The effects of smoking on whisker movements: a quantitative measure of exploratory behaviour in rodents

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Abstract

Nicotine, an important component of cigarette smoke, is a neurotransmitter that contributes to stress, depression and anxiety in smokers. In rodents, it increases anxiety and reduces exploratory behaviours. However, so far, the measurements of exploratory behaviour in rodents have only been semi-quantitative and lacking in sufficient detail to characterise the temporal effect of smoking cessation. As rodents, such as mice and rats, primarily use whiskers to explore their environment, we studied the effect of 3 months smoking with 1 and 2 weeks smoking cessation on whisker movements in mice, using high-speed video camera footage and image analysis. Both protraction and retraction whisker velocities were increased in smoking mice ($p<0.001$) and returned to normal following just one week of smoking cessation. In addition, locomotion speeds were decreased in smoking mice, and returned to normal following smoking cessation. Lung function was also impacted by smoking and remained impaired even following smoking cessation. We suggest that the increased whisker velocities in the smoking mice reflect reduced exploration and impeded tactile performance. The increase in whisker velocity with smoking, and its reduction following smoking cessation, also lends support to acetylcholine being involved in awareness, attention and alertness pathways. It also shows that smoking-induced behavioural changes can be reversed with smoking cessation, which may have implications for human smokers.

1. Introduction

Tobacco smoking is a serious health problem and one of the major causes of death worldwide (Vella and Di Giovanni, 2013). While smoking can reduce anxiety and relieve stress (Piciotto et al. 2002), nicotine in cigarette smoke also has noxious effects, such as increasing anxiety and depression following chronic use and withdrawal (Casarrubea et al., 2015; Piciotto et al. 2002). Despite its potential noxious effects, nicotine intake is reinforced via the dopaminergic system (Corrigall et al., 1992; Di Chiara, 2000; Maskos et al. 2005; Tolu et al. 2013; Faure et al. 2014). It acts by binding with the nicotinic acetylcholine receptors (nAChRs), which mediate dopamine release and other neurotransmittors, such as serotonin and glutamate (Pierucci et al., 2004; Lester, 2014). Different patterns of neurotransmitter release occur depending on the course of nicotine administration (acute, chronic and withdrawal) and this partly accounts for the complex behavioural effects of nicotine on anxiety and depression. In addition, the distribution of nAChRs throughout the brain also means that nicotine administration can cause a variety of behavioural responses in both animals and humans (McDermott et al., 2013; Casarrubea et al., 2015).

In rodents, the administration of nicotine to regions of the brain that are associated with reward, such as the central amygdala (Zarrindast et al., 2008), lateral septal nucleus (Ouagazzal et al., 1999), dorsal raphe nucleus (Cheeta et al., 2001) and different areas of the mesolimbic dopaminergic system (Picciotto et al., 2002), has induced behaviours associated with anxiety, including a reduction in exploratory behaviours (Battig et al. 1976; Casarrubea et al., 2015; Mesa-Gresa et al. 2013). Exploratory behaviours are usually approximated by measuring the duration and frequency of a range of movements, including rearing, head-dipping, grooming, climbing, sniffing and licking, during open field or hole-board tests (Casarrubea et al., 2015). In particular, head-dipping has been found to reduce significantly in

rodents treated with nicotine in hole-board tests (Casarrubea et al., 2015; Piri et al. 2011; ve Yontem et al. 2014), and is thought of as a reduction in exploration of the holes and floor. In healthy rodents, head-dipping (or "dabbing") has been associated with whisker exploration of the floor (Arkley et al., 2014; Grant et al., 2009; 2012b), as the whiskers are the primary tactile organ in nocturnal rodents (Roohbakhsh et al. 2016). Measuring duration and frequencies of exploratory behaviours, such as head-dipping is thought to not be sufficient to wholly characterise the complex effects of smoking and smoking cessation on behaviour (Casarrubea et al., 2015). Rather, an enhanced quantification of exploration is needed, and we propose that measuring precise changes in whisker movements in rodents might well offer this alternative.

Whiskers in rats and mice move backwards and forwards in a behaviour known as whisking, which occurs up to 25 times per second (Vincent, 1912). Studies have found that rodents use their whiskers to guide many tasks such as locomotion, navigation, foraging and hunting (Grant & Arkley 2016). With the development of high-speed video cameras and analysis programs, it has become apparent that rodents do not just make simple sweeping movements with their whiskers. Rather, they can precisely change the amplitude, velocity and position of their whiskers during locomotion and object exploration (Arkley et al., 2014; Carvell & Simons, 1995; Grant et al., 2009; Hartmann, 2001; Kleinfeld et al., 2006; Mitchinson et al., 2007; Szwed et al., 2003; Towal & Hartmann, 2008; Welker, 1964). For example, object exploration is generally associated with slower whisker movements at lower amplitudes (Carvell & Simons, 1995; Grant et al. 2009). Following an object contact, sensory information from the whisker shaft, such as force and direction, is transmitted in the follicle and passed through multiple neural pathways to the cortex (Grant & Arkley 2016). The organisation of cholinergic neurons throughout whisker-related sensorimotor areas in rodents (Beak et al., 2010), including brainstem, thalamus, (Timofeeva et al., 2005; Bosman et al.,

2011), cortex (Bosman et al., 2011), cerebellum (Timofeeva et al., 2005), zona incerta and amygdala (Bosman et al., 2011) indicates that nicotine may well have an effect on whisker sensorimotor integration.

