the NK1 antagonist probably also affected these synapses and the motor aspects of behavior that they control.

It is believed that some of the neurobiological substrates and mechanisms used to implement innate fixed action patterns, such as the grooming chain or foraging patterns, could have served as the foundation on which evolution built more flexible mechanisms which allowed animals to adapt to environmental demands (Berridge and Whishaw, 1992; Grillner and Waller, 2004; Kolodny et al., 2015; Dhawale et al., 2019). Neuronal ensembles similar to central pattern generators, the basic circuits known to control many innate motor patterns in lower species, have been suggested to arise in the striatum and other brain areas such as cortex, after a skill has been acquired (Yuste et al., 2005; Carrillo-Reid et al., 2008;

Yin et al., 2009). This suggests that network arrangements have been preserved by evolution, where more primitive neural systems are found in the context of the flexible processing underlying learning. Thus, it is possible that the results from our experiment with innate and spontaneous patterns, could be relevant for other sequential behaviors including learned ones.

5 Conclusions

It is known that the execution of action sequences is accompanied by specific activity patterns in striatal MSNs; however, how these striatal activity patterns arise and are maintained is not fully understood. Our results suggest that substance P, a neuropeptide abundant in the striatum, plays a key role in regulating the transitions between behaviors of highly ordered sequences, which could be due to its direct facilitatory effects on MSNs, or due to interactions with corticostriatal afferents and/or striatal dopamine.

Declaration of competing interest

None.

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CRediT authorship contribution statement

Natalia Favila: Methodology, Investigation, Software, Formal analysis, Data curation, Writing -original draft. **Paul Overton**: Conceptualization, Methodology, Investigation, Writing – review & editing, Project administration, Funding acquisition**. Kevin Gurney**: Methodology, Supervision, Validation, Writing – review & editing.

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Figure captions

Fig 1. Experimental design and apparatus. (A) The open field box and arrangement of cameras used. (B) Drug timeline and experimental groups according to dose (2 and 4 mg/kg).

Fig 2. Prediction Suffix Tree (PST) example. Visual representation of how previous behavioral sequence affect the transitions probabilities of the next behavior in a simple example consisting of 3 behaviors: A, B and C. This PST is composed of three first order Markov chains (A, B, C) and two second order Markov chains (A-B and C-B).

Fig 3. Effects of NK1 antagonist L-733,060 on general properties of behavior. (A) Total time spent inactive per session. (B) Mean duration of the inactive states. (C) Distribution of general open field behaviors. (D) Mean number of grooming chains. (E) Mean duration of the grooming chains. Data represent Mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

Fig 4. Effect of NK1 antagonist L-733,060 on transition probability from active to inactive states. Data represent Mean ± SEM.

Fig 5. Prediction suffix trees (PST) showing first and higher order relationships between general behaviors. Top diagrams, (A) saline and (B) 2mg/kg, show the PSTs found in the low dose group. Bottom diagrams, (C) saline and (D) 4 mg/kg, show the PSTs for the high dose group.

Fig 6. Transition diagrams showing first order probabilities between the grooming chain phases. Top diagrams, (A) saline and (B) 2mg/kg, show the probabilities found in the low dose group. Bottom diagrams, (C) saline and (D) 4 mg/kg, show the probabilities for the high dose group. Red arrows indicate the probabilities that changed by 0.10 or more when L-

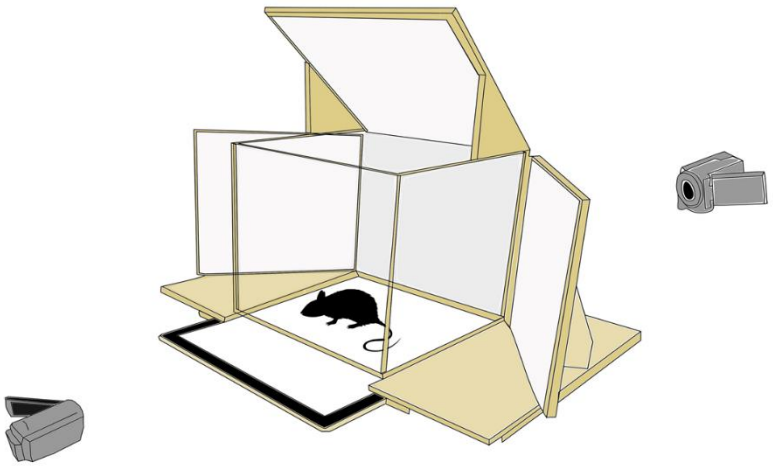
733,060 was injected. (E) Effect of L-733,060 on transition entropies of the grooming chain. Data represent Mean ± SEM. * $p < 0.05$.

Fig 7. Prediction suffix trees (PST) showing first and higher order relationships between grooming behaviors. Top diagrams, (A) saline and (B) 2mg/kg, show the PSTs found in the low dose group. Bottom diagrams, (C) saline and (D) 4 mg/kg, show the PSTs for the high dose group.

Fig 8. Effect of L-733,060 on temporal patterns. Number of temporal patterns found in grooming bouts when saline and L-733,060 were injected at the low (A) and high dose (B).

