Universidade do Minho
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Social behavior and chronic pain conditions- The role of oxytocin

Abstract

Chronic pain (CP) is a debilitating condition affecting 20% of the adult population. Neuropathic pain is one of the most severe manifestations of CP being frequently associated with comorbidities such as sleep disturbances, depression, anxiety and cognitive deficits. In preclinical studies these manifestations have been explored aiming to understand better the pathophysiology of CP but also as potential proxies of pain. Social behavior has in this regard remained largely unexplored. The work in this thesis aims to study how chronic pain affects different dimensions of social behavior and the putative involvement of oxytocin (OXT).

Rats with chronic neuropathic pain, spared nerve injury model (SNI), were evaluated regarding social exploration behavior in two different paradigms, neutral arena (NA) and three chambers paradigm (3-CH) 1 month after pain onset. Ultrasonic vocalizations (USVs) were recorded in the targeted chambers of the 3-CH. After this test, blood was collected and OXT quantified by ELISA. Anxiety- and depressive-like behaviors were also evaluated in the elevated-plus maze and forced swimming test, respectively.

SNI animals, presented social exploration deficits in NA paradigm. In the 3-CH, both SHAM and SNI animals presented a clear preference toward exploration in the occupied chambers. Contrary to SNI animals, SHAM presented a tendency to visit SNI targets suggesting the manifestation of an empathy-like behavior. Indeed, SHAM presented not only higher systemic OXT concentrations, but these positively correlated with social exploration time. Regarding USVs, SNI and SHAM emitted in the range of 50KHZ; 14 clusters were isolated and in 2, SNI and SHAM differed significantly. Also, during encounters, the number of vocalizations increased significantly in both groups. No differences were found in any of the clusters but SNI-SNI, SHAM-SHAM, SNI-SHAM and SHAM-SNI (explorer-target) presented specific patters of cluster transition probability. No impairments in anxiety- or depressive-like behavior were observed.

In conclusion, this work demonstrates that chronic pain is associated with decreased sociability and with an OXT deficit. The absence of anxiety- and depressive-like manifestations guarantees the specificity of the effect. Data is in line with previous studies of the group employing different experimental conditions indicating that the phenotype reported is robust and may provide an interesting proxy of chronic pain in rodents.

Key words: Chronic pain, Neuromodulation, Neuropathies, Oxytocin, Social behavior

Comportamento social sob condições de dor crónica- O papel da Oxitocina

Resumo

A dor crónica é uma condição debilitante que afeta cerca de 20% da população mundial. A dor neuropática é uma das manifestações mais severas da dor crónica e está frequentemente associada a consequências como perturbações do sono, depressão, ansiedade e défices cognitivos. Estudos pré-clínicos têm vindo a explorar estas comorbidades com o objetivo de entender melhor a patofisiologia da dor crónica assim como o seu potencial para avaliar dor. O trabalho desta tese procura estudar a forma como a dor crónica afeta as diferentes dimensões do comportamento social a o envolvimento da oxitocina (OXT).

Animais com dor crónica neuropática (SNI), foram avaliados ao nível do comportamento de exploração social com dois paradigmas diferentes, arena neutra (NA) e o paradigma das três áreas, um mês após a instalação da dor. Vocalizações ultrassónicas (USVs) foram gravadas no paradigma 3-CH. Após este teste, recolheu-se sangue aos animais para medir OXT. O comportamento ansioso e depressivo, foram avaliados através dos paradigmas "elevated-plus maze" e "forced swimming test", respetivamente.

Animais SNI apresentaram défices de exploração social no paradigma NA. No 3-CH, todos os animais (controlos e SNI) apresentaram uma preferência clara pela exploração social em relação ao isolamento. Ao contrário dos SNI, os controlos, revelaram uma tendência para interagir mais com animais SNI, o que sugere a existência um mecanismo empático. De facto, os animais controlo apresentaram maiores níveis de OXT sistémica, que estão associados a um maior tempo de interação social. As USVs foram classificadas em 14 grupos nos quais, os animais SNI e controlos apresentaram diferenças significativas (em 2). Nos encontros sociais o número de USVs aumentou, no entanto, não foram encontradas quaisquer diferenças entre as condições sociais testadas. Ainda assim, os padrões de comunicação dos animais nas diferentes condições sociais testadas, revelaram-se diferentes. Por último, não foram observadas diferenças no comportamento ansioso e/ou depressivo.

Em conclusão, este trabalho demonstra que a dor crónica está associada a uma diminuição da sociabilidade assim como a um défice de OXT. A ausência de comportamentos ansioso e depressivo garante a especificidade do efeito. Ainda que, com diferentes condições experimentais, os resultados estão de acordo com estudos anteriores do grupo indicando que fenótipo reportado é robusto e poderá ser uma medida de dor crónica mais precisa em modelos animais.

Palavras chave: Comportamento social, Dor crónica, Modulação, Neuropatias, Oxitocina

Índex

List of Abbreviations

ACC: Anterior Cingulate Cortex

Amy: Amygdala

ASD: Autism Spectrum Disorders

AVP: Arginine Vasopressin

BC: Blood Collection

CIP: congenital insensitivity to pain

3CH: 3 Chambers paradigm

CNS: Central Nervous System

CP: Chronic Pain

DLF: Dorsolateral Funiculus

DRG: Dorsal Root Ganglia

DRt: Dorsal Reticular nucleus

EPM: Elevated Plus Maze

FST: Forced Swimming test

IASP: international association for the study of pain

IBS: Irritable Bowel Disease

IS: Insular Cortex

LDCV's: Large Dense Core Vesicles

LC: Locus coeruleus

magnOT: magnocellular oxytocinergic neurons

MC4R: Melanocortin 4 receptor

Nac: Nucleus Accumbens

NE: Neutral arena paradigm

NK1: Neurokinin1

NK1R: Neurokinin receptor

OXT: Oxytocin

OXTR: Oxytocin receptor

PAG: Periaqueductal Grey

parvOT: parvocellular oxytocinergic neurons

PFC: Prefrontal cortex

PSL: Partial Sciatic Nerve Ligation

rACC: rostral Anterior Cingulate Cortex

RVM: Rostral Ventral Medulla

SC: Spinal Cord

SEM: Standard Error of the Mean

SI: Somatosensory cortex

SII: Secondary somatosensory cortex

SNI: Spared Nerve Injury

SNL: Spinal Nerve Ligation

SP: Substance P

V1AR: Vasopressin receptor 1A

VF: Von Frey

Illustration índex

1. Introduction

1.1 Pain

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey & Bogduk, 1994) (see also Williams & Craig, 2016). Pain is a vital component of organisms' response to noxious stimuli, triggering a set of responses aiming primarily to avoid tissue damage or, in case of injury, to favour recovery (Burma, Leduc-Pessah, Fan, & Trang, 2017). Congenital insensitivity to pain (CIP), a condition characterized by the inability to process nociceptive stimuli, is an extreme example of its importance. CIP patients die at a young ages normally as consequence of repeated traumas and injuries (McMurray, 1950, 1975; Nagasako, Oaklander, & Dworkin, 2003; Thrush, 1973). While a fundamental part of the response to aggression, pain can also be a serious health problem if persistent (David Borsook, Andrew M Youssef, Laura Simons, Igor Elman & Eccleston, 2018; Heinricher, 2016; Johnson, 2019; Marchand, 2008; Woolf, 2010). Chronic pain (CP) serves no useful biological purpose, severely affecting the quality of life being frequently comorbid with emotional and cognitive deficits (N. N. Burke, Finn, McGuire, & Roche, 2017; Kwok & Trang, 2017; McWilliams, Cox, & Enns, 2003). Endogenous mechanisms of pain control both at central and peripheral levels are therefore essential for a strict control of pain.

1.1.1 The nociceptive stimulus and pain

Pain is a complex phenomenon involving multiple nodes of the peripheral and central nervous system (Marchand, 2008). A nociceptive stimulus, i.e. a noxious stimulus capable of depolarizing the primary afferent neurons (nociceptor), is conducted from the periphery to the spinal cord and then to the brain through ascending pathways, resulting in perceived pain experience (Bingel & Tracey, 2008). Classically, $A\delta$ and C fibers are considered to be involved in the conduction of nociceptive information. The former, are moderately myelinated fibers involved in fast and precise location perception of pain whereas the latter are unmyelinated fibers associated with slower, dull and diffuse sensation of pain (Dubin &

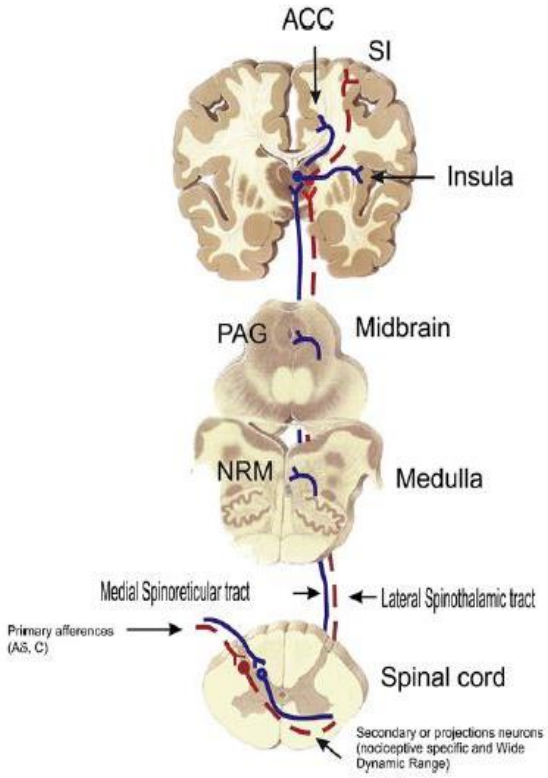


Figure 1: **Schematic representation of nociceptive pathways**. Affective-motivational (blue) and sensory-discriminative (red) ascending pathways (see also main text). *Adapted from Marchand 2008.*

Patapoutian, 2010; Marchand, 2008). However, there is evidence that the large myelinated fibers Aβ fibers can also be involved (Djouhri & Lawson, 2004).

Afferent fibers project to specific laminae of the spinal cord, C fibers to superficial laminae I and II, $A\delta$ to laminae I and V, and Aβ fibers to deeper laminae III, IV and V (Basbaum, Bautista, Scherrer, & Julius, 2009). Spinal cord projection neurons conduct the stimulus to the brain. Classically, two different ascending pathways are considered, the sensory-discriminative involving the lateral nuclei of the ventrobasal thalamus and somatosensory cortex and a affective-motivational involving the medial thalamus and limbic areas (Basbaum et al., 2009; Marchand, 2008; Willis, 1985; Willis, Kenshalo, & Leonard, 1979).

1.1.2 Pain modulation

Pain perception is sensitive to emotional, environmental and cognitive factors (Neugebauer, Galhardo, Maione, & Mackey, 2009; Ossipov et al., 2010; Seymour, 2019). For instance, Rossi and colleagues recently showed that music influences pain perception of healthy newborns (Rossi et al., 2018). Also, sleep deprivation affects pain in healthy subjects; according to the authors of a meta-analysis the effect size being comparable with those achieved through CP treatments (Schrimpf et al., 2015). Among other brain regions the periaqueductal grey (PAG)/rostral ventromedial medulla (RVM)/spinal cord axis plays a pivotal role in pain modulation (Bingel & Tracey, 2008). Different areas such as hypothalamus, amygdala (Amy), or rostral anterior cingulate cortex (rACC) project to the PAG which then projects to spinal cord via RVM. Indeed, Fields and colleagues showed that the RVM possess 2 populations of neurons that respond differently after a nociceptive input. ON-cells, are normally silent and activate right before the nociceptive reflex whereas OFF-cells present tonic activity which is suppressed upon the impending nociceptive stimuli and immediately before the motor response (Fields, Bry, Hentall, & Zorman, 1983). This and subsequent works demonstrated the bidirectional nociceptive control of RVM, i.e. inhibition and facilitation (Bingel & Tracey, 2008; De Felice & Ossipov, 2016) Other regions also participate in pain modulation like the Dorsal Reticular nucleus DRt (Lima, 2002) ,dorsolateral funiculus (DLF) (Ossipov et al., 2010) and locus coeruleus (LC) (Boadas-Vaello, Homs, Reina, Carrera, & Verdú, 2017) but only the RVM has simultaneously inhibitory and facilitatory valences. Alterations in descending modulatory mechanisms are thought to be relevant in context of CP (Kuner, 2010) as well as in analgesic's pain-relieving efficiency (Ossipov et al., 2010). One of the most clinically relevant phenomenon associated with alterations in descending modulatory circuits is the placebo analgesia. For instance, in an early study it was shown that patients expecting an analgesic drug after a molar removal, showed lower pain scores after a placebo injection (Levine, Gordon, & Fields, 1978) – see also for review (Schafer, Geuter, & Wager, 2018). On the other hand, nocebo effect is the expectation of worsening after a treatment. Multiple studies showed that patients expecting an outcome of pain responded to nonpainful stimuli (F. Benedetti, Lanotte, Lopiano, & Colloca, 2007; Fabrizio Benedetti et al., 2003; Colloca & Benedetti, 2007; Ossipov et al., 2010).

1.1.3 Pain characterization

Pain can be classified based on its etiology in nociceptive, inflammatory and neuropathic as well as in its duration in acute if resolving after the cessation of the stimulus or healing of the injured tissues or chronic

if prolonged (Treede et al., 2008; Woolf, 2010; Zilliox, 2017). In clinical settings, fixed time periods are used according to the pathology (e.g. 3 months for post-herpetic neuralgia; 6 months for chronic back pain). (Apkarian, Baliki, & Geha, 2009; Lavand'Homme, 2011; Marchand, 2008; Woolf, 2010).

1.1.3.1 Nociceptive pain

Nociceptive pain results from the direct activation of nociceptors by external nociceptive stimuli. In these conditions pain threshold is below tissue damage and therefore pain is considered protective. Upon cessation of the stimulus and in the absence of tissue damage, pain normally resolves after seconds or minutes (Marchand, 2008; Millan, 1999).

1.1.3.2 Inflammatory pain

Inflammatory pain is also a natural protective process that results from the infiltration of immune cells after tissue injury or infection. It promotes repair through the hypersensitization of the injured area which will discourage contact and/or movement. Typically, inflammatory pain resolves upon recovery though, in some circumstances it might perpetuate as in rheumatoid arthritis (Millan, 1999; Woolf, 2010).

1.1.3.3 Neuropathic pain

Neuropathic pain results from a lesion to the somatosensory system. The prevalence of this condition is 7%-10% of those with CP manifesting in conditions like diabetic neuropathy, trigeminal neuralgia, fibromyalgia, spinal cord injury or even multiple sclerosis. Neuropathic pain patients have either sporadic or continuous episodes of pain that appear in different forms such as burning, itching or shocking. It is considered chronic and debilitating condition that can last from months to years (Johnson, 2019; Marchand, 2008; Treede et al., 2008; Zilliox, 2017).