Finding a quantitative way to measure exploratory behaviours, by measuring whisker movements, would offer the ability to capture the complex effects of smoking and nicotine administration on rodents. As nicotine has been found to affect general exploratory behaviours in rodents (Battig et al. 1976; Casarrubea et al., 2015; Mesa-Gresa et al. 2013), it is to be expected that whisker movements, being the primary mode of exploration, will also be affected by nicotine and smoking. This study will, for the first time, explore the effect of chronic smoking, the most important source of nicotine in humans, on whisker movements in mice. Previous studies have documented that nicotine results in a reduction in general exploratory behaviours in rodents (Battig et al., 1976; Casarrubea et al., 2015), which we predict to be represented here by faster moving whiskers (Carvell & Simons, 1995; Grant et al., 2009; Mitchinson et al., 2007). A novel behavioural system that tracks and non-invasively measures whisker movements (Grant et al., 2013) will be used to obtain a quantitative measure of the impact of smoking and smoking cessation on exploratory whisker movements in mice.

2. Methods

All experimental procedures were approved by the Ethical Committee of Animal Experiments of the KU Leuven.

2.1 Animals

Forty male C57Bl6 mice were used in this study. Animals were housed on a 12-hour light-dark cycle and supplied with pelleted food and water *ad libitum*.

2.2 Smoking Procedures

Animals were randomly assigned to the following groups: Control (C: n=10), Smoking (S: n=11), Smoking cessation for 1 week (S1W: n=9) and Smoking cessation for 2 weeks (S2W: n=10). Smoking was selected as the nicotine administration technique, as it is the most common way people are exposed to elevated levels of nicotine. Smoking animals were exposed to cigarette smoke (3R4F research cigarettes with filter purchased from Kentucky Tobacco Research and Development Center, University of Kentucky) using a nose-only exposure system (InExpose System, Scireq). Mice were placed in soft restraints and connected to an exposure tower. A cigarette puff was generated every minute, leading to 10 seconds of cigarette smoke exposure followed by 50 seconds of fresh air. Mice were acclimatized to the cigarette smoke exposure during the first week of the experiment. Afterwards, animals were exposed daily to four cigarettes, twice a day, 5 days per week, over 3 months (Rinaldi et al., 2012). Control animals were treated similarly, but were exposed to filtered air for the same duration. Animals in the smoking cessation groups stopped smoking for 1 or 2 weeks. As nicotine withdrawal behaviours are usually absent from 5-6 days (Damaj et al. 2003), the one-week time-point was selected as a minimum, and the two-week time-point was selected as an additional measure. Smoking and control mice were exposed to cigarette smoke or filtered air, respectively on the morning of their behavioural assessment and tested approximately 2 hours after the smoking or filtered air treatment. Any stress caused by restraint in the experimental set-up was, therefore, equivalent between the smoking and control groups. The total particle density concentration of the cigarette smoke in the tower was measured weekly and was on average 149.5 mg total particulate matter per m^3. Mice were weighed weekly to ensure they maintained a healthy body mass for inclusion in the study. Two mice in the S1W group did not survive the smoking protocol.

2.2 Recording and Measuring Behaviour

Each mouse was placed in to a transparent, Perspex, rectangular arena (20 x 30 x 15 cm) (Fig. 1a), which was lit from below by a bright, normal-spectrum light box (PHLOX LEDW-BL-400/200-SLLUB-Q-1R-24V). The mouse was filmed from above using a digital high-speed video camera (Phantom Miro ex2) recording at 500 frames per second with a shutter-speed of 1 ms and a resolution of 640x480 pixels. Multiple 1-s video clips were collected opportunistically (by manual trigger) when the animal moved in the field of view of the camera. Approximately 16 clips were collected from each animal. Four to six clips from each mouse were selected and trimmed based on to the following selection criteria developed in Grant et al. (2013): i) the mouse was clearly in frame; ii) both sides of the face were visible; iii) the head was level with the floor (no extreme pitch or yaw); iv) the whiskers were not in contact with a vertical wall; and v) the mouse was clearly moving forward. Six of the eleven smoking animals (S) could not be included in the study as their whiskers were barbered by a conspecific and thus could not be imaged. Barbering is not usually associated with stress, but rather caused by a particularly dominant animal in the home cage (Bresnahan et al. 1983). While barbering is relatively rare, to overcome this in future studies it is recommended to remove the dominant individual from the home cage, or to house mice singularly, a month before filming. This left a sample size of 32 animals (C: n=10, S: n=5, S1W: n=7, S2W: n=10), which is reflected in the individual averages in Figure 3.