Fig. 1

A



B

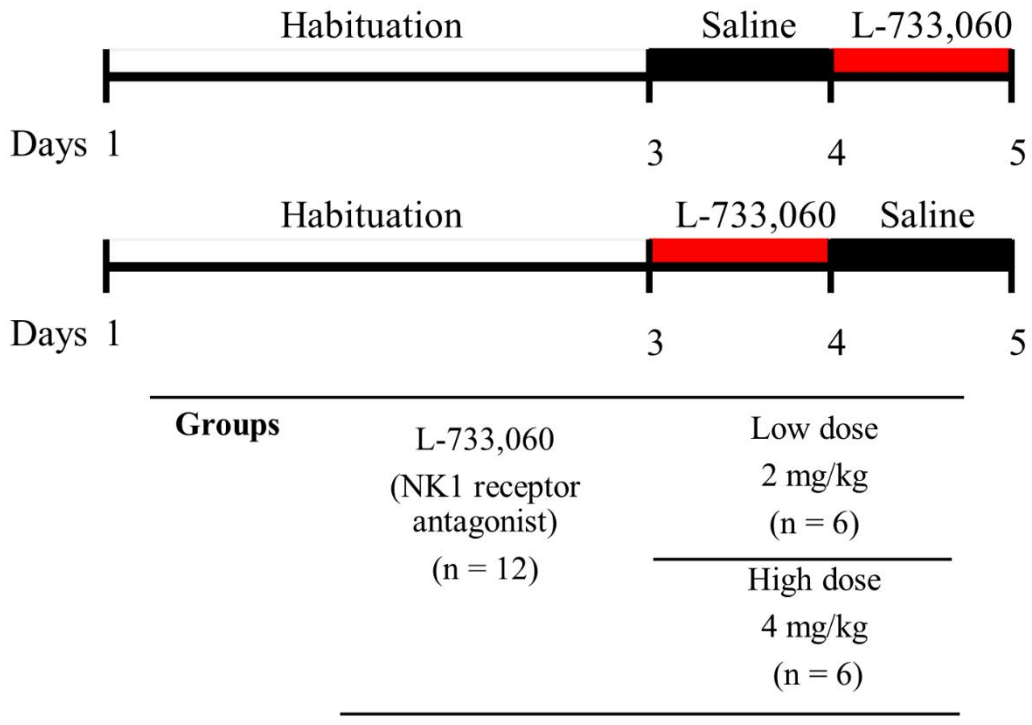

Habituation
Saline
L-733,060
Days 1
3
4
5
Habituation
L-733,060
Saline
Days 1
3
4
5
Groups
L-733,060
(NK1 receptor antagonist)
(n = 12)
Low dose
2 mg/kg
(n = 6)
High dose
4 mg/kg
(n = 6)

Fig. 2

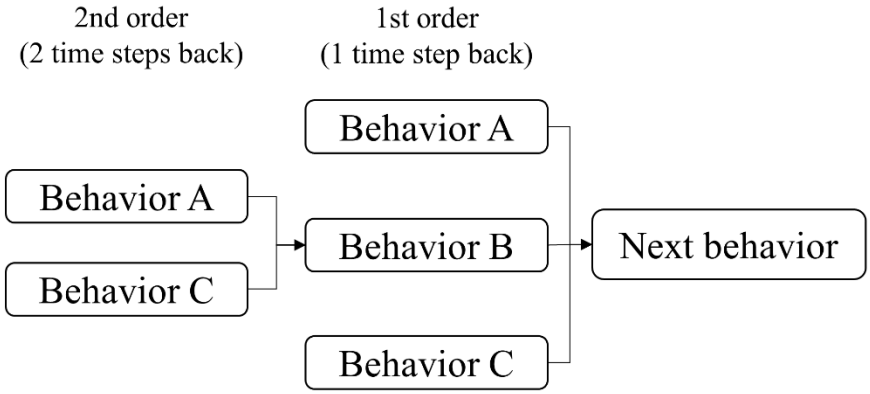

2nd order
(2 time steps back)
1st order
(1 time step back)
Behavior A
Behavior A
Behavior C
Behavior B
Behavior C
Next behavior