1.2 Chronic pain

While acute pain is a protective mechanism relevant for homeostasis, CP is pathological and has no biological relevance. CP affects 20 % of adult population regardless of sex, age, income, ethnicity or geography (Azevedo, Costa-Pereira, Mendonça, Dias, & Castro-Lopes, 2012; Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006; IASP, 2004; Sorge & Totsch, 2017). However, CP prevalence increases with age and it is more frequent in females and in those with strenuous work or less education (Azevedo et al., 2012; Breivik, Eisenberg, & O'Brien, 2013). In Portugal, CP prevalence is about 36.7% in adult population (Azevedo et al., 2012). Etiology of CP is diverse being frequently associated with cancer, osteo and rheumatoid arthritis, viral infections, metabolic diseases (e.g. diabetes), viral injections (e.g. herpes zoster), drugs (e.g. taxol and other cytostatic agents) and injuries particularly affecting in the nervous

system (Breivik et al., 2013; IASP, 2004). It is highly debilitating affecting patients' well-being and work capacity as well as of their relatives (Baron, Binder, & Wasner, 2010; Lavand'Homme, 2011; Marchand, 2008; Woolf, 2010). CP has also a substantial socioeconomic impact. Associated costs have been estimated to be up to 3-10% of gross domestic product (GDP) in Europe, and $635 billion in the USA (Azevedo et al., 2012; Breivik et al., 2013; Johnson, 2019). In Portugal the associated costs (in 2010) were estimated in 2.71% of the national GDP (Azevedo, Costa-Pereira, Mendonça, Dias, & Castro-Lopes, 2016). Such results from lower work productivity, absenteeism, medication, job loss or early retirement.

CP is frequently comorbid with other debilitating conditions such as mood disorders, anxiety, depression and even sleep disturbances (Bair, Robinson, Katon, & Kroenke, 2003; Breivik et al., 2013; Moriarty, McGuire, & Finn, 2011; Nicholson & Verma, 2004; Tsang et al., 2008; Wasan, Anderson, & Giddon, 2010). Indeed, CP patients are up to 3 to 5 times more likely to develop depression than heathy people, but the prevalence of depression increases accordingly to multiple factors namely pain severity, duration, frequency and number of symptoms (Bair et al., 2003). Meanwhile, depressed patients tend to have more pain than people without depression suggesting a bi-directional relation between the two pathologies (Bair et al., 2003; Croft et al., 1995; Gureje, Von Korff, Simon, & Gater, 1998; Leino & Magni, 1993; Wang et al., 1999). Anxiety is also a comorbidity of CP with significant prevalence in adults and in adolescent (Jastrowski Mano, 2017; Tsang et al., 2008).

Manifestations of anxiety-, depressive-like behavior (Bravo et al., 2012; Fukuhara et al., 2012; Gonçalves et al., 2008; Leite-Almeida, Pinto-Ribeiro, & Almeida, 2015; Narita et al., 2006; G. J. Norman et al., 2010; Tajerian et al., 2013) and cognitive deficits such as attention, learning, memory, information processing or executive function (Kodama, Ono, & Tanabe, 2011; D. M. Lee et al., 2010; Moriarty et al., 2011; Oosterman, Derksen, van Wijck, Veldhuijzen, & Kessels, 2011) have also been observed in preclinical studies particularly in rodents. However, regarding anxiety and depression, conflicting results have been described (Hasnie, Wallace, Hefner, Holmes, & Rice, 2007; Kontinen, Kauppila, Paananen, Pertovaara, & Kalso, 1999; Pitzer, La Porta, Treede, & Tappe-Theodor, 2019; Urban, Scherrer, Goulding, Tecott, & Basbaum, 2011). Such might be related with the differences in the experimental conditions. Indeed several studies in rodents have shown that pain duration (Yalcin et al., 2011), the age of the experimental subject (Leite-Almeida et al., 2009) and the location of pain (Leite-almeida, José, Wei, & Ribeiro-costa, 2012) critically influence the manifestation of anxiety- and depressive-like behaviors as well as impairments cognition.

The observation of behavioral alterations in CP models while reinforcing the face value of the rodent model in the context, also permitted additional readouts of the ongoing pathology. Indeed, the use of evoked

measures for pain assessment has been criticized for years as it does not reflect the ongoing nature of CP and many authors suggest that it might be one important factor limiting translation (see also section 1.5 below). However, as stated above, behavioral manifestations of anxiety- and depressive-like behaviors are conditioned by a number of biological and experimental factors that are not fully understood. Therefore, researchers have turned their attention to spontaneous behaviors [e.g. burrowing (Rutten et al., 2014) or facial expressions assessment (Langford et al., 2010)] that might offer an etiologically more relevant proxy of animals' well-being and therefore of CP impact.

In this line, social behavior while remaining relatively unexplored in the context of CP is advantageous in several aspects. First, both humans and rodents are social species; moreover hyposociability has been observed in both species in the context of CP (Mogil, 2015) (see also 1.3). Second, contrary to anxiety and depression it can be directly quantified in rodents. Third, social behavior manifests spontaneously but can also be stimulated in a variety of experimental contexts. Fourth, it can be accessed longitudinally which is a major advantage in comparison with classical paradigms of rodent anxiety and depression. Finally, the neurobiological substrates of social behavior are sufficiently explored to study pain-related impact on its physiology.

1.2 Social behavior and chronic pain

Social behavior is a fundamental part of rodents and human's behavior reportoire; however, it is much less studied in context of CP. Increasing evidence have been showing that social behavior modulates/is modulated by pain conditions both in humans and laboratory animals (Chen & Hong, 2018; Z. Li et al., 2014; Mogil, 2015; G. Norman, 2010; Smeester, Lee, & Beitz, 2017). Indeed, Brown and colleagues show that in the presence of either active or passive support given by a friend or a stranger, pain is less reported, in the cold pressure test, than in an isolation condition (Brown, Sheffield, Leary, & Robinson, 2003). In rodents, Fanselow showed that naïve rats exposed to odors from stressed rats displayed significantly less pain after injections formalin (Fanselow, 1985). On the contrary, it has also been described that odors, visual cues, empathy or social company can modulate pain expression (Martin, Tuttle, & Mogil, 2014; Mogil, 2015). For instance, Tuttle and colleagues described that long-lasting pain, namely from spared nerve injury model, SNI, in outbred mouse siblings, abolished the familiarity effect of propinquity on the tube co-occupancy test (TCOT) (Tuttle et al., 2017). In a subsequent study, they showed that relative dominance status was reversed if neuropathic pain was installed in the dominant mice (Tansley et al., 2019). Moreover, Kavaliers and Choleris showed that naïve animals that had

witnessed an attack by biting flies to other mice, displayed analgesia and self-burying behavior 24h later, when they were exposed to harmless flies (Kavaliers, Choleris, & Colwell, 2001). Visual cues play a role in this social contagion since it has been described that placement of opaque physical barriers between the mice blocks the phenomenon (Langford et al., 2006), see (Martin et al., 2014) for extensive review.

There is a need for a better assessment of emotional dimension in pain throughout evaluation of spontaneous behaviors like burrowing or even the facial expressions assessment. Therefore, the role of social behavior under ongoing pain is an important but unexplored dimension that could be used as an emotional measure in CP conditions. Oxytocin (OXT) has been described as possible mediator of the pain-social relation (Agren, Uvnäs-Moberg, & Lundeberg, 1997; Robinson et al., 2002). In these perspectives, it is worthy to explore the role of OXT in the interplay between social behavior and CP.

1.4 Oxytocin

1.4.1 General

Oxytocin (OXT) is a highly conserved molecule across species, composed by 9 amino acids organized in a cyclic sequence and with neuromodulatory properties (Marlin & Froemke, 2017). It was described for the first time by Henry Dale in the context of its uterine-contracting properties (Dale, 1906) and nowadays is broadly used in the clinics in childbirth or in autism spectrum disorders (ASD) treatments (Feldman, Monakhov, Pratt, & Ebstein, 2016) This neuropeptide is produced manly at the paraventricular nucleus (PVN) but also at the supraoptic nuclei (SON) of the hypothalamus. Arginine vasopressin (AVP) is also produced in these areas especially in the PVN. In fact, some authors report that the production of these two molecules is cross balanced, meaning that high circulating OXT is associated with low circulating AVP and vice versa (Gimpl, Fahrenholz, Physiol, & Metab, 2009; Mitre, Minder, Morina, Chao, & Froemke, 2018; Stoop, 2012). OXT producing neurons, magnocellular (magnOT) and parvocellular (parvOT) neurons, are structurally different and project to specific areas within the brain but also to the periphery. Specifically, magnOT neurons are larger projecting directly to the periphery through the posterior lobe of the pituitary gland. Besides their role in periphery projections, magnOT neurons also project directly to forebrain regions such as nucleus accumbens (Nac) and central amygdala (Amy). ParvOT neurons are smaller and project mainly to the brainstem (Johnson & Young, 2017; Meyer-Lindenberg, Domes, Kirsch, & Heinrichs, 2011)

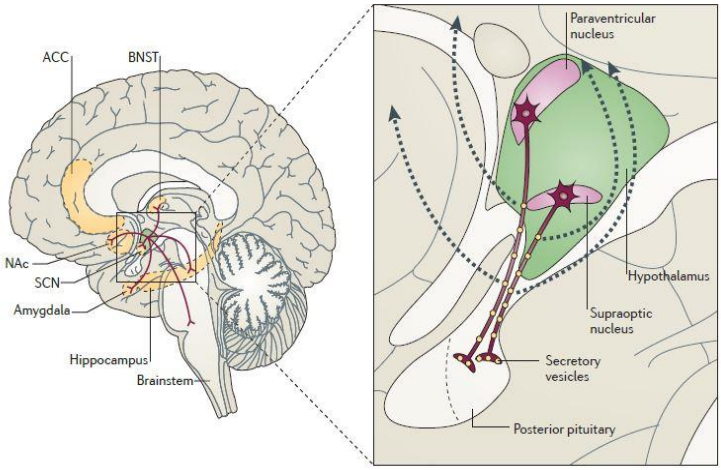


Figure 2: Illustration of OXT neurophysiology. OXT is produced by magnocellular and parvocellular neurons at paraventricular nucleus and supraoptic nuclei of hypothalamus. It is processed across their axons and then secreted to multiple brain regions for which theses neurons have projections. While magnocellular neurons project directly to forebrain regions like amygdala, parvocellular neurons project at longer distance such as the brainstem. Adapted from Lindenberg and colleagues 2011

Upon production OXT is stored in large dense core vesicles (LDCV's) distributed throughout axons, soma and dendrites of magnOT and in less extent in the parvOT neurons. OXT is released by magnOT neurons throughout their axons' terminals triggering an action potential with the increase of intracellular calcium levels (Ca^{2+}) (Johnson & Young, 2017). While magnOT neurons seems to have multiple axons ramifications thus projecting to multiple areas, parvOT neurons project to more distant areas in the brain and they are also responsible for the extracellular fluid release around producing zones. Axonal release in magnOT neurons happens in an *en passant* regime whereas somatodendritic release happens by increase in intracellular Ca^{2+} levels where melacortin 4 receptor (MC4R) and CD38 act and are considered key elements for neuronal modulation namely at behavioral level (Chu, Sun, Xu, Niu, & Xu, 2012; Higashida, Yokoyama, Kikuchi, & Munesue, 2012; Jin et al., 2007; Z. X. Li, Liu, He, & Xiang, 2017; Lopatina, Inzhutova, Salmina, & Higashida, 2012; Mastinu et al., 2018; Young, 2007). However, OXT release can also happen in the absence of afferent inputs since theses neurons (magnOT) express OXT receptors that are able to control OXT release without interfering with intracellular Ca^{2+} calcium levels (Baribeau & Anagnostou, 2015; Thomas R. Insel, 2010; Johnson & Young, 2017; Ludwig & Leng, 2006; Meyer-Lindenberg et al., 2011; Stoop, 2014). Both axonal, somatodendritic and extracellular fluid release of OXT by magnOT and parvOT neurons are known to be equally relevant for behavioral modulation. While axonal release acts on specific brain regions triggering faster modulations, extracellular fluid release is responsible for slower and transient dimensions of behavioral modulation. Glutamatergic networks couple magnOT neurons activity synchronizing OXT release into the periphery (Johnson & Young, 2017). Eliava and colleagues described that parvOT and magnOT neurons also communicate, the former controlling

the latter, i.e. a small population of parvOT in addition to the release of OXT, it also stimulates magnOT neurons release into the periphery leading to nociception suppression (Eliava et al., 2016). Also, central and peripheric OXT release are to some extend independent systems – see for review (Ludwig & Leng, 2006). In fact, OXT concentration in extracellular fluid at SON is 100-1000-fold higher than in the blood. Moreover, in response to sucking in lactating rats, hypothalamic OXT levels are higher before any detectable arise in the blood stream (Moos et al., 1989). Thus, dendritic and axonal release of this peptide, i.e. central and peripheral, respectively, can be independently regulated (Baribeau & Anagnostou, 2015; Johnson & Young, 2017; Landgraf & Neumann, 2004; Ludwig & Leng, 2006).

Besides the specific and well-known role of OXT at the peripheric level, the modulation at the central nervous system is also extremely relevant but not so well understood at the behavior level. It is implicated in many behavior modulations such as maternal behavior, fear memory or even social recognition (Chini, Verhage, & Grinevich, 2017; Ferguson, Young, & Insel, 2002; Stoop, 2014). As so, its possible role across other behavioral dimensions is worthy to explore.

1.4.2 Oxytocin and social behavior

In 1992, Insel and Shapiro reported that prairie voles a typically monogamous specie presents significant differences in OXT receptor density and distribution in the brain when compared to montane vole a typically polygamic specie of voles (T R Insel & Shapiro, 1992). Thus, revealing an important role in social bonding and affiliative behavior. Moreover, Roger-Carter and colleagues showed OXT receptor mediation of social affective stimuli. In a social affective paradigm, experimental animals approached stressed ones depending upon their age, but after the blockage of insular OXT receptors these social affective behaviors were disrupted (Rogers-Carter et al., 2018). In the last decades, much has been explored in the OXT field and its implications in behavioral modulation – see for instance for review (Chini et al., 2017; Stoop, 2014). Indeed, dysfunction in the so called “love hormone” has been being directly linked with autism (LoParo & Waldman, 2015; Yamasue & Domes, 2017; Young & Barrett, 2015) and schizophrenia (Veras et al., 2018) specifically at the OXT receptor polymorphisms level. Social hierarchies in rodent models (W. Lee et al., 2019), maternal behavior (Marlin, Mitre, D’Amour, Chao, & Froemke, 2015; Toepfer et al., 2017), aggressiveness (de Jong & Neumann, 2017), social recognition (Ferguson et al., 2002; Oettl et al., 2016), fear memory (Knobloch et al., 2012) or even social bonding (T R Insel & Shapiro, 1992) have also been increasingly associated with OXT. For instance, Lee and colleagues showed that socially dominant males have higher OXTR binding and lower V1AR binding in socially relevant brain regions (W.