In each selected clip, the mouse snout and whiskers were tracked using the BIOTACT Whisker Tracking Tool (Perkon et al., 2011). The tracker semi-automatically finds the orientation and position of the snout, and the angular position (relative to the midline of the head) of each identified whisker. Tracking was validated by manually inspecting the tracking annotations overlaid on to the video frames (Fig 1b) and a total of 166 clips, each of around 0.5 seconds in length, were included in the analysis (C: n=51, S: n=33, S1W: n=35, S2W: n=47).

159 The movement of the entire whisker field was determined from the unsmoothed mean of all
160 the tracked whisker angular positions for each side frame by frame (Grant et al. 2012; Figure
161 1c, termed *naïve mean angle (nma)*). The following variables were calculated from the
162 whisker angular position data. *Offset* is the mean angular position. To estimate the *amplitude*,
163 the offset was removed from the whisking angle time series and the root mean square value
164 was computed to give the root-mean-square (RMS) whisking amplitude. These time series
165 were approximately sinusoidal, so the ‘‘peak-to-peak whisking amplitude’’ was estimated by
166 multiplying the RMS whisking amplitude by $2\sqrt{2}$ (Chatfield, 2003). This estimate of
167 amplitude is reasonably robust to accommodate departures from a purely sinusoidal pattern.
168 Whisk *frequency* was calculated using a discrete-fourier transform (FFT function in Matlab),
169 with a peak frequency cut-off of 50 Hz, as anything above this would not be expected
170 (Mitchinson et al. 2011). An auto-correlogram fitted each FFT curve to the original angular
171 position signal and provided an indication of fit, or power; the FFT curve with the highest
172 power was selected as the best frequency fit. Mean angular *retraction* and *protraction*
173 *velocities* were calculated as the average velocity of all the backward (negative) and forward
174 (positive) whisker movements, respectively. Offset, amplitude, retraction and protraction
175 velocities were calculated individually for each whisker side, and then averaged between the
176 left and right sides to give one value of each per clip.

177 As locomotion is a common behavioural measure, average *locomotion speed* was also
178 calculated on a per-frame basis by tracking the nose tip and calculating the average number
179 of metres moved per second. Each day the arena was calibrated, by taking an image of a
180 ruler, to make the pixel to mm conversion.

181 **2.3 Pulmonary mechanics**

To verify that the dose and duration of smoking was such that it had physiological effects we also investigate pulmonary mechanics. The pulmonary system is directly exposed to cigarette smoke and effects should be seen there. Thereto, after filming, the mice were anesthetized with a intraperitoneal injection of a mixture of xylazine (8.5 mg/kg, Rompun®, Bayer, Belgium) and ketamine (13 mg/kg, Anesketin®, Eurovet, Belgium) and tracheotomized. Mice were then placed in a body plethysmograph and connected to a computer-controlled ventilator (Buxco-Force Pulmonary Maneuvers) to measure lung compliance (Cchord). Lung compliance, or more specifically chord compliance, measures the linear section of the lung Pressure-Volume Curve, and is strongly associated with lung volume. It has been suggested as a way of diagnosing a range of respiratory disorders (Harris 2005).

All the mice, including the barbered smoking mice were included in this section of the study. However, three control mice, one smoking mouse, one smoking cessation week 1, and one smoking cessation week 2 mouse were euthanized during procedures unrelated to this study prior to the extraction of these measurements, leaving a sample size of 32 (C: n=7, S: n=10, S1W: n=6, S2W: n=9), which is reflected in the individual averages in Figure 2.

2.4 Statistical considerations

All data was distributed normally. Differences between groups for whisking measures and locomotion speed were analysed with linear mixed models. The treatment groups of mice (smoking, controls, smoking cessation week 1 and smoking cessation week 2) was a fixed between factor, and the individual mouse ID was a random between factor. Lung function data was analysed using a univariate ANOVA, with treatment group as a between factor.

As whisking variables can be altered by locomotion speed (Arkley et al. 2014; Grant et al. 2012a), locomotion speed was also added as a covariate to the linear mixed models, but did not have a significant effect on the results and, therefore, was not included here.

A significance level of < 0.05 was selected for all analyses. Tukey post-hoc tests were carried out on significant results and indicated with a * on the subsequent graphs. Partial Eta Squared (η^2p) values are quoted for effect sizes throughout.