Fig. 3

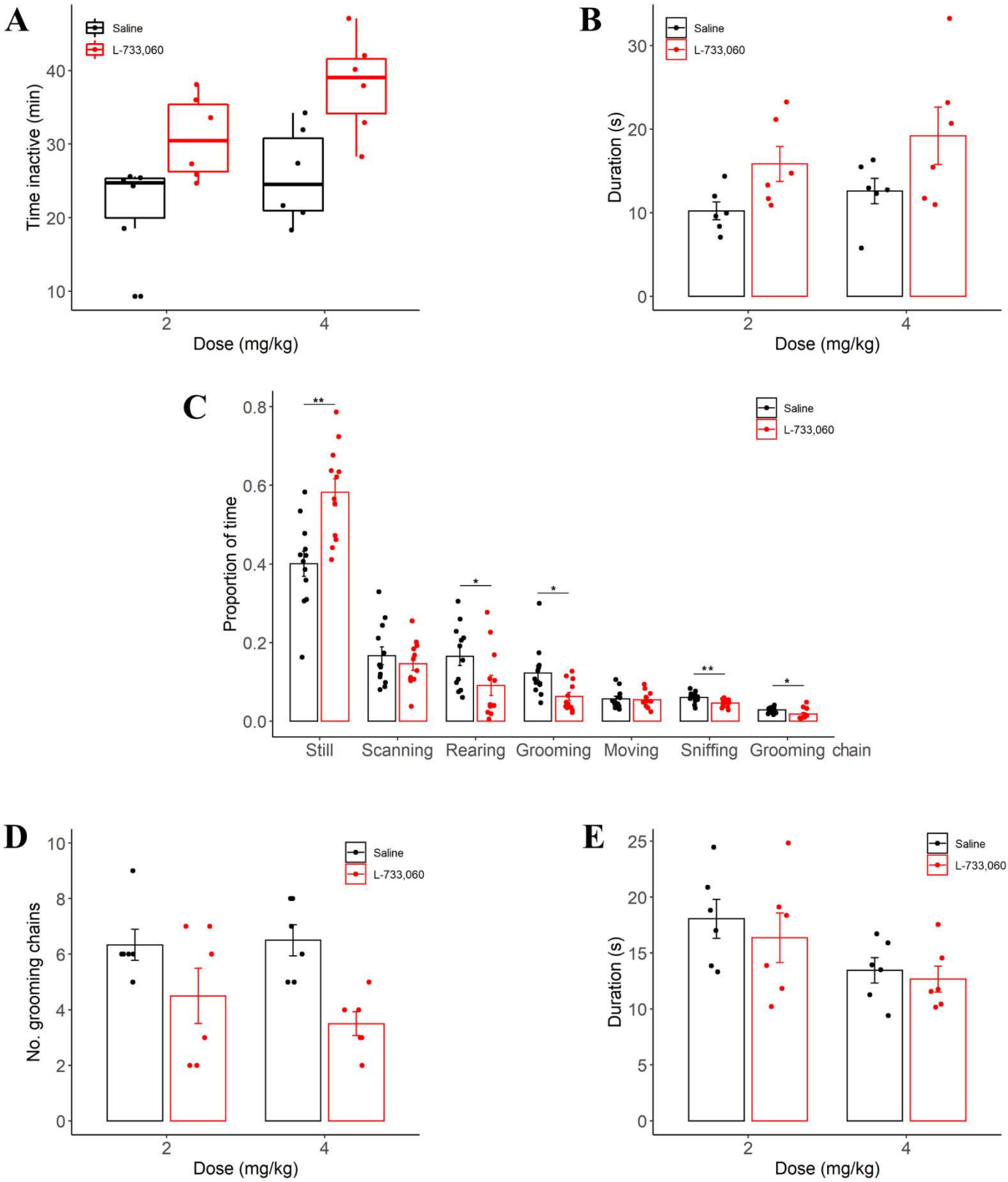

A
Saline
L-733,060
Time inactive (min)
40
30
20
10
2
4
Dose (mg/kg)
B
Saline
L-733,060
Duration (s)
30
20
10
0
2
4
Dose (mg/kg)
C
Saline
L-733,060
Proportion of time
0.8
0.6
0.4
0.2
0.0
**
*
*
**
*
Still
Scanning
Rearing
Grooming
Moving
Sniffing
Grooming chain
D
Saline
L-733,060
No. grooming chains
10
8
6
4
2
0
2
4
Dose (mg/kg)
E
Saline
L-733,060
Duration (s)
25
20
15
10
5
0
2
4
Dose (mg/kg)

Fig. 4

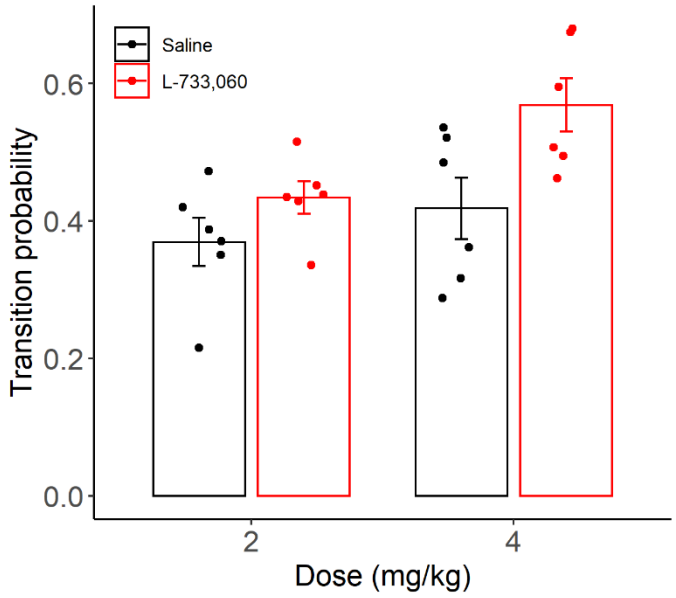

Saline
L-733,060
Transition probability
0.0
0.2
0.4
0.6
2
4
Dose (mg/kg)