Lee et al., 2019). Moreover, Marlin and colleagues described that OXT enables pup retrieval in female mice by auditory cortical pup call responses (Marlin et al., 2015).

Many other factors like early-life stress (ELS) or past experiences can modify our social behavior and it seems that OXT is also involved at these levels in female offspring rats, Champagne and colleagues showed that low levels of maternal care are associated with lower OXTR expression in the PVN, Amy and also in the lateral septum (Champagne, Diorio, Sharma, & Meaney, 2001; Toepfer et al., 2017). This neuropeptide has not only a direct involvement on neuromodulation but also an interference with other processes that latter on will lead to social deficits. In fact, it was described that ELS such as neglect care or child abuse, could compromise OXT system at DNA methylation level leading to higher susceptibility of embedded alterations that will therefore change OXT signaling causing intergenerational transmission (Toepfer et al., 2017).

In addition to the mentioned behavioral dimensions, there are many others that also showed significant impairments in the OXT system. For instance, social reward evokes activity from Nac OXT (Mitre et al., 2018) as well as OXT signaling pathway at the amygdala is required for sex-specific cues in males but not in females (Yao, Bergan, Lanjuin, & Dulac, 2017). Indeed, sex has been reported as a regulatory factor in processes modulated by OXT (Borland, Rilling, Frantz, & Albers, 2018). In clinical research, there are already multiple studies using OXT intranasal treatments that have been showing promising results in autism spectrum disorders (ASD) (Yamasue & Domes, 2017). Additionally, the literature states that OXT is also implicated in pain (Freund-Mercier & Uhl-Bronner, 2007; Tracy, Georgiou-Karistianis, Gibson, & Giummarra, 2015).

1.4.3 Oxytocin in chronic pain

It has been described that nociception and pain modulation are affected by OXT levels. When administered systemically, OXT displays a potent analgesic effect. Such has been demonstrated in multiple studies – see for instance (Condés-Lara, Maie, & Dickenson, 2005; Juif & Poisbeau, 2013; Miranda-Cardenas et al., 2006; Nishimura et al., 2019); see also for review (Rash, Aguirre-Camacho, & Campbell, 2013). Also, OXT receptors are expressed in relevant pain processing areas such as PAG, superficial layers of the spinal cord or even medial amygdala (Poisbeau, Grinevich, & Charlet, 2017). However, Petcu and colleagues also showed that in knock-out mice for OXTR, OXT-mediated analgesia was also observed but was abolished in AVP- 1A (V1AR) receptor null mutant mice suggesting a complex

interplay between the two systems (Schorscher-Petcu et al., 2010). At the periphery, OXTR have also been described to contribute for OXT mediated analgesia – see for review (Poisbeau et al., 2017).

Oxy involvement in the nociceptive process has also been described by Eliava and colleagues. They demonstrate that parvOT neurons exert an inhibition of nociceptive processing at the SC throughout the activation of OXTR and NK1 receptor (NK1R), present in the same SC neurons, and a slower action on magnOT neurons of the SON leading to OXT release into to the blood. They also described a strong analgesia given by parvOT neurons on inflammatory pathological condition buts not in neuropathic pain (Eliava et al., 2016). More recently, Nishimura and colleagues reported that neuropathic pain induced by partial sciatic nerve ligation (PSL) increases OXT production at the hypothalamic region as well as OXT conduction to the pituitary tissue (Nishimura et al., 2019).

1.5 Rational and aims

In the past decades, successful advances have been taken in pain research regarding anatomical pathways, mechanisms of chronification and possible therapeutically targets for pain management. However, despite the efforts, treatments for CP are still very limited and many authors contest the reliability of animal models (Blackburn-Munro, 2004; Bowen & Casadevall, 2015; Klinck et al., 2017; Mogil, Davis, & Derbyshire, 2010). A paradigmatic example is Substance P (SP) and NK1R that were described to be highly distributed in SC layers and having an important role on nociception in animal models (Zubrzycka & Janecka, 2000). It was reported that thermal hyperalgesia and mechanical allodynia was greatly diminished in NK1 ablated neuropathic rats (Nichols, 1999). However, in clinical trials no analgesic effect of NK1 antagonists was found (Hökfelt, Pernow, & Wahren, 2001). Reasons for such are not entirely clear. Experimental conditions play certainly a role. Indeed, typically young, male rodent animals are used in most experiments whereas most frequent clinical pain patients are women of middle age (Klinck et al., 2017). Addition of factorial designs introducing genotype, sex or age can favor better approaches to improve translation (Blackburn-Munro, 2004; Hay, Thomas, Craighead, Economides, & Rosenthal, 2014; Klinck et al., 2017; Le Bars, Gozariu, & Cadden, 2001; Mao, 2009; Negus et al., 2006; Whittaker & Howarth, 2014). Pain assessment itself is a problem in rodents. CP induces emotional and cognitive deficits both in humans and in rodents (Andrews et al., 2012; Bair et al., 2003; Breivik et al., 2013; Moriarty et al., 2011) that might be used as a proxy for CP. Much less explored, social behavior might offer an etiologically relevant measure of pain-related effect. As so, the main goal of this work is to give insights about the interplay between CP and social behavior as well as the involvement of the

oxytocinergic system. The project comprises an extensive characterization of animals' social behavior and interspecific communication in CP as well as associated alterations in the oxytocinergic system. Previous unpublished results from the lab showed that after chronic neuropathic pain induction, rats present social deficits in the neutral arena paradigm. This phenotype was reverted after the treatment with analgesic but more importantly after an acute injection of OXT. More specifically, this work intends to:

1. Evaluate the impact of CP on different paradigms of social behavior: NA and 3-CH;
2. Evaluate the impact of CP on classical paradigms of anxiety and depression;
3. Monitor OXT levels in CP and control subjects;
4. Analysis of ultrasonic vocalization in CP and control conditions both in social and isolation settings;

2. Materials and Methods

2.1 Study-design

To study the impact of neuropathic CP on social behavior, animals were induced with the spared nerve injury (SNI) model of neuropathic pain (see 2.3) and performed a battery of behavioral tests,30 days after, (Figure 3). EPM and FST test were performed before the NA and 3-CH to decrease their influence on the molecular analysis. Mechanical allodynia was measured with Von Frey monofilaments every week during the experience and blood collection was performed in 3 different time points (Figure 3). All the behavioral assessment was done during the night period of the cycle (8:30pm- 3am).

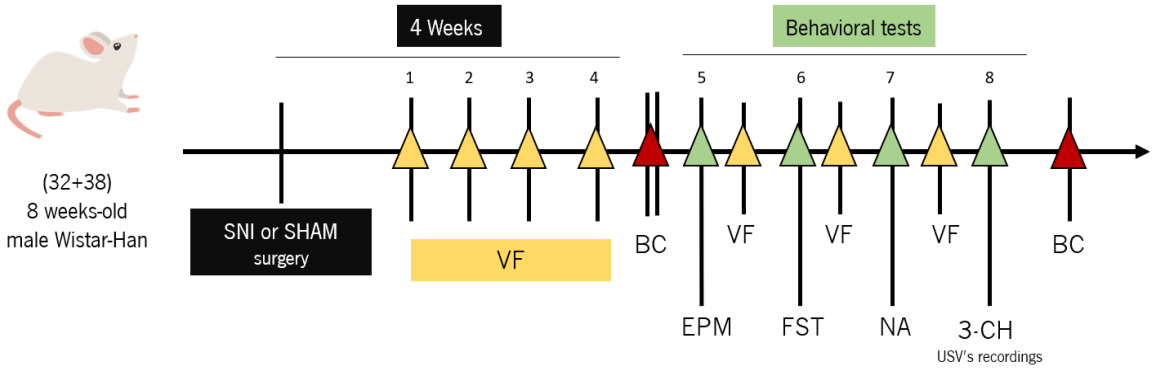


Figure 3: Study-design. Behavioral assessment started 30 days after SNI. VF monofilaments were used to measured nociception every week during the experiment. BC was performed in three time-points: 1. 8am before behavioral assessment; 2. 8pm before behavioral assessment; and 3. Immediately after the last behavioral test. 3- CH: 3-Chamber paradigm; BC: Blood collection; EPM: Elevated plus maze; FST: Forced swimming test; NA: Neutral arena; SNI: Spared nerve injury; VF: Von Frey.

2.2 Animals

Seventy male Wistar-Han rats (Charles River, France) with 8 weeks of age at the beginning of the experiment were used. Of those, 32 were evaluated and 38 (10 SNI, 10 SHAM and 18 Naïve) were used as elements on the behavioral assessment. Animals were housed in pairs in a 12h light/dark cycle (lights on at 8 a.m.) with controlled temperature (21±1°C) and humidity (50%-60%). Water and standard diet (4RF21; Mucedola SRL, Settimo Milanese, Italy) were available *ad libitum*. Handling was performed every day for one week before the surgery and at least once a week thereafter. Animal's weight was monitored weekly during the experience. Of the 70 rats, 32 were evaluated in the protocol described in figure 3 and the remaining 38 were used as targets in the 3-chamber (3-CH) experimental setting (see below). They were housed in pairs of the same group. All the experimental protocols were performed in accordance with the guidelines of the European Communities Council directive 2010/63/EU and efforts were made to minimize animals' discomfort.

2.3 Spared Nerve Injury (SNI)

For the induction of neuropathic pain, the SNI model was used, as described previously (Decosterd & Woolf, 2000). Animals were anesthetized with an intraperitoneal injection of a 1:1.5 mix of Sededorm® (VetPharma Animal Health, Spain) (Medetomidine Hydrochloride) and Ketamidor® (Richter Pharma AG, Austria) (Ketamine) (1mg/kg). After shaving the right posterior paw, a longitudinal incision was made, the muscle and the sciatic nerve were then exposed. The peroneal and the tibial branches were ligated with a 4-0 non-absorbable suture (Silk Suture Thread, FST, Germany) and a distal axotomy was performed. Care was taken to avoid damage to the sural nerve that was spared. The same procedure was applied to the SHAM animals, except that none of the nerves was lesioned. In the end, muscle and skin were sutured separately and subcutaneous injection of Antisedan® (Atipamezole Hydrochloride, ORION Corporation, Finland) was given to each animal to revert the anesthesia effect. Then, animals were left in their home cages to recover. Animals were monitored in the following days to check for open wounds and signs of infection.

2.4 Nociception assessment

Assessment of mechanical allodynia was done by the up-and-down method with Von Frey monofilaments (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). The habituation to the test was performed on the day before the first assessment. Animals were left for 5 min in the experimental setting, which consists of an elevated mesh wire that allows complete access to the paws and where the animals are confined to a plastic box (22x13.5 cm). In the test, the right posterior paw of each animal was touched with a series of 8 Von Frey monofilaments being the minimum 0.4g and moving progressively to 0.6g, 1g, 2g, 4g, 6g, 8g, 10g and 15g (North Coast Medical Inc., USA). The filaments are presented perpendicularly to the lateral part of the injured paw (sural dermatome) for 6-8 seconds. The test started with the central monofilament (2g) and the test advanced upward if no response was produced or downward if a withdraw of the limb was seen until 6 measurements were obtained around the threshold point. Stimuli were presented in intervals of several seconds allowing the resolution of behavioral responses to previous stimuli. The 50% threshold of each animals was then calculated with the following formula:

$$50\%\ \text{threshold} = \frac{10^{(xf+k\delta)}}{10000}$$

where xf = logarithmic value of the last filament used, k = tabular value for the pattern of responses and δ= mean difference between stimuli (0.224) (Chaplan et al., 1994).

2.5 Behavioral assessment

2.5.1 Elevated Plus Maze

The elevate plus maze (EPM) paradigm was used to assess anxiety-like behavior (Pellow, Chopin, File, & Briley, 1985). The apparatus (ENV-560; Med Associates, USA) consists of two illuminated open arms crossed with two closed arms (50.8x10.2 cm) elevated 72.4 cm from the floor. Each animal started the test in the central area (10x10 cm) explored the maze for 5 minutes. The experimental setting was cleaned with alcohol 10% between trials. Each trial was recorded with a video camera for posterior analysis using the video tracking software Ethovision XT (Noldus, Netherlands). The ratio between the time spent in the open arms and closed arms was used as a readout of anxiety-like behavior.

2.5.2 Forced Swimming Test

The forced swimming test (FST) was used to assess learned helplessness, a manifestation of depressive behavior (Porsolt,1977). The test is based on the effort of the animals to swim in a situation that they cannot escape. Habituation consisted in a 5-minute session in which the animals were introduced in a circular recipient (65x27 cm) with water (22-24°C), from which they cannot escape. The same procedure was repeated on the following day, but the session was recorded in video and immobility time was measured. Data was analyzed using the video tracking software Ethovision XT (Noldus, Netherlands).

2.5.3 Neutral Arena

To evaluate social interaction the neutral arena (NA) was used. A square black box (47x47x40 cm) in which animals had direct contact with each other was used. Three individual habituation sessions of 5 minutes each, were performed in 3 consecutive days. For the test, 2 animals were positioned face to face in different dyads: SHAM-SHAM, SNI-SNI and SHAM-SNI animal for 15 minutes. Nose to nose, nose to genitals or nose to body interactions were quantified (Giancardo et al., 2013). All sessions were video recorded, and data was analyzed manually with the Observador v0.2.7® software.