3. Results

Lung compliance was significantly increased in the smoking mice and remained impaired even after 2 weeks smoking cessation (ANOVA: $F(3,164)=7.258$, $p=0.001$, $\eta^2p = 0.500$, Tukey Post-hoc: C<S,S1W,S2W). This can clearly be seen in Figure 2a, where the control mice have a significantly lower average Cchord compliance value than the smoking and smoking cessation groups. Indeed, the lowest Cchord compliance values can be seen in Figure 2b in the C2 and C5 control mice, and the highest values in the S9, S11 and S6 smoking mice.

The smoking mice locomoted significantly slower than the control mice, however, after 1-week smoking cessation this difference had disappeared (mixed model: $F(3,25.4) = 9.981$, $p<0.001$, $\eta^2p = 0.173$, Tukey Post-hoc: S<C,S1W,S2W). This can clearly be seen in figure 3a, where the smokers have a significantly slower average locomotion speed than the control mice, and those in the smoking cessation conditions. Specifically, Figure 3b show that mouse S11 in the smoking condition had the lowest locomotion speed overall, with control mouse C10 having the fastest locomotion speed overall.

Example whisking traces from a smoking and control mouse can be seen in Figure 4. From Figure 5 it can be seen that smoking mice move their whiskers faster than all the other treatment groups in both the protraction and retraction stages of the whisk (protraction velocity mixed model: $F(3,29.5) = 7.055$, $p=0.001$, $\eta^2p = 0.092$, Tukey Post-hoc: S>C,S1W,S2W; retraction velocity mixed model: $F(3,31.6) = 6.486$, $p=0.002$, $\eta^2p = 0.100$, Tukey Post-hoc: S>C,S1W,S2W). Table 1 shows that the control mice held their whiskers

slightly further forward (with higher offset values) than those in the smoking cessation treatments, however this was not significant (mixed model: $F(3,26.5) = 2.498$, $p=0.081$, $\eta^2p = 0.055$). Likewise, smoking mice did tend to have larger amplitudes than control mice, however, this was also not significant (mixed model: $F(3,26.8) = 2.417$, $p=0.088$, $\eta^2p = 0.064$) (Table 1). Frequency was also not significantly altered between the smoking groups (mixed model: $F(3,159) = 1.711$, $p=0.167$, $\eta^2p = 0.038$).

4. Discussion

Results from this study show that there are measureable changes in exploratory behaviour in smoking mice, compared to control and smoking cessation conditions. In particular, whisking protraction and retraction velocities were both significantly increased (Fig. 5 and Table 1) and locomotion speed was significantly reduced (Fig. 3) in smoking mice (two hours post-smoking) and returns to normal following smoking cessation of just one week. Lung compliance was significantly increased in smoking mice, and did not recover following smoking cessation (Figure 2).

Smoking mice locomoted slower than non-smoking mice (Figure 3). Specific changes in locomotion have not yet been found in rodents treated with nicotine (Casarrubea et al., 2015); however, general activity has been found to decrease (Mesa-Gresa et al., 2013), which offers support for our observation. Other studies have reported increases (Battig et al., 1976; Calderone et al. 2008; Slawecki et al., 2003), or no changes (Casarrubea et al., 2015; Piri et al. 2011) in physical and locomotor activity levels in nicotine-treated mice, which differ from our own findings. Indeed, the association of nicotine and locomotion is complex in the literature and can be affected by gender (Calderone et al. 2008), and probably dosage as well. Whatever the cause of these discrepancies, the reduction in locomotion speed in our study was reversed after only a one-week period of smoking cessation (Figure 3). It is interesting to

note that smoking in humans is also often associated with reduced activity levels (Kaczynski et al. 2008; Larsson & Orlander 1984) and if our data in mice can be translated to humans, they suggest that a reduced drive for physical activity can be readily reversed by smoking cessation.

A decrease in exploratory behaviour following nicotine administration is a robust finding in rodents (Battig et al., 1976; Casarrubea et al., 2015; Slawecki et al., 2003). Specifically, nicotine-treated mice have been found to spend more time away from open areas and reduce the amount of time spent rearing and head-dipping (Casarrubea et al., 2015; Slawecki et al., 2003). Many studies have found a reduction in head-dipping during a hole-board task, following nicotine administration (Casarrubea et al., 2015; Piri et al. 2011; ve Yontem et al. 2014). Head-dipping in exploring, healthy rodents has been found to be associated with whisker exploration of the floor (Arkley et al., 2014; Grant et al., 2009; 2012b), and we propose here that measuring whisker movements directly, rather than head movements, can offer a way to quantitatively measure exploratory behaviour in freely moving rodents.