Fig. 5

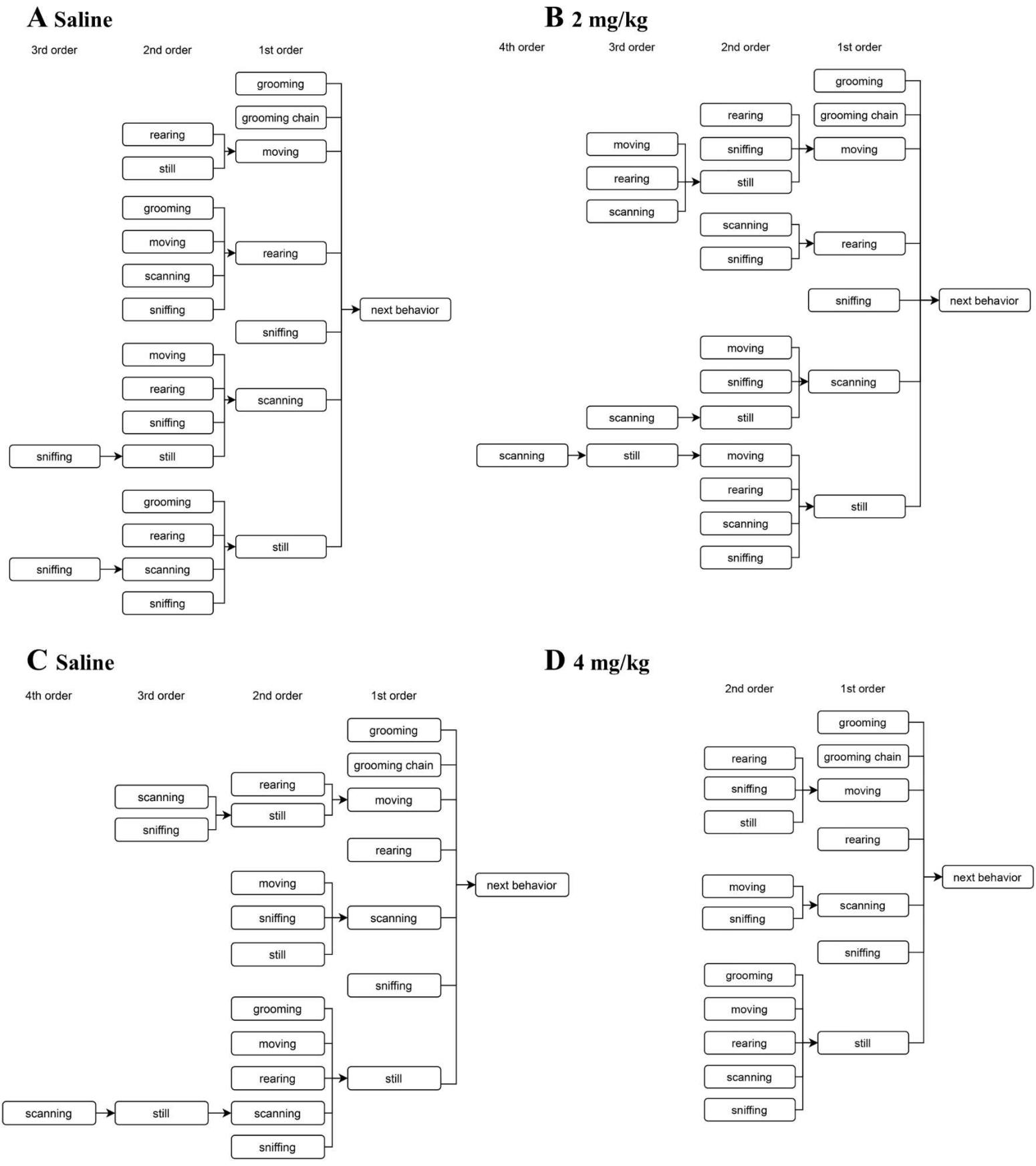
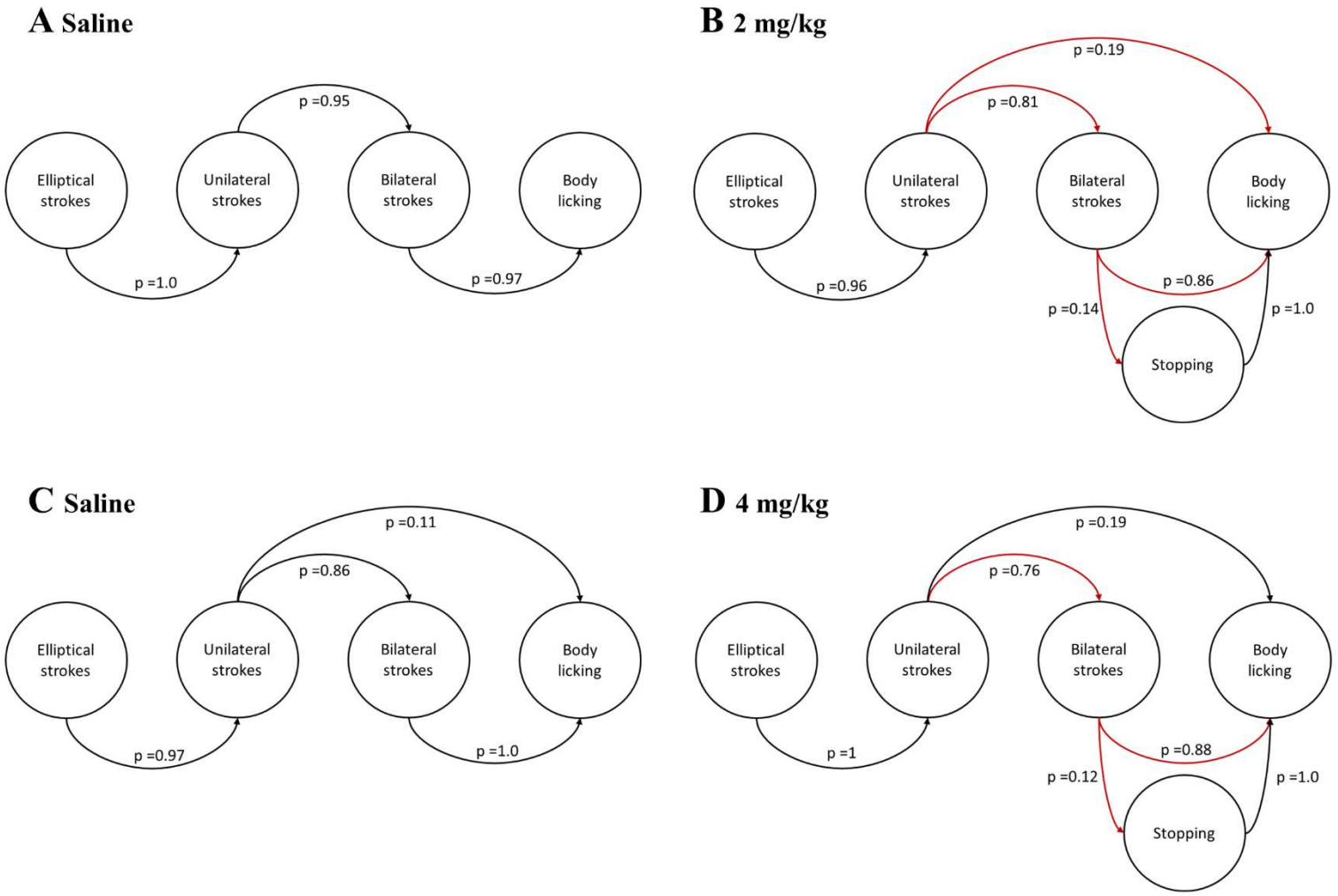