2.5.4 Three-Chamber paradigm

To evaluate social preference, a modified version of the 3-chamber (3-CH) paradigm was used (Nadler et al., 2004; Yang, Silverman, & Crawley, 2011). In this case, assessed animals (explorers) had the choice to interact with 2 animals, either SHAM or SNI (targets), both unfamiliar. As in the original 3-CH, the experimental setting consisted of a transparent plastic arena (150x50 cm) symmetrically divided in 3 different areas (50x50cm). Within the side areas there was a smaller round and closed area (8 cm in diameter) covered with mesh grid to allow the contact between the explorer and target animal (Figure 4A). Habituation was done in 3 consecutive days with 10-minutes sessions. In the first habituation session target animals were allowed to explore the entire arena. In the second session, these same animals were placed in the closed areas. Finally, in the third day of habituation, the explorer animals were able to explore the arena (closed areas were empty). On the first day of test the explorer animals were assessed regarding their social preference. For that, explorers were positioned in the center area and for 10 minutes they were able to explore SHAM or SNI target animals. Preference index was calculated by the following formula in which *intSHAM* and *intSNI* represents the interaction time of the explorer animals with SHAM and with SNI animal, respectively.

$$Preference\ index = \frac{intSHAM - intSNI}{intSHAM + intSNI}$$

In the second day of test, the assessed animals were able to choose between interaction with a naïve unfamiliar animal or no animal (no interaction). To eliminate bias from position or from retest, the side of the target animals was balanced between sessions and between groups. All the sessions were recorded by video for further analysis. Moreover, it is important to notice that target animals were used two or three times in the test to cover all the explorers. Data was extracted from manual analysis of the videos with the use of the Observador v 0.2.7® software

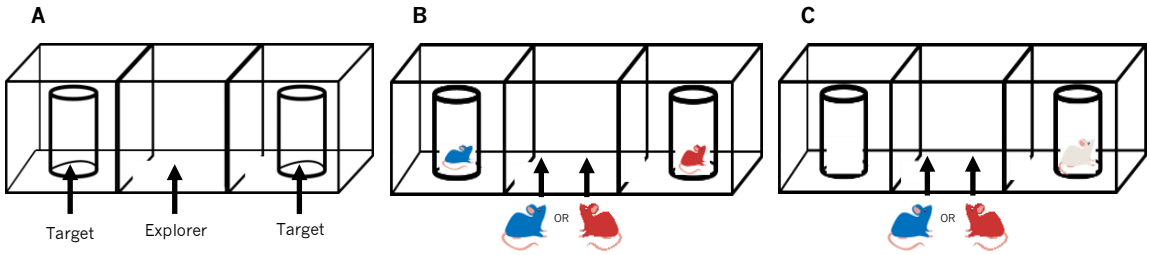


Figure 4: Schematic representation of experimental setting. Animals that were closed in the smaller area each side area were called target animals and the ones that were assessed were called explorers (a); First day of test in which social preference was assessed. SHAM (collared in blue) and SNI (collared in red) animals both as targets and explorer animals (b); Second day of test in which naïve animals were used as targets (colared in white) and both SHAM (colared in blue) and SNI (colared in red) were used as explorers (c).

2.6 Ultrasonic vocalizations recordings

Ultrasonic vocalizations (USVs) were recorded during day 1 of the 3-CH assessment (see 2.5.4). 2 microphones (CM16 Ultrasound Gate, Avisoft Bioacoustics, Germany) were placed on the left and right-side arenas of the 3-CH. Recordings' data was latter synchronized with the video images by the Ethovision XT software (Noldus, Netherlands) and processed on Matlab R2018b software. For the processing of the USVs a deep learning-based software (toolbox of the Matlab) called Deep Squeak (Coffey, Marx, & Neumaier, 2019) was used. The number of calls, duration and frequency were calculated and grouped in clusters. Number of vocalizations in each cluster and transition probability between clusters, which reflects the articulation of calls from different clusters, was also calculated. As referred above (see section 2.5.4) target animals were used more than once during the test to cover all the explorers. As so, each file from isolated animals were not a truly independent animal. Two or three of those files were from the same animal recorded in different times. However, the same happened with social encounters files, since each dyad of animals was composed by one explorer and one target animal.

2.7 Blood Collection

As mentioned before (see 2.1) blood was collected in 3 different times points: two days before the behavior start (8am), on the day before the behavior (8pm) and immediately after the last behavioral paradigm. This were only performed on the explorer animals. For blood collection, a little incision on the tail vein of the animals was performed around 100µl of blood were collect to a microtube. The samples

were then centrifuge at 13000 rpm for 15 min and the supernatant was collected to a new microtube and kept at -80°C until use.

2.8 Euthanasia

All the explorer animals were sacrificed 90 minutes after their last session of behavior. For that, they were deeply anesthetized with an intraperitoneal injection of Eutasil® (Pentobarbital, CEVA SANTE ANIMALE, France) at 100mg/kg and perfused with saline (NaCl 0.9%). Half of the animals were also perfused with Paraformaldehyde (PFA) 4% to fix the tissues. The brains were then removed and stored at 4°C in a PFA 4% solution for 72 hours and then changed for a 30% Sucrose solution. After decapitation the skull of the non-fixed animals was emerged on liquid nitrogen and then the brain was removed and Nac, Amy, prefrontal cortex (PFC) and pituitary tissue were macrodissected on cold conditions. The samples were stored first on dry ice and then at -80°C, except pituitary tissue that was preserved in a phosphate buffer 0.1 M + 0.1% pH=7.2 glutaraldehyde solution and stored at 4°C. for future analysis.

2.9 Oxytocin measurements

Systemic OXT levels were measured with an ELISA Kit (CSB-E14197R, CUSABIO, USA). In this work only the samples taken right after the behavioral assessment were analyzed. All the samples were analyzed in duplicates and samples dilution (1:2) was based on the standard curve previously tested. 50 µl of sample and 50 µl of HRP-conjugated were added to each well. After mixing, the ELISA plate was incubated for 1hour at 37°C, in the end of which 50µl of each substrate were added, followed by a new incubation at 37°C for 15 min. All the samples were protected from the light during the procedure. The reactions were then stopped, and optical density was measured at 450nm (Varioskan Flash, Thermo Fisher).

2.10 Statistical analysis

JASP Team (2018) JASP v0.10.0.0 software was used for the statistical analysis and GraphPad Prism v7.04 (GraphPad software, Inc., USA) was used for the graph's elaboration. Normality was assumed for all the statistical analysis. Weight and mechanical allodynia evolution were compared with repeated-measures ANOVA while unpaired t-tests were used to compare SNI vs SHAM in the EPM, FST and

systemic OXT measures. Paired t-test was also used in this context specifically when the same subjects are in the two groups as in the 3 Chambers paradigm. Moreover, when comparing between 3 or more groups one-way or two-way ANOVAs were used, if there were one or two intergroup comparisons, respectively. Correlation analysis were done by Pearson's test. Sphericity was tested with Mauchly's test and corrected with Greenhouse-Geiser. For the post hoc analysis Tukey test was used. Data were considered significant if $p<0.05$. All results are represented as mean ± SEM.

3. Results

The present work intended to thoroughly characterize social behavior of rodents under chronic neuropathic pain conditions. For that, male Wistar-Han rats were induced with chronic neuropathic pain model, SNI, and a battery of behavioral paradigms was performed 4 weeks after the surgery (Figure 3).

3.1 Animals Weight

Animals' weight was controlled weekly along the experiment. All animals had a regular evolution regarding their weight and no statistically significant differences were registered between groups ($F(1,49) = 1.128$, $p= 0.293$, $\eta^2= 0.023$) (Figure 5).

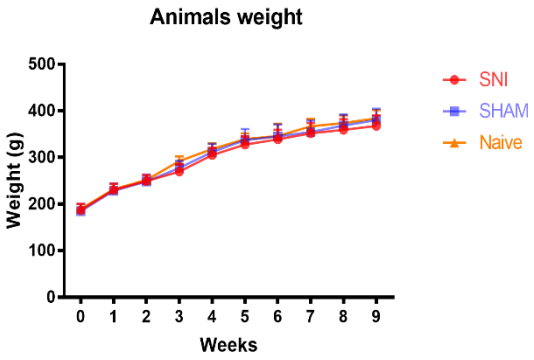


Figure 5: Animals well-being. Weight measurements of all the animals throughout the experiment. No differences were found between groups, not even after the surgery (week 1). All of them presented a regular evolution regarding their weight. Data presented as mean ± SEM, ***p< 0.001, n=70.

3.2 Mechanical Allodynia assessment

Starting one week after the SNI surgery, mechanical allodynia was measured weekly using VF monofilaments. As expected, SNI animals presented a significant decrease in their 50% thresholds when compared to SHAM animals (F (1,49) = 536.194, p<0.001, η^2= 0.916) (Figure 6).

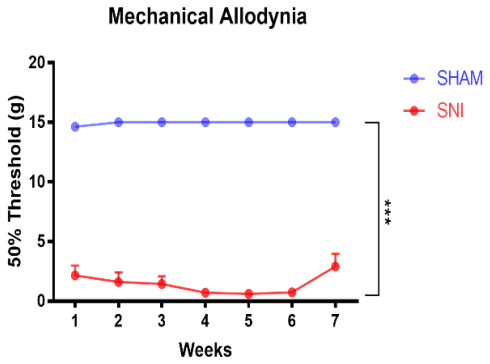


Figure 6: Nociceptive assessment. Mechanical allodynia was measured weekly, starting one week after the SNI surgery. All SNI induced animals presented a significant decreased in their thresholds, revealing a hyper sensibility state, in comparison with SHAM controls. Data presented as mean ± SEM, ***p< 0.001, n=52.

3.3 Anxiety-like behavior

EPM was used to evaluate anxiety-like behavior. As seen in Figure 7, SNI did not induce anxious-like behavior, as SNI and SHAM animals do not present any statistically significant difference regarding the ratio between the time spent in the open and in the close arms (T (29)= -1.112, p= 0.275, *d*= -0.400).

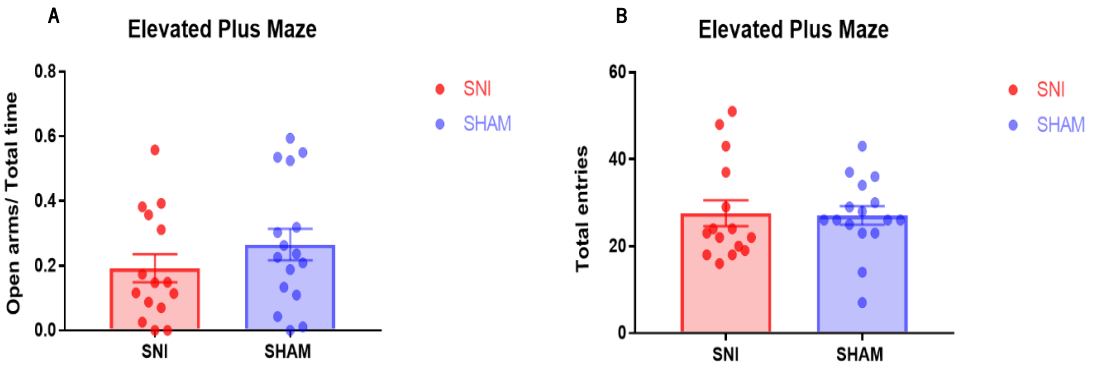


Figure 7: Anxiety-like behavior evaluation. SNI and SHAM animals do not present statistically significant differences in the anxiety-like behaviour in the EPM. (A) Ratio of time spent on the open arms and total time of the test. (B) Total number of entries in the EPM arms (open + close). Data presented as mean ± SEM, n= 31.

Similarly, no deficits on locomotion/exploration, as observed by the number of total entries in the arms, were observed T (29) = 0.148, p 0.883, d= 0.053).

3.4 Depressive-like behavior

Concerning depressive-like behavior, SNI animals do not present statistically significant differences when compared to SHAM animals in the immobility time during the FST paradigm (T (29) = 0.086, p = 0.932, d= 0.031), Figure 8.

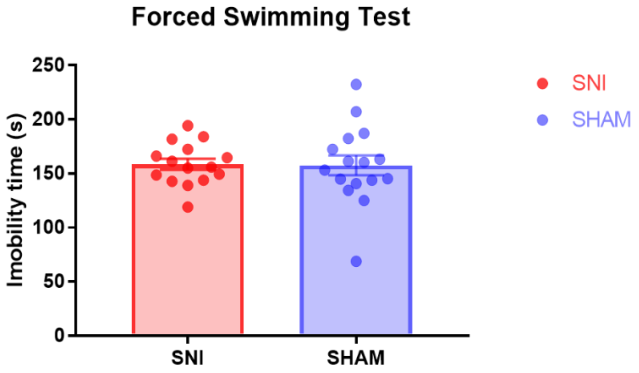


Figure 8: Depressive-like behavior evaluation. SNI and SHAM animals do not present statistically significant differences in the depressive-like behavior. None of the groups showed a depressive-like phenotype 5 weeks after SNI induction. Data presented as mean ± SEM, n=31.

3.5 Social behavior (NA)

To study social behavior two tests were used: the NA and the 3-CH (Figure 3). The NA evaluates social interaction between two animals.

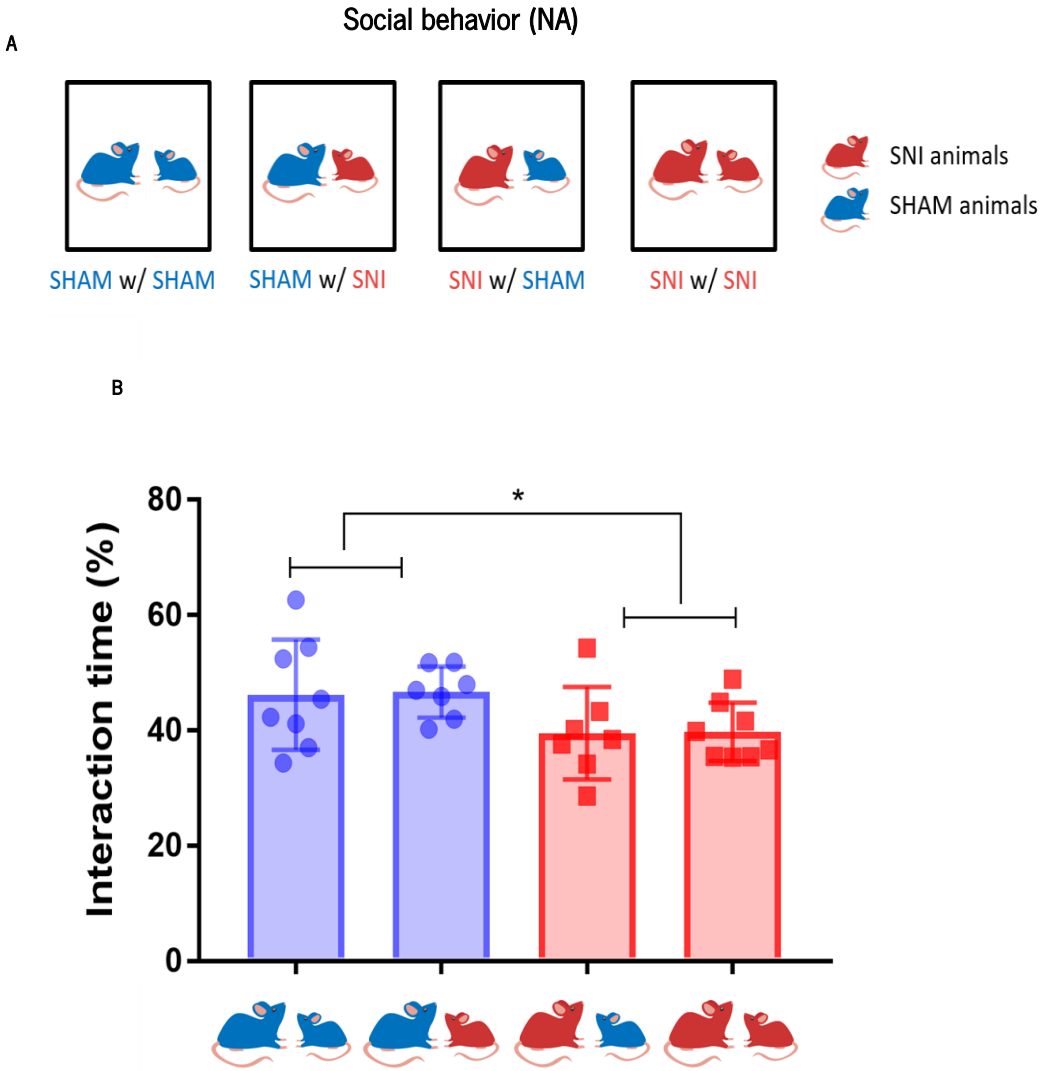


Figure 9: Neutral arena paradigm. Schematic representation of the different social dyads tested (A) SNI animals interact less than SHAM. However, for both SHAM and SNI, the percentage of interaction with an animal in a painful condition or not, is not different (B). Note that, representation of SNI animals is always the illustration of a "red rat" as well as the representation of SHAM animals is always a "blue rat". Moreover, in each dyad the bigger illustrated rat represents the evaluated animal (explorer). Data presented as mean ± SEM, *p=0.015, n=30.