Exploration in rodents, such as mice and rats, is primarily guided by their sense of whisker touch (Grant & Arkley 2016). Just like other sensory systems, tactile sensitivity is enhanced by moving the sensor in a certain way over an object (Carvell & Simons, 1995; Mitchinson et al., 2007; Grant et al., 2009; Towal & Hartmann, 2008). In particular, good performance on tactile tasks are often associated with slower whisker movements (Carvell & Simons, 1995), which allow the whiskers to contact surfaces for longer durations (Carvell & Simons, 1995; Grant et al., 2009). Rats and mice have the ability to change the velocity of their whiskers on a per-whisk basis, so they can respond quickly with changes in their whisking profiles (Towal & Hartmann, 2008). They are even able to speed up and slow down different phases of the whisk cycle, so that they can contact an object at an optimum speed (Moxon, 2008). The speed and amplitude of a whisker contact elicits different response profiles in thalamic and

cortical neurons (Pinto et al., 2000); for example, high velocity contacts elicit more spikes particularly in the thalamic and cortical neurons (Pinto et al. 2000; Shoykhet et al., 2000). As both velocity and amplitude information are used to code object position (Szwed et al., 2003; Ahissar & Arieli, 2001) the increased whisker speeds in the smoking mice may indicate that exploration abilities and tactile performance are somewhat impeded in these animals.

Whisker positions (offset) showed large inter-individual variability (Figure 5b) and did not differ significantly between smoking and non-smoking mice. Also amplitude and frequency were not significantly affected by smoking (Figure 5, Table 1), although amplitude did tend to show a general trend to be larger in the smoking mice than in controls and after smoking cessation. Whisking amplitude is usually decreased during close exploration of a surface (Carvell & Simons, 1995; Grant et al., 2009; Mitchinson et al., 2007); therefore, a reduction in exploration might well have caused the small increase in amplitude that we observed in smoking mice. In addition, perhaps the small sample numbers of smoking mice (n=5) have also contributed to the lack of significance in this result.

That whisking behaviour recovered in mice that have stopped smoking for only a week (Fig. 5), without recovery of normal lung compliance (Fig. 2), suggests that behavioural effects are likely to improve well before lung recovery. In addition, the mechanism for the increase in whisking velocities is likely to be the interaction of nicotine with neuronal structures, rather than any change in lung function during smoking (Fig. 2). While smoking was selected as the nicotine administration technique in this study, as it is the most common way that people administer nicotine, and provides an efficient way of delivering it to the brain (Henningfield & Keenan 1993), future work could carry this study out using a direct nicotine delivery system, such as a patch. While the number of smoking mice included in the study was less than in the other conditions, we are confident that our statistical analyses represent our

findings, and we have manually examined the video footage and whisker traces to corroborate our findings for both locomotion and whisking data.

4.1 Links to brain and behaviour

Due to the distribution of nAChRs throughout the brain and the complexity of behavioural pathways, it is hard to make strong inferences linking the effect of smoking and nicotine to any specific brain areas. Delivery of nicotine to specific brain areas, such as brainstem nuclei, cerebellum, primary motor cortex or primary somatosensory cortex, might help to improve understandings of the role of nicotine, and acetylcholine, on behaviour. A study by Shao and Feldman (2001) found that applying nicotine to the pre-Bötzinger complex (the brainstem pattern generator area for both breathing and whisking) caused neurons to fire at higher frequencies with lower amplitude spikes. Furthermore, Casarrubea et al. (2015) found that lesioning the lateral habenula, a structure associated with negative motivational signals, reversed nicotine-induced anxiety and reductions in exploratory behaviour. Cholinergic projections have been found to enhance whisker responses in primary motor cortex (M1) (Berg et al., 2005) and primary somatosensory cortex (Oldford & Castro-Alamancos, 2003; Eggermann et al. 2014), especially during alert states. Indeed, Eggermann et al. (2014) suggest that nicotinic signalling during whisking contributes to active states in the Primary Somatosensory Cortex. That exploration behaviours are reduced in smoking mice, may indicate a lack of attention to their surroundings, and gives support to the suggestion that acetylcholine is involved in awareness, attention and alertness pathways (Bosman et al., 2011).

5. Conclusions

We quantified whisker movements in mice as a measure of exploratory behaviours following chronic smoking and cessation. We present here a quick, yet quantitative, method of

recording whisker movements, that does not require any animal training. We found that both protraction and retraction whisker velocities were significantly increased in smoking mice, and recovered following just one week of smoking cessation. As whisker velocities are linked with active sensing and object coding, we suggest that the smoking-induced increase in whisker velocity indicates a reduction in exploratory behaviour. The quick normalisation of smoking-induced changes in behaviour following smoking cessation may have implications for human health, as smoking-related anxiety behaviours may also recover in humans following cessation. As anxiety is strongly linked to the successfulness of smoking cessation (Pomerleau et al. 1978), an anxiety assessment conducted soon after smoking cessation may inform help to inform further smoking cessation plans.