A Saline
3rd order
2nd order
1st order
grooming
grooming chain
rearing
moving
still
grooming
moving
scanning
sniffing
rearing
sniffing
moving
rearing
sniffing
sniffing
still
scanning
grooming
rearing
sniffing
scanning
sniffing
still
next behavior
B 2 mg/kg
4th order
3rd order
2nd order
1st order
grooming
grooming chain
rearing
sniffing
moving
moving
rearing
still
scanning
scanning
sniffing
rearing
sniffing
next behavior
moving
sniffing
scanning
scanning
still
scanning
still
moving
rearing
scanning
sniffing
still
C Saline
4th order
3rd order
2nd order
1st order
grooming
grooming chain
scanning
rearing
sniffing
still
moving
rearing
moving
sniffing
scanning
still
sniffing
grooming
moving
rearing
still
scanning
still
scanning
sniffing
next behavior
D 4 mg/kg
2nd order
1st order
grooming
grooming chain
rearing
sniffing
moving
still
rearing
moving
sniffing
scanning
sniffing
grooming
moving
rearing
scanning
sniffing
still
next behavior

Fig. 6


A Saline
Elliptical strokes
Unilateral strokes
Bilateral strokes
Body licking
p =0.95
p =1.0
p =0.97
B 2 mg/kg
p =0.19
p =0.81
p =0.96
p =0.86
p =0.14
p =1.0
Stopping
C Saline
p =0.11
p =0.86
p =0.97
p =1.0
D 4 mg/kg
p =0.19
p =0.76
p =1
p =0.88
p =0.12
p =1.0
Stopping