Two-way ANOVA analysis reveal that SNI animals interact less than SHAM regardless of who they are interacting with (Figure 9B) ($F(1,26) = 6.768$, $p = 0.015$, $\eta^2 = 0.206$). Furthermore, no statistically significant differences were found within each group, SHAM or SNI, when they were interacting with pain or pain-free animals.

3.6 Social behavior (3-CH)

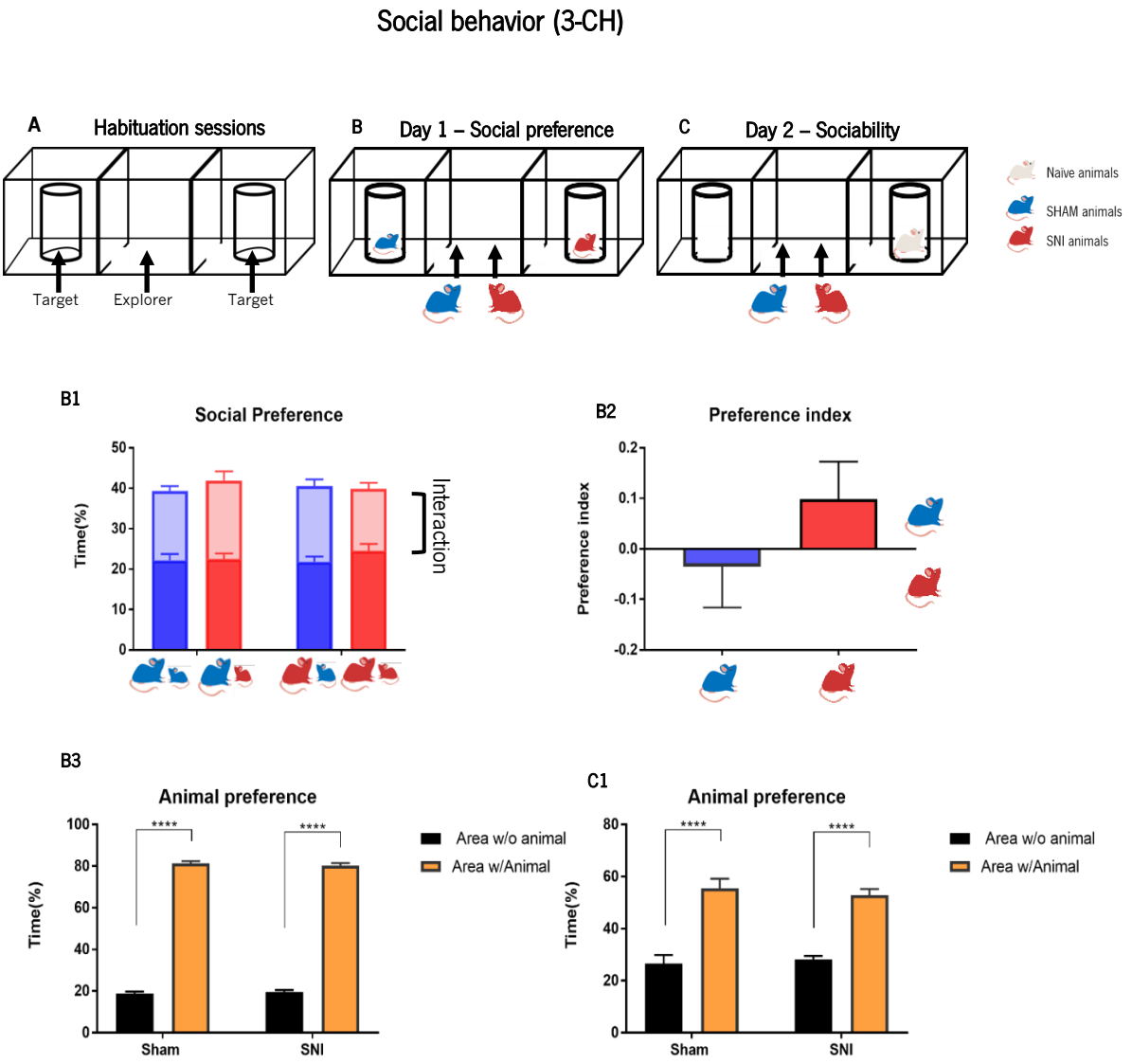


Figure 10: 3 Chambers paradigm for social preference assessment. Experimental design for the 3 sections of 3- CH paradigm. Habituation day (A), social preference day (B) and sociability day (C). No evidence of differences was found regarding the time spent by the animals in each one of the social areas (B1). However, the data suggest that SHAM animals interact more with SNI while the SNI animals seem to interact more with SHAM (B1). Moreover, the preference index shows a opposite choice between the two groups (B2). Clear evidence was found regarding the social repertoire of rodents. All the animals present a clear preference for social encounter rather than isolation in both days of the paradigm, day 1 (B3) and day 2 (C1) respectively) Note that, representation of SNI animals is always the illustration of a "red rat" as well as the representation of SHAM animals is always a "blue rat". Moreover, in each dyad the bigger illustrated rat represents the evaluated animal (explorer). Data presented as mean ± SEM, ****p<0.0001, n=30.

The 3-CH test intends to evaluate the rodent's social preference, for one animal over the other, when they have the opportunity to choose. To evaluate social preference, explorer animals were able to interact, in the same trial, with animals in painful or not painful conditions. The time in each arena and the time of interaction between each one of these dyads was measured. No statistically significant differences were found regarding the time spent by the explorer animals (SHAM or SNI) in each interacting area of the paradigm neither the time of interaction with SHAM nor SNI targets (Figure 10B1). However, there is a tendency to SHAM animals interact more with SNI, while SNI animals tend to interact more with controls (Figure 10B1-2).

Finally, results show that both SHAM and SNI have a clear preference for social encounter over isolation, both in day 1 (SHAM animals: $T(14)= 28.442$, $p<0.0001$, $d= 7.344$; SNI animals: $T(13)= 29.963$, $p<0.0001$, $d= 8.008$) as well as day 2 (SHAM animals: $T(14)= 4.159$, $p<0.0001$, $d= 1.074$; SNI animals: $T(13)= 6.473$, $p<0.0001$, $d= 1.730$) of the paradigm (Figure 10B3 and C1, respectively).

3.7 Systemic Oxytocin

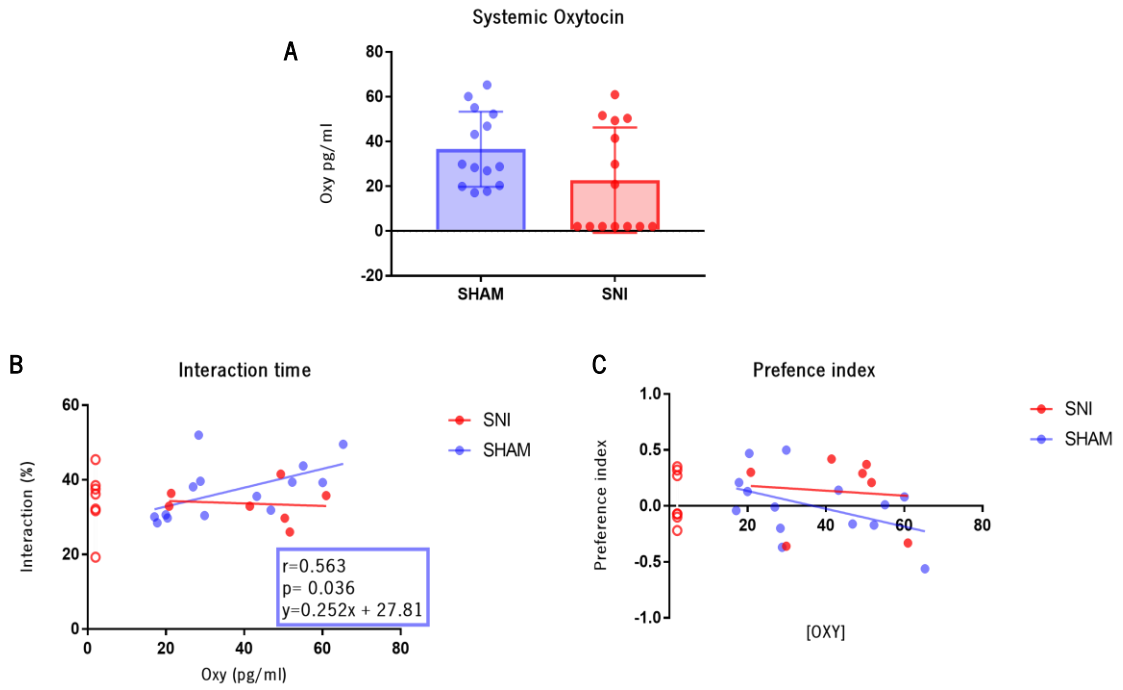


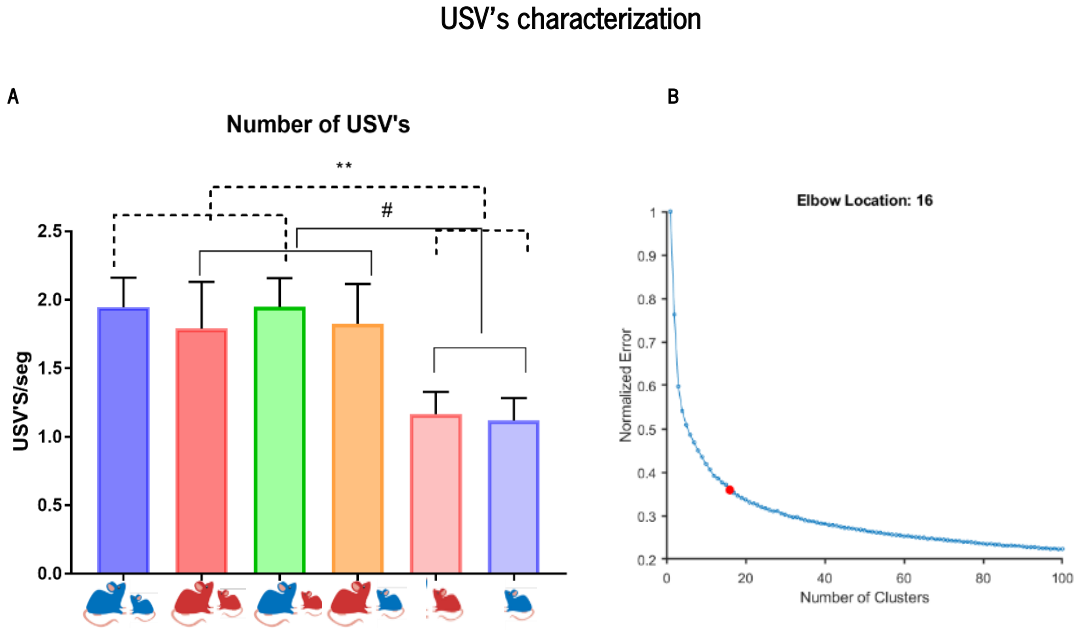
Figure 11: Systemic oxytocin measurements. Using an ELISA KIT, OXT was measured from animal's blood. SNI animals do not present significant lower levels of systemic OXT when compared to SHAM animals. However, 7 animals presented undetectable OXT levels (A). Furthermore, there is a positive correlation between interaction time on the 3 Chambers paradigm and OXT levels for SHAM animals (B), however, this correlation is lost in SNI animals. Regarding the preference index and the systemic OXT levels, no correlation was found (C) Note that red points not colored in both graphs B and C represents the SNI animals whose values of OXT were undetectable and therefore no correlation was tested. Data presented as mean ± SEM, p=0.036, r=0.563, n=30.

To understand the role of OXT in the context of neuropathic CP and social behavior, blood was collected from all explorer animals right after day 1 of 3 Chamber paradigm (Figure 10B) and systemic OXT levels were measured by ELISA. No statistically significant results were found between SHAM and SNI animals ($T(26) = 1.792$, $p = 0.085$, $d = 0.677$) (Figure 11A). However, this neuropeptide was not detected in half of SNI animals (vs 1 SHAM), because its concentration was below the detection levels of the kit. In that way, SNI animals seem to be divided in animals with normal levels of OXT and animals with very low concentration of this peptide (see Figure 11A). Further analysis of these group separately reveals no differences at the behavioral level (results not shown).

Correlation analysis showed a positive correlation in SHAM animals between the levels of OXT and the interaction time of the explorer animals with both targets ($P=0.036$; $R= 0.563$), but not in SNI animals (Figure 11B). On the other hand, no statistically significant correlations were found between preference index and OXT levels (Figure 11C).

3.8 Ultrasonic vocalization analyses

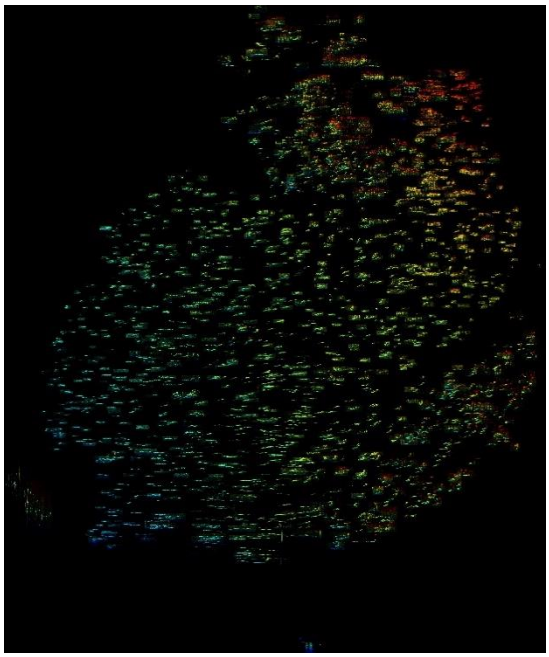
To study if the communication between SHAM and SNI animals is different during a social encounter or in isolation, USVs were recorded on the social preference day of the 3-CH (see section 2.1.5.4). As referred before, SHAM and SNI animals were used as explorers as well as target animals thus leading to 4 social conditions to compare. Furthermore, USVs recordings when target animals were alone were also analyzed.