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FIGURE CAPTIONS

Figure 1 Recording and tracking mouse behaviour. a) The experimental set-up. The high-speed video camera above the arena, which was illuminated from below by a light box. b) The tracked video footage showing head and whisker traces (left whiskers in red and right whisker in blue). c) Example of recording of whisker angles (nma: naïve mean angle) of the left (in red) and right (in blue) whisker fields.

Figure 2: Lung compliance in control, smoking and smoking cessation (one or two weeks) mice. a, is the mean plot for all animals with standard error bars, and b presents the data for individual mice. * indicates difference of control from all other treatments, at $p=0.001$.

Figure 3: Locomotion speed in control, smoking and smoking cessation (1 or two weeks) mice. a, is the mean plot for all animals with standard error bars, and b presents the data for individual mice. * indicates difference of smoking mice from all other treatments, at $p<0.001$.

Figure 4: Example of traces of whisker movement in smoking (a) and control (b) mice. The whiskers of the smoking mice move faster than those of the control mice. The blue trace shows the mean whisker movements on the right hand side, and the red trace corresponds to mean whisker movements of the left hand side.

Figure 5: Whisker velocities in control, smoking and smoking cessation (one or two weeks) mice. a and c and e show the mean plot for all animals with standard error bars, and b and d presents the data for individual mice. a,b: protraction velocity; c,d: retraction velocity. * indicates a difference in smokers from all other treatments, at $p<0.001$;

TABLE

Table 1. Measurements of whisker offset, amplitude and frequency in control, smoking and smoking cessation (one or two weeks) mice. Table shows the Mean±standard error data for the remaining whisker measurements where no significant effect of smoking treatment was observed. Velocity results can be seen plotted in Figure 5.

Whisker Variables	C	S	S1W	S2W
Offset	95.43±0.98	93.16±1.50	90.11±1.06	89.36±1.23
Amplitude	36.88±1.12	43.61±1.95	37.88±1.06	40.27±1.86
Frequency	13.37±0.72	11.32±0.74	11.38±0.66	12.42±0.78

Figure 1

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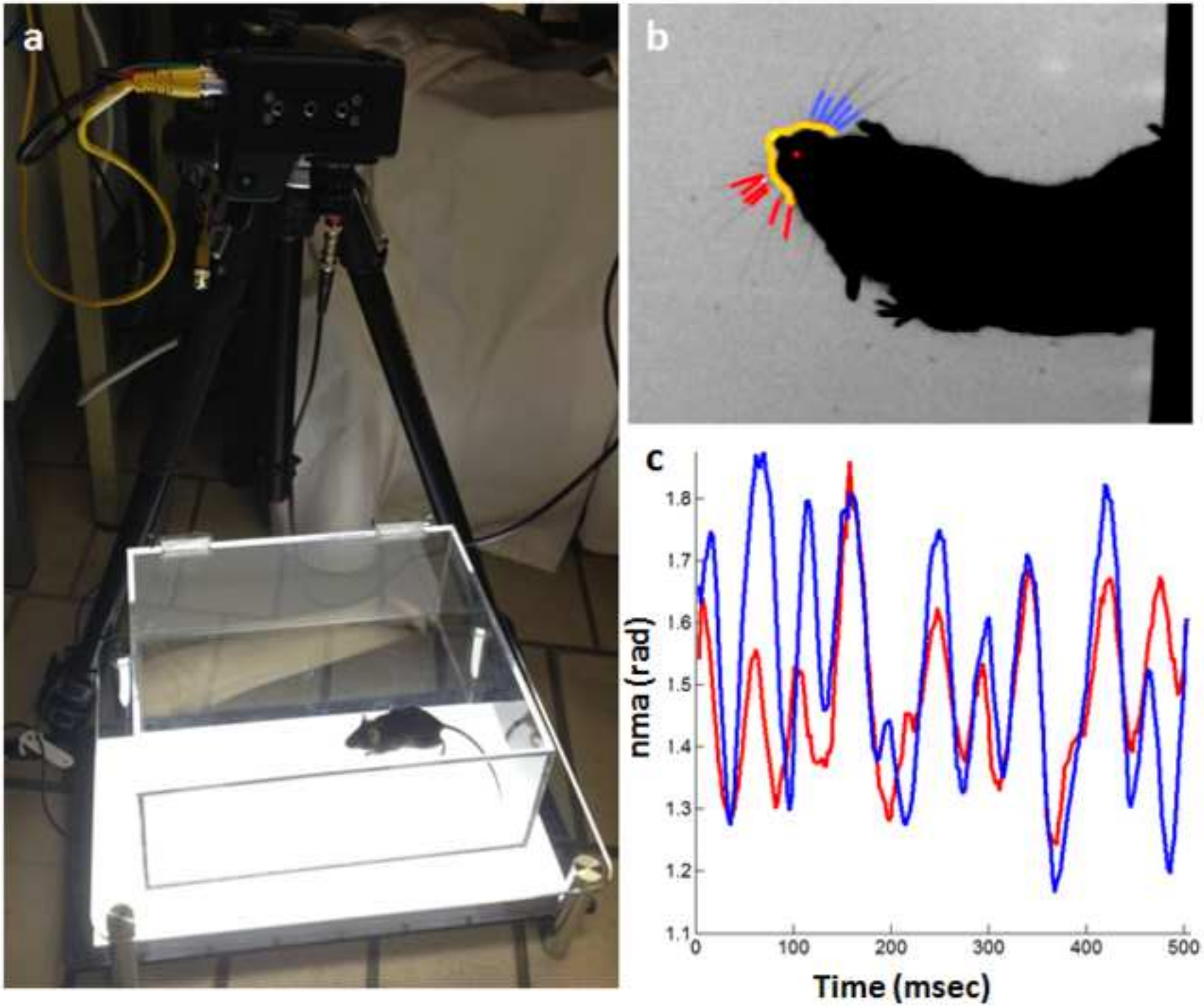