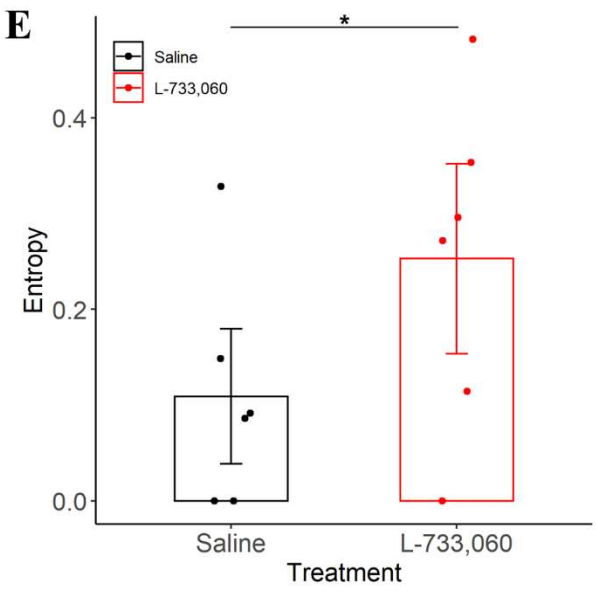

E
*
Saline
L-733,060
Entropy
0.4
0.2
0.0
Saline
L-733,060
Treatment

Fig. 7

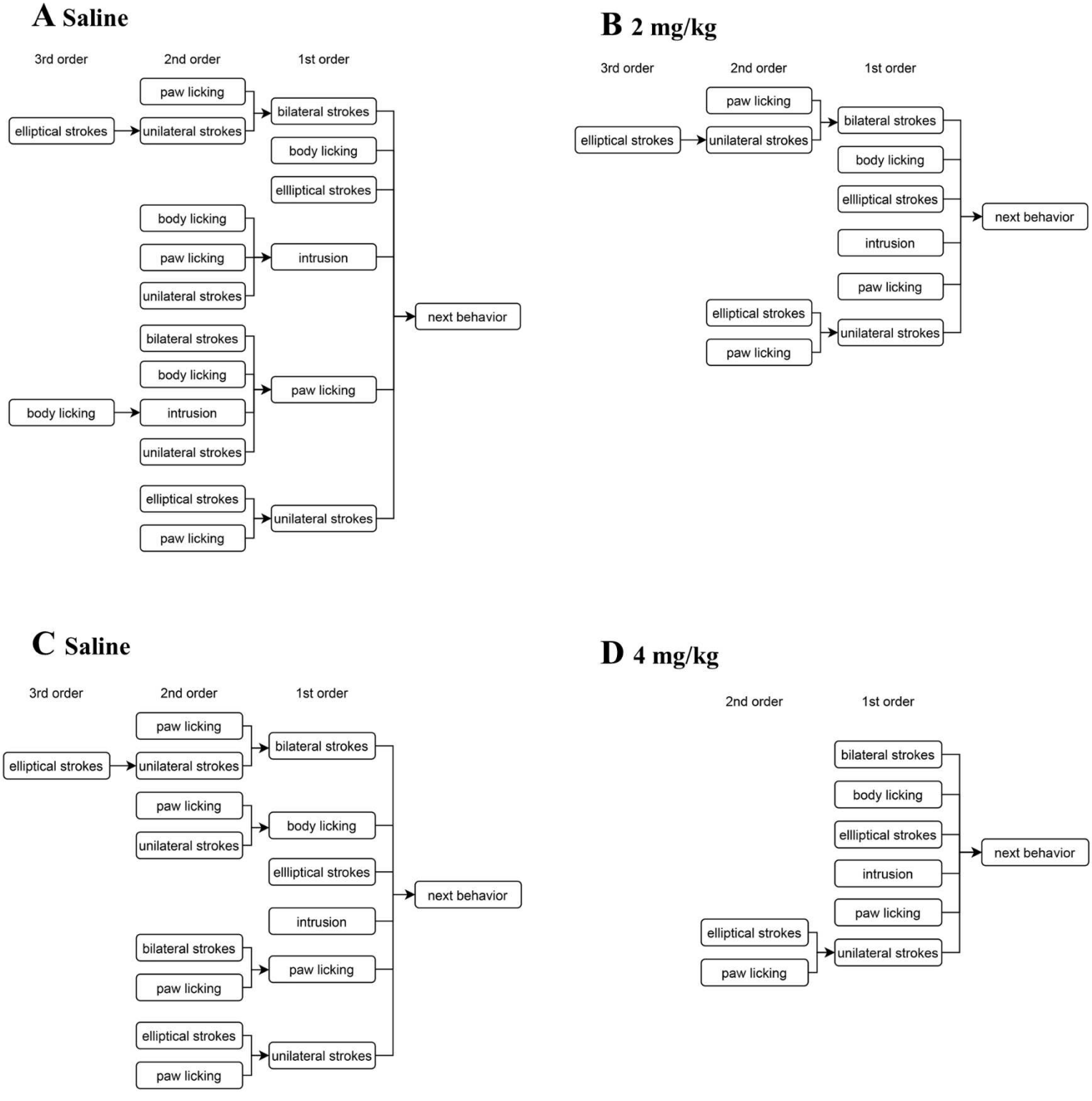
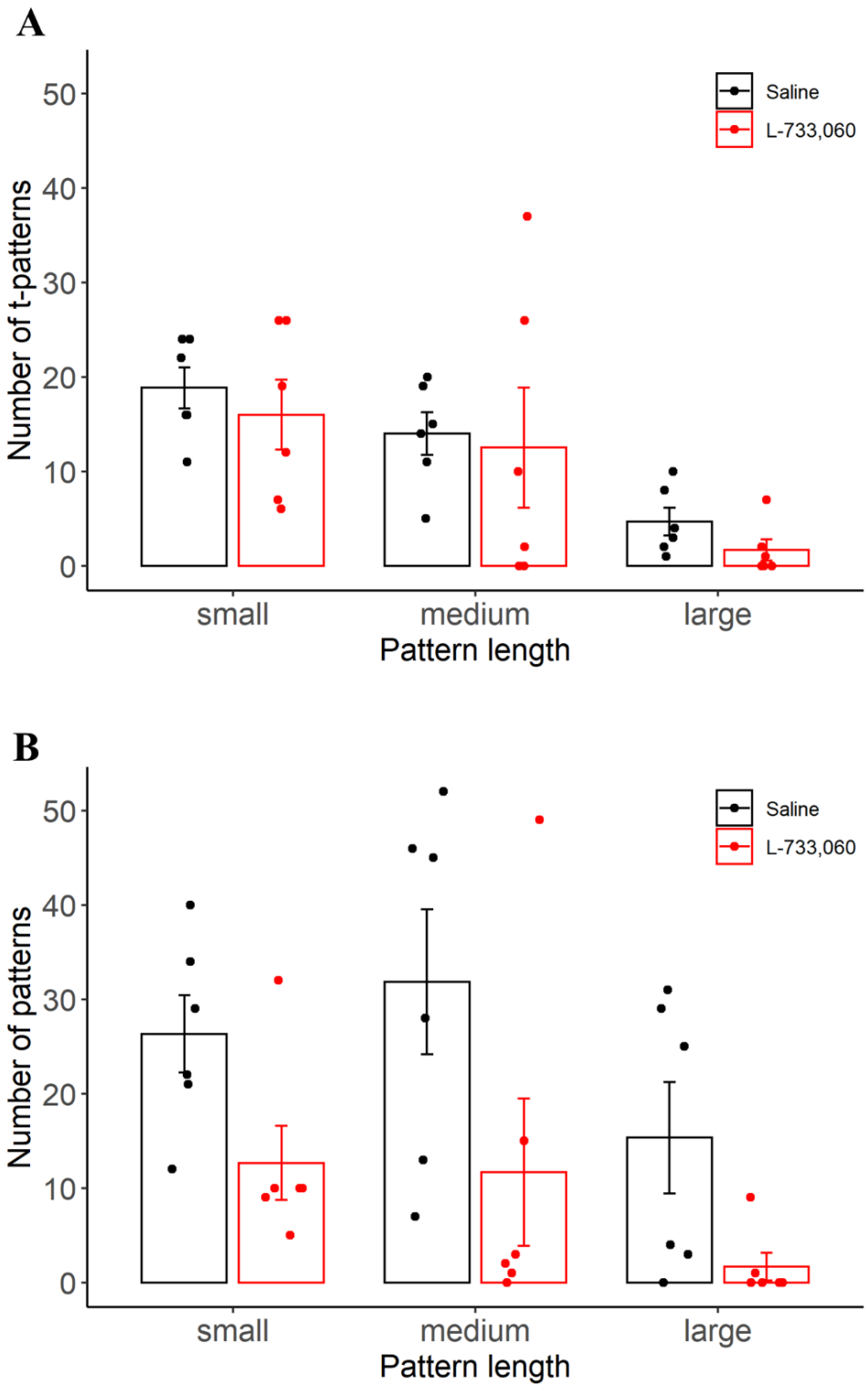