C

CLUSTERS	Call Duration(s)	Frequency (KHZ)	Slope (KHZ/seg)	SHAPE Sinuosity	SHAPE Tonality
1	0,0269±0,0149	59,9617±1,9451	164,1825±247.3116	1,4099±0.4200	0,4866±0.1020
2	0,0467±0,0246	55,7643±1.8729	62,1266±1890.0481	1,3265±0.4722	0,5133±0.1071
3	0,5723±0,1256	59,0941±4.2161	-6,0588±18.3261	6,0992±3.1060	0,4758±0.0620
4	0,0529±0,0271	72,6354±6.4715	343,2452±479.8600	2,9351±1.2721	0,4456±0.0648
5	0,0506±0,0341	69,6424±6.9154	597,1431±537.5612	2,7679±1.1772	0,4609±0.0709
6	0,0517±0,0287	72,3916±7.1767	469,2324±481.5968	2,9592±1.3168	0,4571±0.0686
7	0,0554±0,0311	72,6707±6.8129	139,9265±375.6441	3,1386±1.4956	0,4482±0.0692
8	0,3677±0,0660	59,1770±4.5765	-5,0585±28.2836	4,7235±2.9511	0,4610±0.0631
9	0,0280±0,0188	67,0548±3.1987	160,2912±301.0635	1,6241±0.6343	0,4600±0.0947
10	0,0514±0,0371	63,3140±5.6418	-336,4573±335.6391	2,6807±1.3732	0,4853±0.0918
11	0,0436±0,0247	60,5608±4.6907	522,1537±365.4262	2,1378±0.8690	0,5173±0.0927
12	0,0446±0,0271	62,4089±5.2638	714,1751±528.3999	2,5199±1.1720	0,4831±0.0804
13	0,0363±0,0214	80,5390±5.3614	96,9584±338.9127	2,0846±1.1547	0,4154±0.0533
14	0,0374±0,0213	49,2805±3.9718	50,4563±240.0387	1,2480±0.4670	0,4869±0.1013
15	0,0468±0,0327	63,2320±5.7103	682,9920±537.9906	2,6765±1.1773	0,4765±0.0763
16	0,0413±0,0220	60,8926±4.9776	693,4700±426.6432	2,3743±1.0221	0,5087±0.0895

D



E

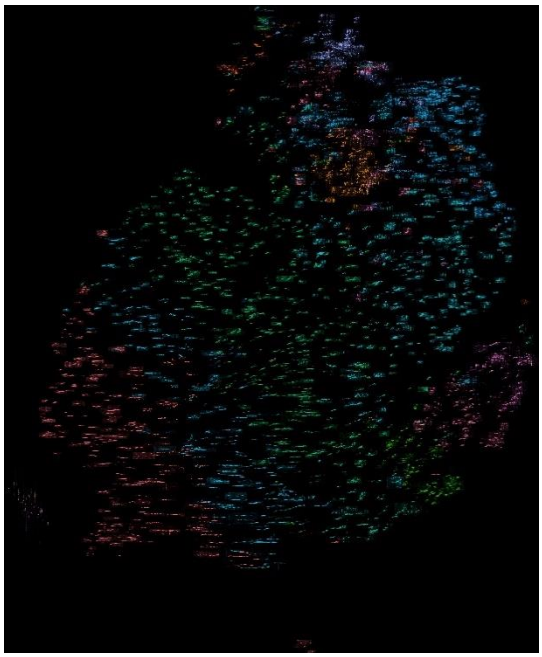


Figure 12: Ultrasonic vocalizations analysis: Clusters characterization. Total number of USV's emitted by the explorer animals in each situation of social encounter(A). Target animals were recorded during the time in which the explorer animal was in the opposite side of the arena. Total number of target animal's USVs when they were alone is decreased when compared with social dyads (A). For the clustering process, the software uses an Elbow curve to define the optimal number of clusters of USV's. 16 was considered the optimal number (B). Representation of the clusters divided based on their characteristics such as duration, frequency and shape (C) Clusters illustration based on USVs frequency (D). Clusters illustration in which each colour corresponds to one cluster (E). Data presented as means ± SEM. **p=0.011, #p= 0.048, n=30.

As shown in Figure 12A there is a significant difference in the total number of USVs emitted per second between animals in a social encounter and isolated animals (F (2,51) = 5.701, p=0.006, η^2= 0.183). The calls were divided by Deep Squeak software in 16 groups according to its characteristics: duration (sec), principal frequency (KHZ) and shape (Figure 12C). To define these clusters, the elbow method was used in which the program finds the optimal number of clusters to minimize the errors between and within clusters. For these set of data, the optimal number was defined as 16 (Figure 12B).

Comparisons between the 4 groups of social encounters conditions together, explorer-target (SHAM-SHAM, SNI-SNI, SHAM-SNI, SNI-SHAM) and the isolated target animals, for each one of the 16 clusters reveal statistically significant differences in most clusters (Figure 13).

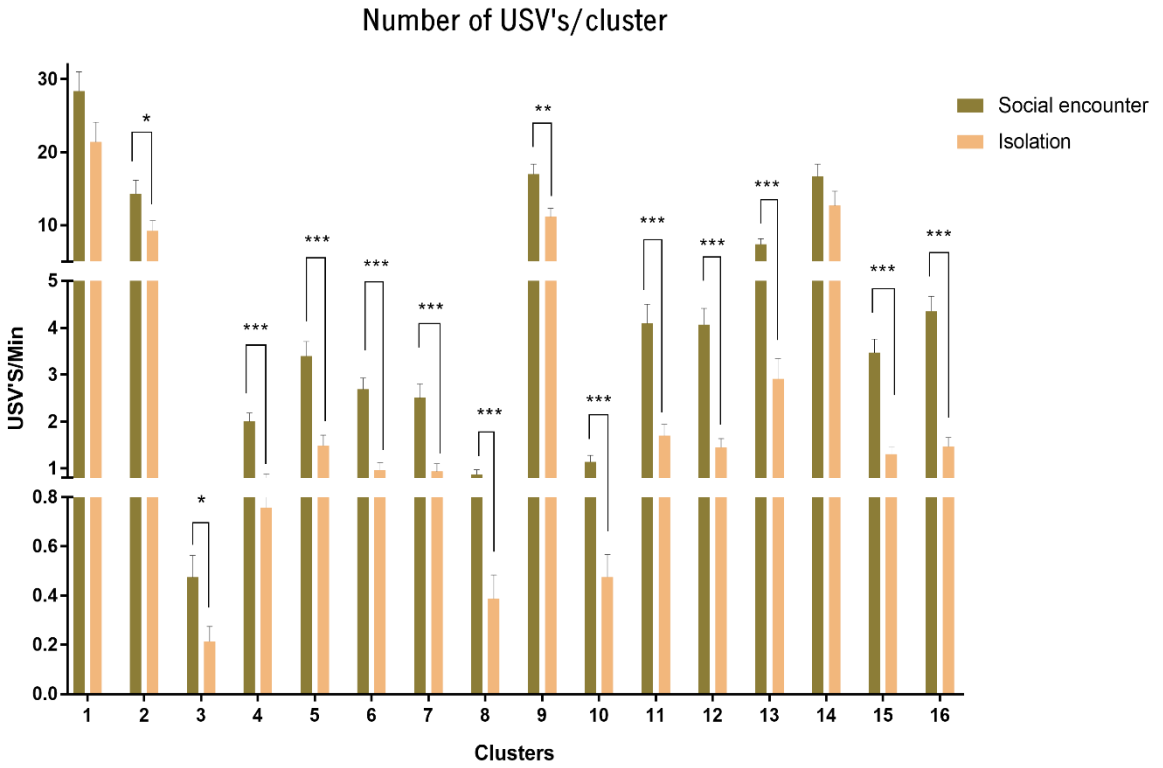


Figure 13: Number of USV's/Min in each cluster: Comparison between social encounter and isolation. The number of USV's in each cluster was normalized to the duration of social encounter or isolation time. Isolation refers to the time that target animals were alone while the explorer was in the opposite side of the arena. Most clusters present significant differences between social encounter and isolation, see section 2 in the Annexes. Data presented as means ± SEM. *p<0,05; **p<0.01; ***p<0.001, n=30.

Apart from clusters 1 and 14, isolated animals emitted less calls/min than animals in a social encounter condition. Complete data of statistically significant differences are described in annexes section (annexes 2). When comparing the number of vocalizations between isolated SHAM and SNI, within the different clusters, significant differences were found in cluster 7 (T (52) = 2.102; p=0.040, *d*= 0.572) and 8 (T (52) = -2.018; p=0.049, d= -0.549) (Figure 14). While in cluster 7 SNI animals have more calls than SHAMs, in cluster 8 it is the other way around (Figure13).

Within clusters, no differences between the 4 groups in social interaction were found (Figure 15). Despite the constant prevalence of cluster 1,2,9 and 14 that have more calls that the other clusters in every social condition, no differences were found.

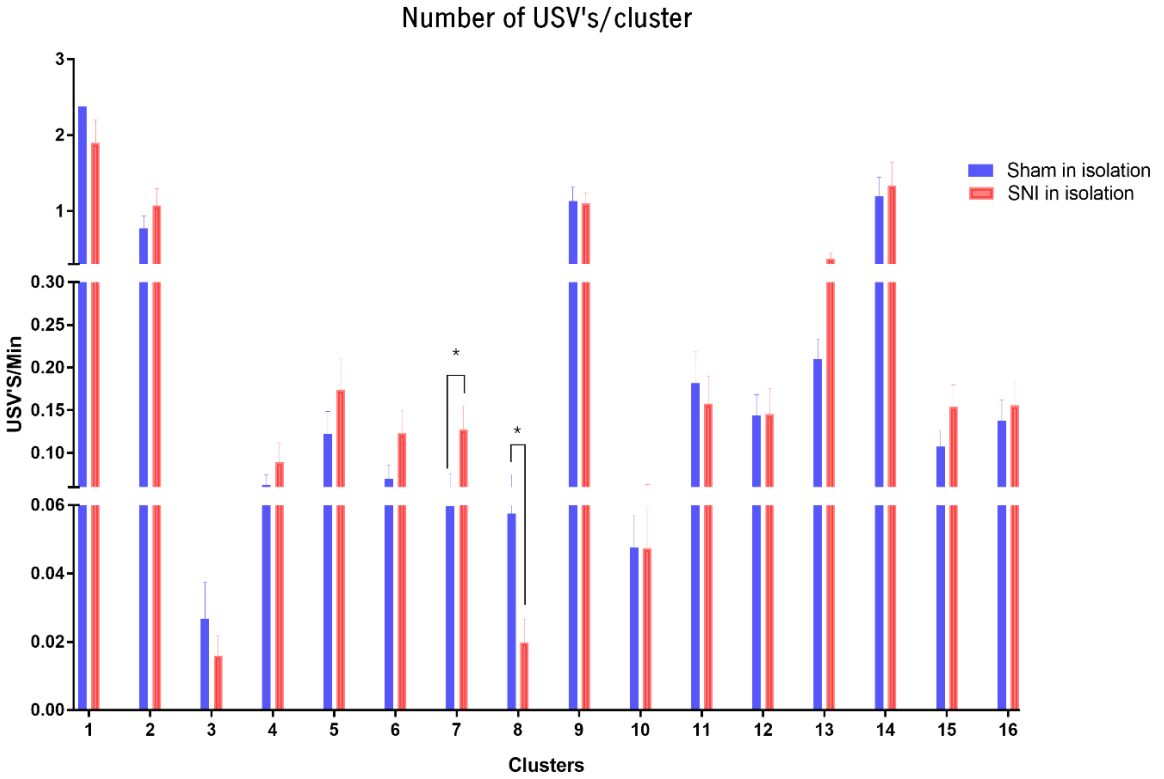


Figure 14: Number of USVs/Min in each cluster: Comparison between target animals in isolation. Every time that explorer animals were in a social encounter with a target, another target animal was alone during that specific time. USVs were recorded and normalized to that specific time of isolation. Clusters 7 and 8 showed opposite differences in the number of USVs between SHAM and SNI animals in isolation. Data presented as means ± SEM. *p<0.05, n=30.

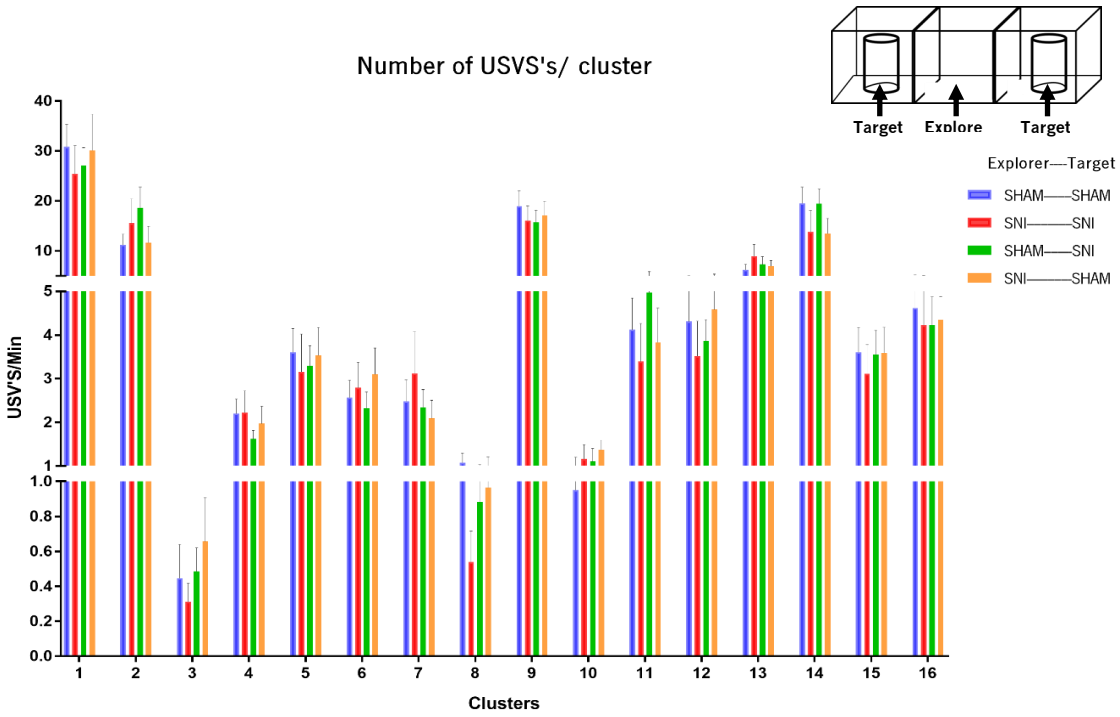


Figure 15: Number of USVs/Min in each cluster: Comparison between different dyads of social encounters. SHAM and SNI animals were used both as explorers and target animals, therefore 4 different dyads of social encounters (represented in the legend) were analysed. No statistical differences were found between groups within each cluster. There is a prevalence of clusters 1, 2, 9 and 14 as the ones with more USVs in all groups but no differences were found between them. Data presented as means ± SEM, n=30.