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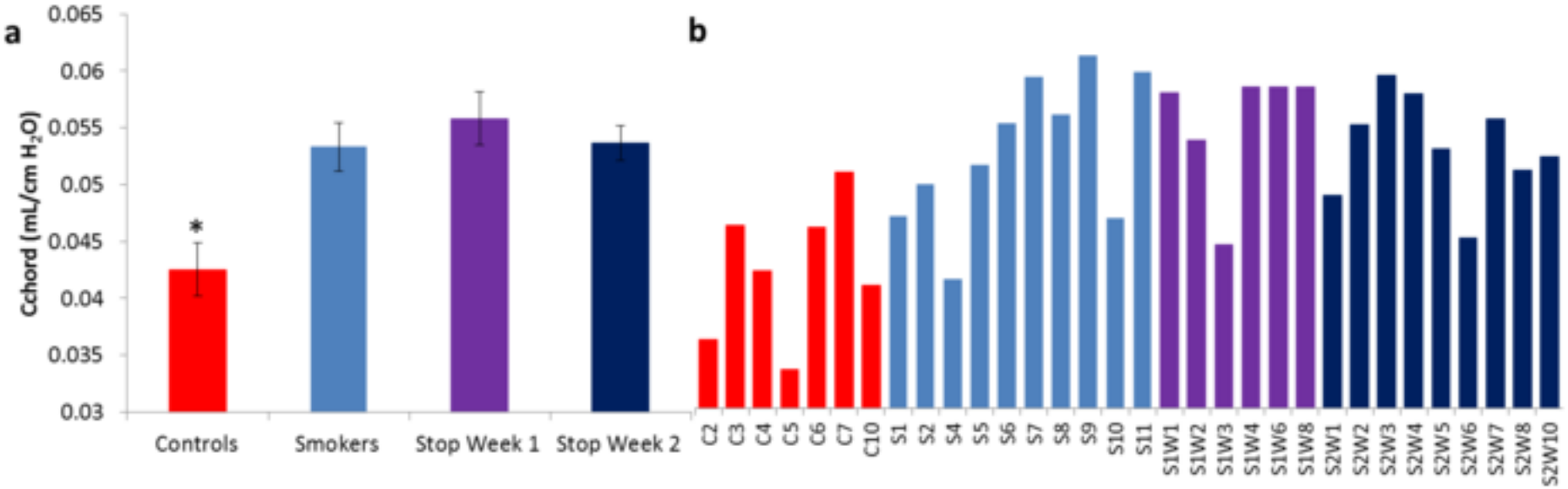