A Saline
3rd order
2nd order
1st order
elliptical strokes
unilateral strokes
paw licking
bilateral strokes
body licking
ellliptical strokes
intrusion
next behavior
B 2 mg/kg
C Saline
D 4 mg/kg

Fig. 8


A
Saline
L-733,060
Number of t-patterns
0
10
20
30
40
50
small
medium
large
Pattern length
B
Saline
L-733,060
Number of patterns
0
10
20
30
40
50
small
medium
large
Pattern length

Supplementary Material

All rats from the high and low dose groups performed similar amounts of behavior under saline conditions, with no significant dose effect on the frequency of behavior: still ($F(1,10) = 0.03$, $p = 0.84$, n. s.), moving ($F(1,10) = 0.80$, $p = 0.39$, n. s.), scanning ($F(1,10) = 0.43$, $p = 0.52$, n. s.), rearing ($F(1,10) = 2.50$, $p = 0.14$, n. s.), sniffing ($F(1,10) = 2.71$, $p = 0.13$, n. s.), grooming ($F(1,10) = 0.13$, $p = 0.72$, n. s.) or grooming chain ($F(1,10) = 0.04$, $p = 0.83$, n. s.). This suggests that there was no prior systematic difference in the activity levels of our two dose groups.

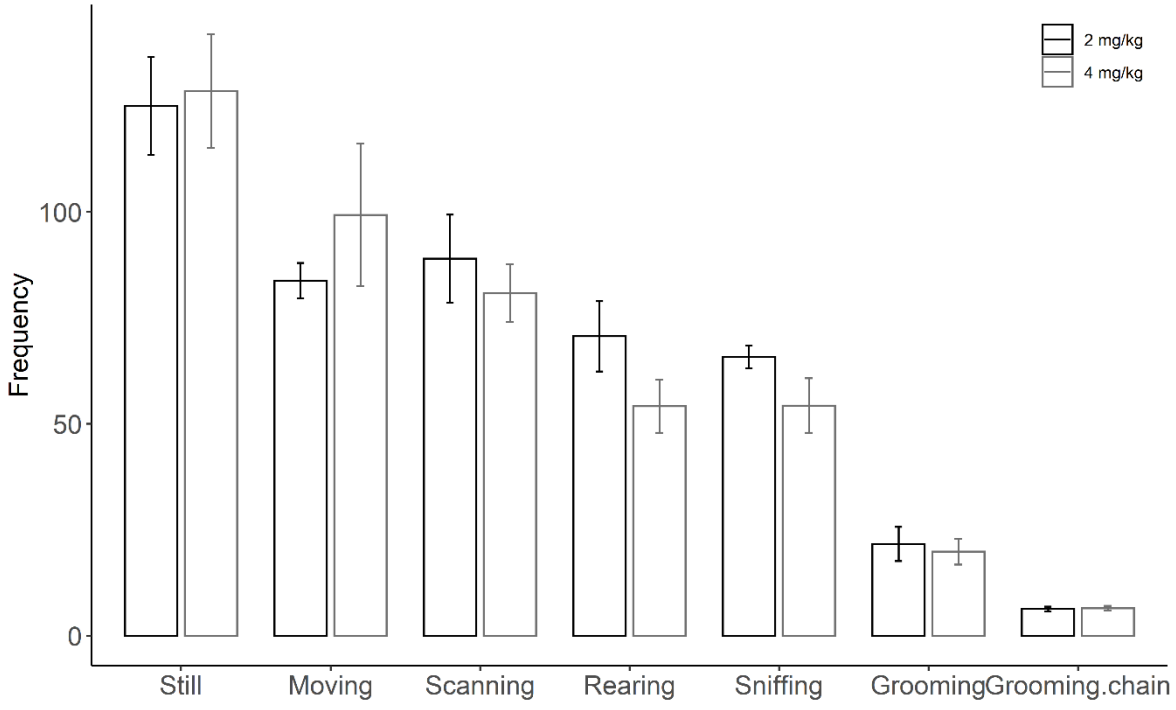


Figure S1. Frequency of open field behaviors in the saline condition for the high (gray bars) and low (black bars) dose groups.