To further characterize these social communications, transition probabilities between clusters were calculated. (Figure 16 and 17) A qualitative analyses of figure 15 shows 4 specific clusters (1, 2, 9 and 14) that have higher transition probabilities, i.e. it is more likely that after the animals emit a call, the next one is from one of these 4 clusters. Moreover, it seems that when target animals are isolated (Figure 16E and F) they have a smaller number of transitions than animals in a social condition, reviling a more homogenous pattern.

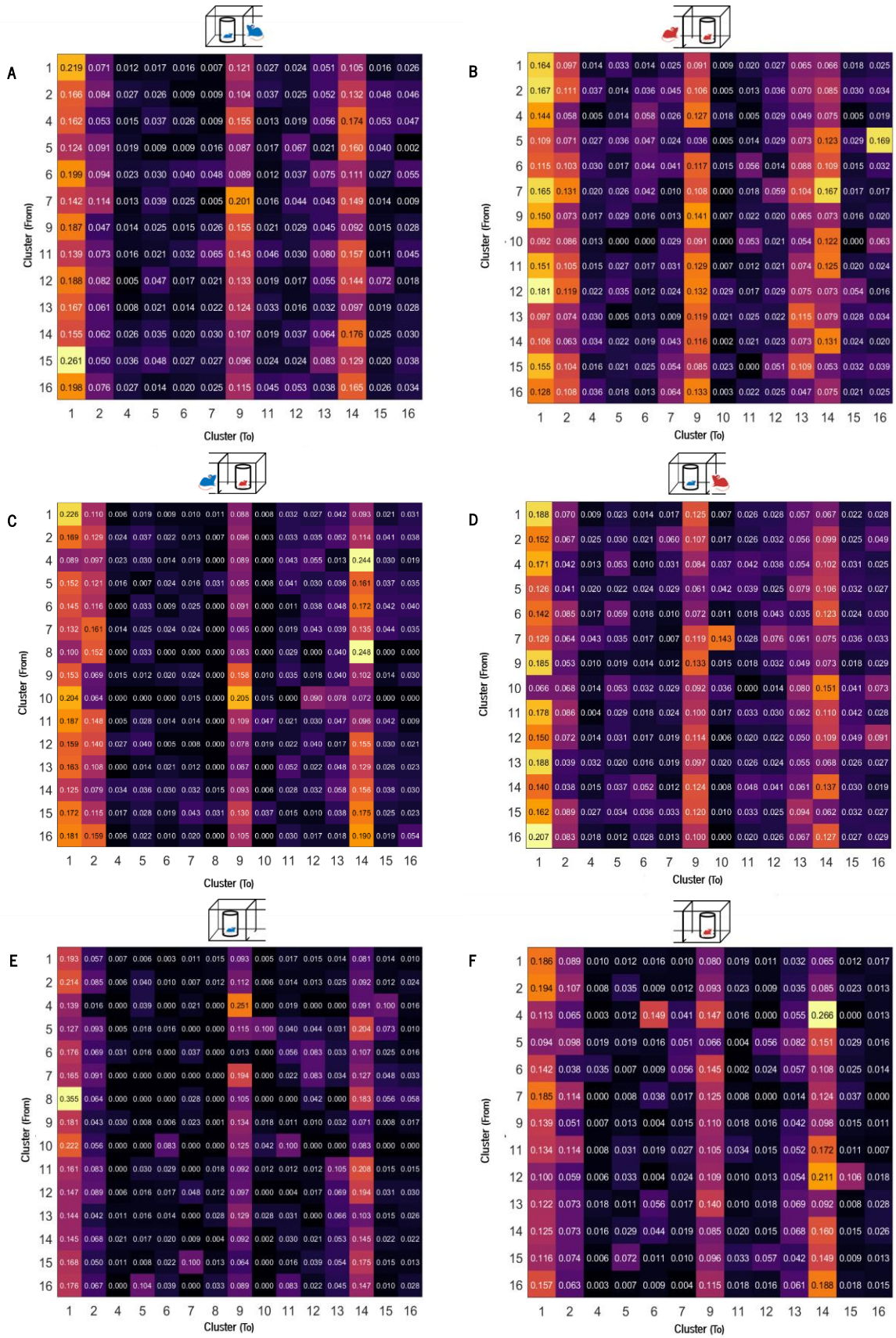


Figure 16: Transition probability between clusters. After the division of the USVs in cluster, the transition probability between clusters was calculated. In all the social conditions (A-F) clusters 1,2,9 and 14 are distinct from the others. No statistical analysis was performed in these domains due to the absence of statistical power. As so, these analyses were only qualitative.

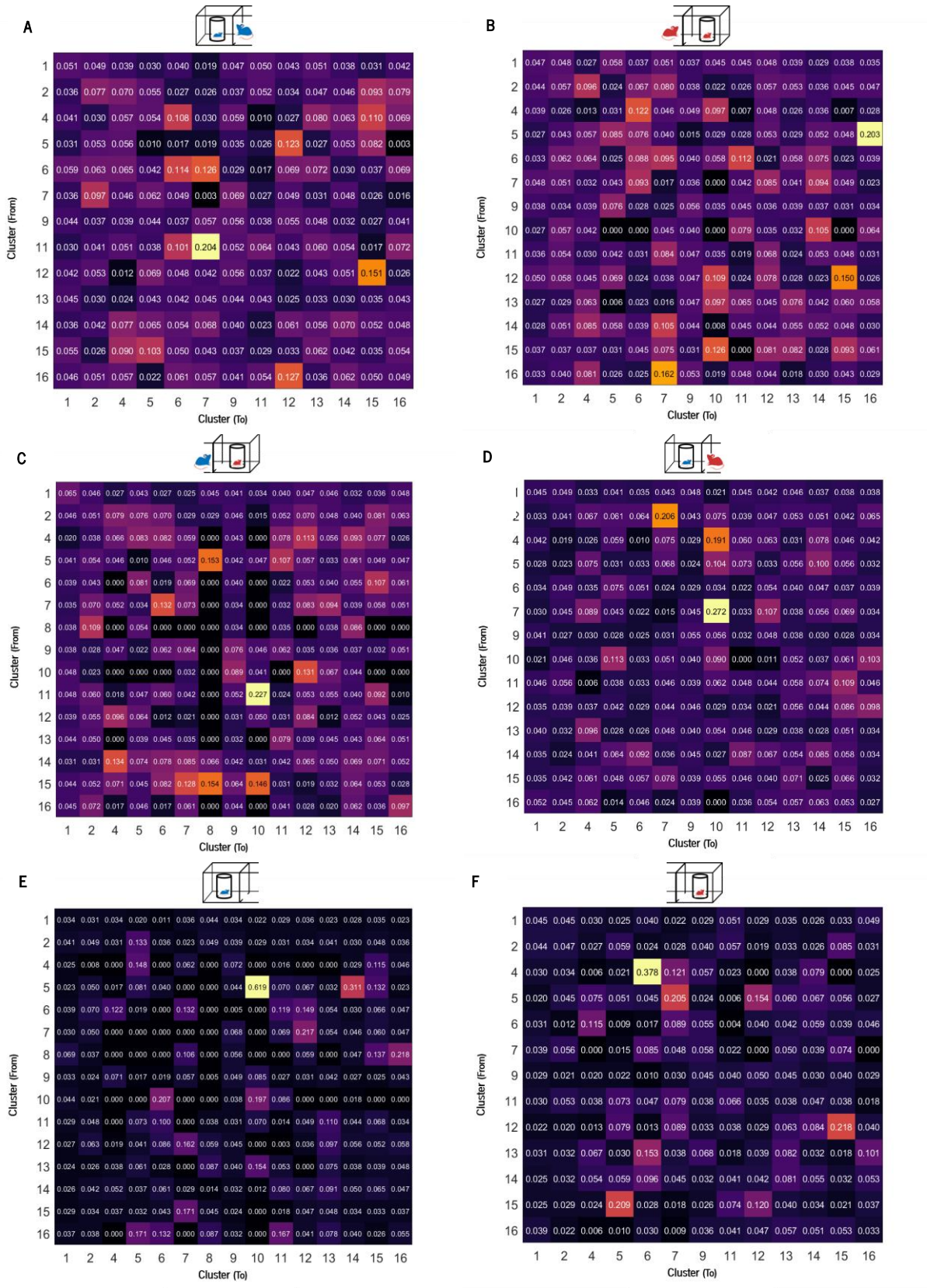


Figure 17: Transition probability between clusters normalized for the number of USVs in each cluster. Since there was 4 clusters with higher number of USVs in comparison with the other, normalization for the number of USVs within each cluster was done in every social condition (A-F) It seems that animals alone (E and F) have a more homogenous pattern of transition than when they are in a social encounter (A-D) It is important to refer that no statistical analysis were performed on these data due to absence of statistical power. As so, these analyses were only qualitative.

In order to eliminate the influence of calls' prevalence, data in each cluster was normalized to the total number of USVs in that cluster (Figure 17). As expected, the higher probabilities for clusters 1,2,9 and 14 (which are the most prevalent) disappear. Interestingly, the more homogeneous pattern of target isolated animals when compared to animal in social conditions was maintained (Figure 17E e F).

4. Discussion

Social behavior in neuropathic rats (SNI model) was analyzed as well as OXT role in this interplay. In a spontaneous social interaction paradigm – neutral arena – neuropathic animals showed significantly less interactions than SHAM operated animals independently of the social dyad tested, SNI-SNI and SNI-SHAM. In a social choice paradigm – 3 chamber – SNI and SHAM animals similarly explored their targets however, results suggest that with a preference towards the opposite phenotype. Curiously, interaction time positively correlates with systemic OXT levels, but only in SHAM animals. Ultrasonic vocalizations analysis show that target SNI and SHAM animals significantly differ in 2 clusters of vocalizations when alone in the 3-CH. Also, the entrance of explorer animals, regardless of their phenotype, is associated with an increasement of USVs emission and the heterogeneity of the USVs pattern when compared to isolation.

The hypossocial phenotype observed in the NA (Giancardo et al., 2013) in CP animals is in agreement with previous unpublished studies of the group. Indeed, we demonstrate that this phenotype is very robust manifesting 1, 2 and 4 months after SNI, in both males and female rats and in dyads of familiar as well as unfamiliar pairs. Curiously, in the 3-CH paradigm (Nadler et al., 2004; Yang et al., 2011) this phenotype was not evident even though both groups presented a marked preference for social interaction over isolation. Also, SNI and SHAM animals presented similar time of exploration, approximately 80 % of the total exploration time. The construct of NA and 3-CH paradigms are inherently different and evaluate social behaviors at different dimensions which might in part explain the differences. In the former, the role of each animal in the dyad is dynamic, whereas in the latter the roles are clearly defined and explorer preference is assessed (Sandi & Haller, 2015). Indeed, the 3-CH results suggest that SNI interact more with SHAM while SHAM have the opposite preference which might reflect a manifestation of empathy-like behavior. Such manifestations have been reported in mice dyads given an identical noxious stimulus; these displayed increased pain behaviors when compared to isolated animals. Interestingly, this increase only happened when the counterparts were cagemates (Langford et al., 2006). Moreover, these authors also showed that this effect was dependent on direct visual observation. Empathic-like behavior in rodents

has also been observed in contexts other than pain – see for instance (Bartal, Decety, & Mason, 2011); see for review (Meyza & Knapska, 2018; Mogil, 2012). Moreover, 3-CH paradigm was used in a subsequent experience to explore the potential of environmental cues to drive animals' social preference (see Annexes, section 1).

Systemic OXT measurements provided an additional piece of evidence distinguishing SNI and SHAM animals in the 3-CH. Higher OXT concentrations in the blood were positively associated with more interaction time in SHAM but not SNI animals. In other words, despite the similar exploration times, these 2 groups are physiologically different. Studies from others have shown that OXT levels influence social exploration drive (Borland et al., 2018; Lukas et al., 2011; Peñagarikano et al., 2015). Of notice, our data does not allow to establish causality between OXT levels and behavior. Measurements of OXT concentration prior to the behavioral assessment would provide critical information on this question. However, part of the SNI group presents abnormally low values of OXT which might suggest an impairment in the regulation of its peripheral secretion. Also, previous data (unpublished) from the group demonstrated that OXT administration in the periphery transiently increased thresholds to mechanical stimulation (and social exploration) in SNI animals in agreement with previous studies (Freund-Mercier & Uhl-Bronner, 2007; Poisbeau et al., 2017; Rash et al., 2013).

Rats and mice emit USVs to communicate emotional states as well as to maintain or to establish social contacts with conspecifics (Chabout, Cressant, Hu, Edeline, & Granon, 2013; Kolacz, Lewis, & Porges, 2018; Simola & Granon, 2018). The number of emitted calls is known to correlate with the duration of social contact leading to the conclusion that an additional analysis, i.e. the time that each animal spent in social interactions and the USVs emitted in that specific time, would be a good complement to better understand USVs significance in behavioral modulation (Chabout et al., 2012). We therefore attempted to identify if in our experimental context USVs played a significant role in the modulation of social preference of explorer rats. Results show that indeed, in social encounters more calls were emitted in all clusters with the exceptions of clusters 1 and 14 that apparently are not relevant for sociability. While not possible to entirely clarify if such resulted simply from the presence of one additional animal in the chamber, the limited range of the microphone and its directionality minimize the interference from the explorer. More importantly, vocalizations became more heterogenous in their characteristics strongly suggesting a shift reflecting the social context. Similar observations were made in other settings. For instance in a mice autism model, hypossociability was associated with a reduction in USVs call rates (Toledo, Wen, Binder, Ethell, & Razak, 2019). In stress-induced conditions (restraint for 3 days) animals revealed significant decrease in their call rate (Grimsley et al., 2016) - see also (Simola & Granon, 2018).