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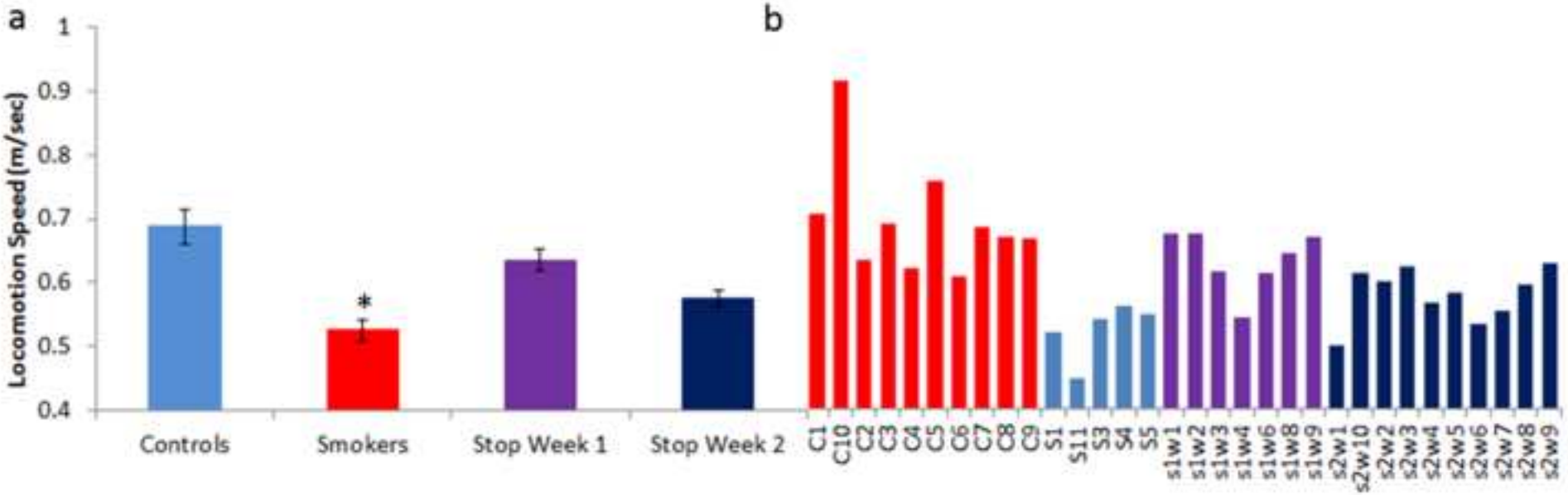

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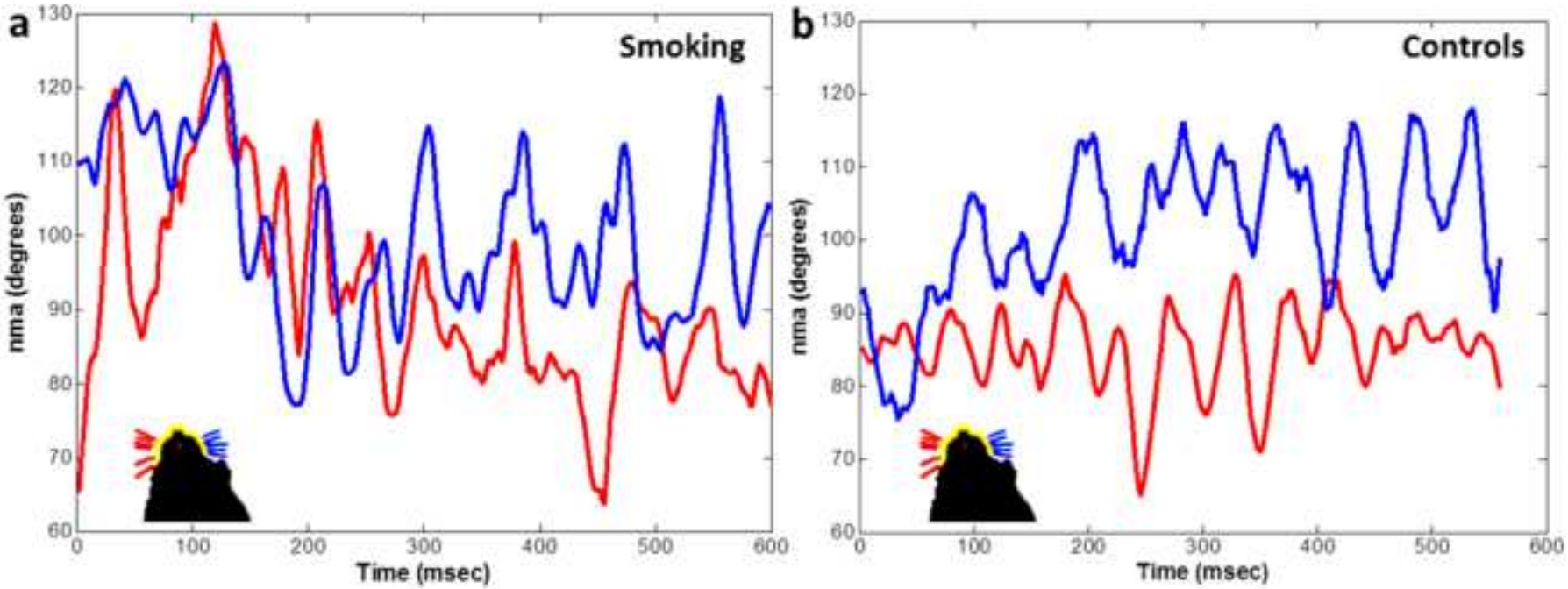

Figure 5

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