For all first order chains of general open field behaviors found in the VLMMs, that is sequences of two behaviors, such as, grooming-moving, sniffing-rearing, moving-scanning, the transition entropy was calculated when saline and L-733,060 were injected. There was no significant treatment ($F(1,24) = 0.13$, $p = 0.71$, n. s.) or treatment×dose effect ($F(1,24) = 0.019$, $p = 0.89$, n. s.), suggesting no effect of L-733,060 on sequences that are already very variable.

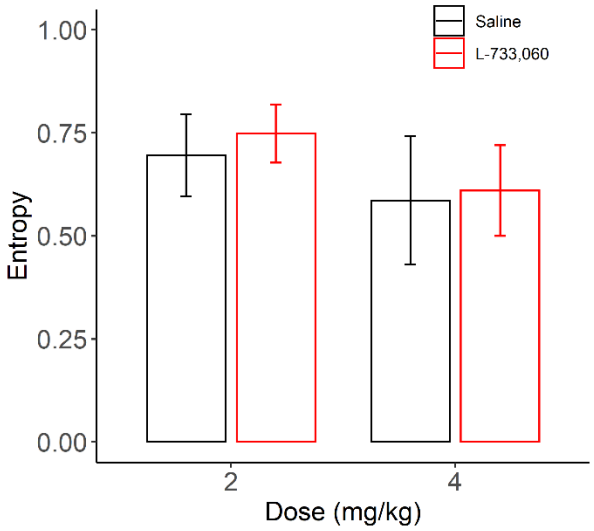


Figure S2. Transition entropies of first order chains found in VLMMs of general behaviors. Data are shown as mean ± SEM.

Here we show the data from the grooming chain transitions at the individual level, rather than by group. There was a significant Treatment×Transition interaction ($F(4,88) = 2.74$, $p = 0.03$), indicating that the effect of the NK1 antagonist was different in each transition of the grooming chain. There was no significant effect on the transition from elliptical strokes-unilateral strokes ($F(1,11)=0.29$, $p = 0.60$, n. s), but there were marginally significant effects of the NK1 antagonist on the transition from unilateral-bilateral ($F(1,11) = 3.96$, $p = 0.07$), bilateral-body licking ($F(1,11) = 3.8$, $p = 0.06$) and bilateral-stop ($F(1,11) = 3.8$, $p = 0.07$).

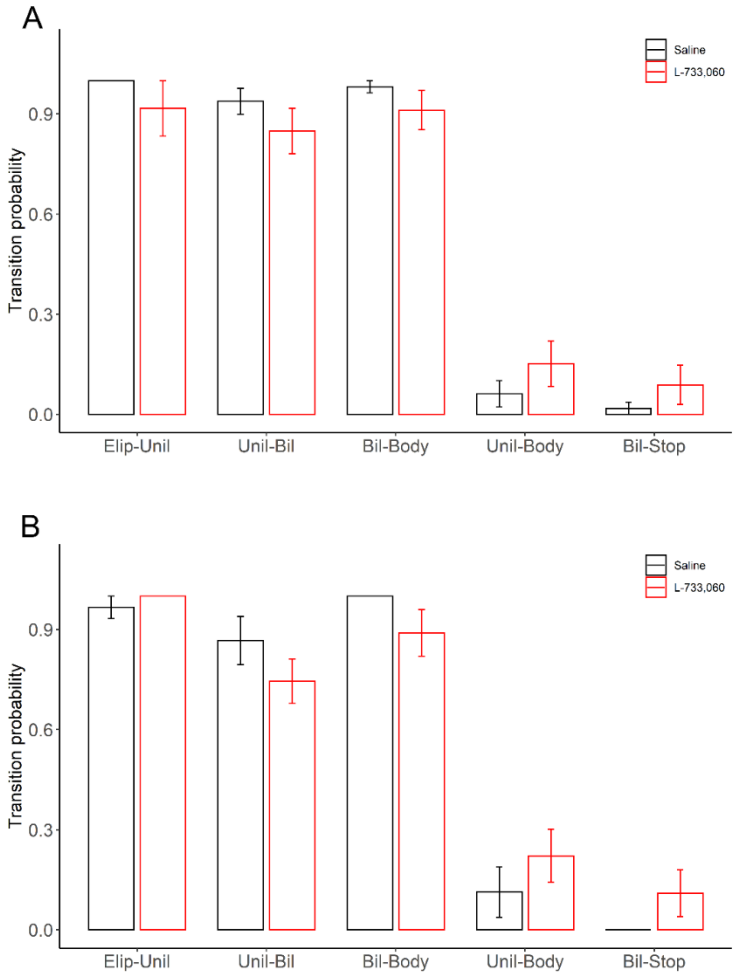


Figure S3. Transition probabilities between the phases of the grooming chain. **A**, transitions probabilities for the low dose group. **B**, transition probabilities for the high dose group. Data are shown as mean ± SEM.