Regarding pain effect on vocalizations, the analysis of SHAM and SNI targets, in the absence of explorer animals revealed 2 clusters of USVs vocalizations (7 and 8) significantly increased/decreased in SNI animals when compared to SHAMs. USVs in the context of CP are largely unexplored and conflicting results have been published. In a spinal nerve ligation (SNL) model USVs were increased after peripheral stimulation (Thompson, Yakhnitsa, Ji, & Neugebauer, 2018) therefore as a response to an allodynic stimulus and not necessarily reflecting ongoing pain. In a study by Kuner's group using the SNI model in mice, an increase on 37 and 50 KHZ USVs was also reported 1 to 4 weeks after lesion and in the absence of peripheral stimulation; however no differences were found after the fourth week which is in general agreement with our observations (Kurejova et al., 2010). It is important to notice that the analysis presented here is unbiased (no specific frequencies were selected) and more refined; indeed, clusters were made based on the overall USVs information collected and then used to compare groups. In a social context, our 4 conditions explorer-target (SHAM-SHAM, SNI-SNI, SHAM-SNI and SNI-SHAM) revealed no significant differences. Again, clusters 1, 2, 9, 13 and 14 were more prevalent than the other clusters. Curiously, clusters 7 and 8 revealed the same trend described before in the SNI-SNI condition reinforcing its potential value as a proxy of ongoing pain. The division in groups affected our statistical power. The cluster transition probability analysis, albeit qualitative, revealed specific patterns for each condition. Their communication patterns were distinct which could be a good indicator of specific communication traits and specific mental states of the emitters (Simola & Granon, 2018). The behavioral significance of these transitions is poorly understood, but the literature has been giving some insights about social communication patterns among rodents, in particular, about USVs patterns alterations in chronic stress, psychiatric disorders, drug addiction or neurological diseases (Brudzynski, 2013; Simola & Granon, 2018). In rodent's literature, USVs are normally classified into two major categories, 22 KHZ and 55 KHZ in the ranges of 20 to 35 KHZ and 35 to 80-KHZ, respectively (Simola & Granon, 2018). While the former in known to be emitted in an unpleasant context (e.g. pain) (Brudzynski, 2013), the latter is commonly associated with pleasant situations (e.g. social encounters) (Brudzynski, 2013). This division, however, has been questioned by several researchers (C. J. Burke, Kisko, Swiftwolfe, Pellis, & Euston, 2017; Chabout et al., 2012; Grimsley et al., 2016; Wright, Gourdon, & Clarke, 2010). As described above, in the context of neuropathic pain model in mice, both types of USVs were elevated in the first weeks after lesion (Kurejova et al., 2010). Indeed, in our work only 55KHZ calls were found though in a great variety as revealed by the number of clusters. Such heterogeneity is aligned with a previous work where up to 14 categories of 55-KHZ USVs were described; authors argued that these might reflect the complexity of emotional communication in rats (Wright et al., 2010) but that is currently speculative. In a recent study authors silenced of PAG-USV neurons preventing male mice to produce USVs and affecting their ability to

attract females. The PAG has a pivotal role in pain modulation as described above suggesting that it might be a critical hub to be explored (Tschida et al., 2019) Indeed, PAG receives oxytocinergic axons from the paraventricular hypothalamic nucleus (PVN) and when OXT is released it decreases the activity of spinal cord nociceptive neurons (Iwasaki & Charlet, 2018).

Depression, anxiety and deficits are frequently comorbid with chronic neuropathic pain (Heinricher, 2016; Neugebauer et al., 2009; Nicholson & Verma, 2004). These manifestations depend on a number of factors including pain duration (Yalcin et al., 2011), injury location (Leite-almeida et al., 2012), age of the experimental subject (Leite-Almeida et al., 2009), sex (Nishinaka, Kinoshita, Nakamoto, & Tokuyama, 2015) among others (Bair et al., 2003; Domonkos, Hodosy, Ostatníková, & Celec, 2018; Leo, 2005; Yalcin & Barrot, 2014). In our experimental conditions, 1 month, right-sided SNI lesion, in male rats no anxiety- and depressive-like behaviors were observed which is in line with previous studies from the group and others (Hasnie et al., 2007; Pitzer et al., 2019; Urban et al., 2011). This eliminates potential confounders reinforcing the specificity of the social behavior in the context. Moreover, such observations strongly support that social behavior is a more robust proxy of CP-related disability than anxiety- and depressive-like behaviors which are apparently more affected by environmental and biological factors in a manner that is not entirely understood. Indeed, unpublished data from the group shows that in the NA hypossociability manifest in both males and females, within dyads of familiar and unfamiliar pairs (SNI-SNI, SNI-SHAM and SHAM), at 1, 2 and 4 months after both left- and right-sided SNI.

5. Concluding Remarks

Overall this work demonstrates that innate behaviors, as social behavior, provides a simple though robust proxy of pain effect in the SNI model. While its generalization to other CP models requires further experimentation, so far, our observations are in line with current views defending more ethologically relevant approaches to CP in animal models (Klinck et al., 2017; Mogil, 2012). Future studies are needed to uncover the role of oxytocin in this interplay, including molecular analysis in brain areas related to pain, social behavior and OXT production such as Nac, Amy, PFC and PVN. Manipulation of OXT circuits would be also important to establish causality relations. Additionally, USVs recording brought new insights about social communication between rodents and uncovering some differences in emotional state between SHAM and SNI animals. However, the knowledge about behavioral significance of USVs is far from satisfactory and it is important to keep finding new insights since it could be an important tool to decipher social behavior modulation, albeit its considerable interindividual variances. (Simola & Granon, 2018; Wright et al., 2010). To sum up, the inclusion of social exploration paradigms, related readouts (USVs) and other ethologically relevant paradigms in the context of CP models will contribute positively to translation in pain research by complementing sensory readouts that are almost exclusively used in preclinical research.

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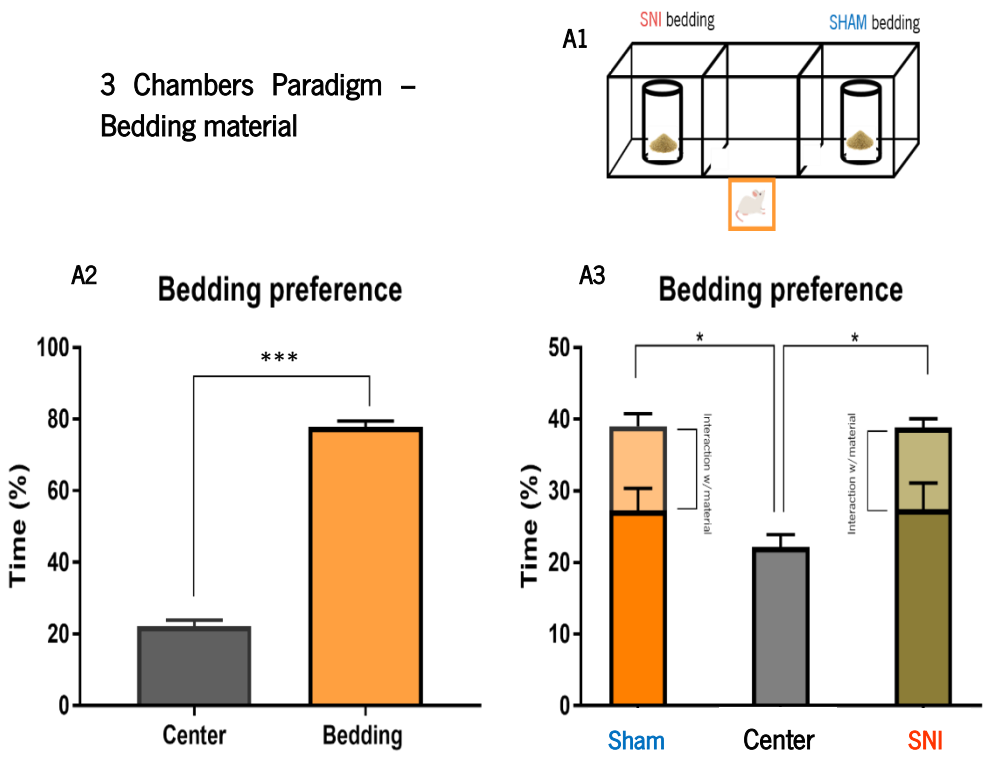
7. Annexes

1. Environmental cues driving social choice of naïve animals

Besides social preference characterization, this work also intended to understand which environmental cues that could drive animals' choice. For this, the 3 chambers paradigm was used. Only naïve animas were used as explorers and the target animals were the same as used in the experiment described in the main work. To define if odors are enough for animals' distinction and thus for driving social choices of the tested animals, SHAM and SNI bedding was put in in the arena as represented in Figure 18A1. As shown in Figure 18A, explores spent more time on bedding material areas than on the empty central area T (17) = 16.310, p<0.001, d= 3.844. No differences were observed regarding SHAM and SNI bedding preferences (Figure 18A3; F (1.153,19.598) = 4.198, p=0.049, η^2= 0.193). Moreover, to understand the implication of pain on the preference, SNI animals treated with gabapentin or vehicle were used as target animals as well as SHAM animals injected with OXT or vehicle (Figure 18B and C, respectively). No evidence of a preference for the treated animals was found in neither one of the conditions (Figure 18B3 and C3). However, naïve animals present a consistent preference for social encounters rather than isolation [SNI animals T (17) = 43.199, p<0.001, *d*= 10.182; SHAM animals T (17) = 20.854, p<0.001, d=4.915, Figure 18B2 and C2, respectively] and [SNI animals F(1.282, 21.800)= 35.071, p<0.001, η^2= 0.673; SHAM animals F(1.434,24.378)= 20.200, p<0.001, η^2=0.543, Figure 18B3 and C3].



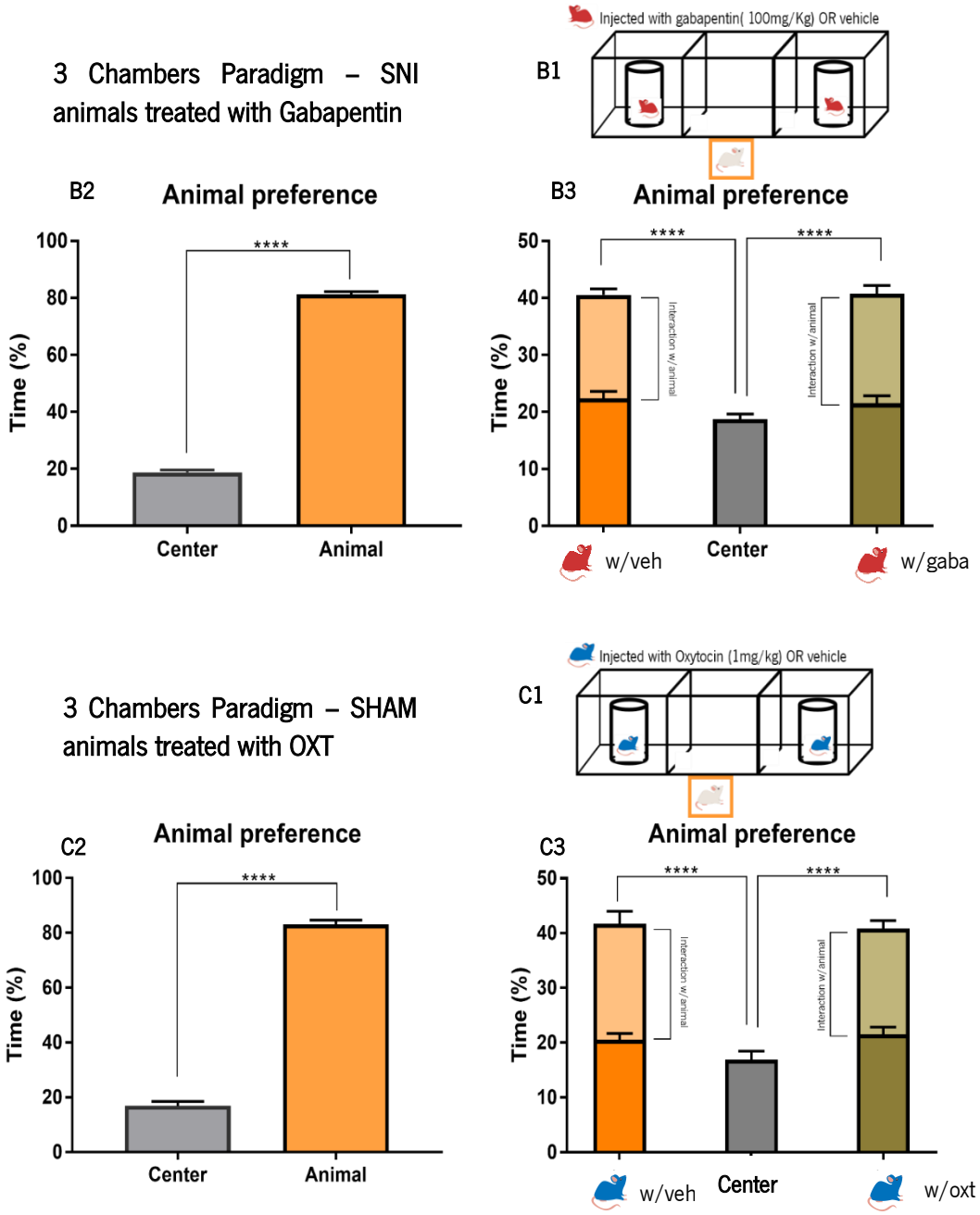


Figure 18: Environmental cues driving animal's social choice. 3 chambers paradigm was used define the influence of environmental cues on animal's social choices. Any influence of odours on naïve animal's social choice was not found. Explorer animals approached SNI and SHAM bedding in the same way (A) When target animals were SNI treated with gabapentin (B) or when they were SHAM animals treated with OXT, the results were very similar. Naïve animals were not driven by any of the conditions tested. However, clear evidence was found for the animal's preference of social encounter over isolation in each one of the conditions tested (A2, B2 and C2). Data presented as mean ± SEM, ****p<0.001, n=18.

In conclusion, the experiment allows to infer that besides consistent preference for social encounter, neither one of the conditions tested was sufficient to drive naïve animal's social choice. Further discussion will take placed on the next section.

2. Statistics of USVs emitted in social encounters vs isolation

Table 1: Additional data of statistically significant differences between social encounter and isolation within each cluster (Figure 12).

CLUSTER	T (STUDENT)	DF	P	*D* (COHEN'S)
2	2.189	106	0.031	0.421
3	2.441	106	0.016	0.470
4	5.644	106	< 0.001	1.086
5	4.985	106	<0.001	0.959
6	6.000	106	<0.001	1.155
7	4.632	106	<0.001	0.891
8	3.439	106	<0.001	0.662
9	3.296	106	0.001	0.634
10	4.060	106	<0.001	0.781
11	5.062	106	<0.001	0.974
12	6.671	106	<0.001	1.284
13	5.027	106	<0.001	0.967
15	6.520	106	<0.001	1.255
16	7.822	106	<0.001	1.505