EFFECTS OF AFFECTIVE TOUCH ON ULTRASONIC VOCALIZATION AND c-FOS EXPRESSION IN RATS

by

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ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

EFFECTS OF AFFECTIVE TOUCH ON ULTRASONIC VOCALIZATION AND c-FOS EXPRESSION IN RATS

The sense of touch has two functional dimensions: discriminative and affective. Discriminative properties of tactile stimuli are relayed via myelinated and fast conducting Aβ fibers while affective properties are transmitted via unmyelinated, slow-conducting C- tactile fibers. Gentle stimulation of CT- fibers was shown to elicit feeling of pleasantness and activate insular cortex. In the present study, hairy skin of male Wistar rats was stimulated with slow, moderate and fast velocities (3, 9, and 18 cm/s, respectively). Affective state was measured with ultrasonic vocalization recordings; neural activity was indicated by c-Fos expressions in primary somatosensory, posterior insular cortex and periaqueductal gray. Fast stimulation was shown to increase the amount and duration of 22- kHz USVs, yet not cause a difference in c-Fos expressions. Furthermore, number and duration of calls emitted in the last minute of stimulation were found to correlate with c-Fos expressions in PAG and contralateral S1. Thus, gentle stroking alters the affective state, albeit in a negative manner. Results of the current study may highlight the importance of the source of gentle touch. Gentle touch originating from con-specifics or familiar other sources may be processed more positively compared to unfamiliar sources. Therefore, future research may focus on this familiarity effect and mimic the setting of con-specific touch to study the processing of CT- afferent stimulation.

Keywords: C-tactile afferent, affective touch, rat vocalization, periaqueductal gray, somatosensory cortex, insular cortex.

ÖZET

Duygusal Dokunmanın Sıçanlardaki Ultrasonik Vokalizasyonlara ve c-Fos Ekspresyonuna Etkisi

Dokunma duyusunun iki boyutu vardır: Ayrımsal ve duygusal. Dokunsal uyaranın ayrımsal özellikleri miyelinli ve hızlı ileten Aβ fiberleriyle taşınırken duygusal özellikler miyelinsiz ve yavaş ileten CT- fiberleri ile taşınır. CT- fiberlerinin yumuşak uyarımının hoşnutluk hissi ve insular korteks aktivasyonuna sebep olduğu gösterilmiştir. Mevcut çalışmada erkek Wistar sıçanlarının kıllı derisi yavaş, orta ve hızlı olmak üzere üç hızla uyarılmıştır (sırasıyla 3, 9 ve 18 cm/s). Afektif durum ultrasonik vokalizasyonlarla; nöral aktivite de periaqueductal grey, primer somatosensoriyel alan ve insular korteksteki c-Fos ekspresyonuyla ölçülmüştür. Hızlı uyarımın 22-kHz seslerin sayısını ve uzunluğunu arttırdığı ama c-Fos ekspresyonlarında bir farka sebep olmadığı bulunmuştur. Buna ek olarak, uyarımın son bir dakikasında yapılan vokalizasyonların sayısı ve uzunluğunun periaqueductal grey ve kontralateral primer somatosensoriyel alandaki c-Fos ekspresyonuyla korelasyonu bulunmuştur. Sonuç olarak, yumuşak uyarım afektif durumu olumsuz yönde etkilemektedir. Mevcut çalışmanın sonuçları yumuşak uyarımın kaynağının önemini vurgulamaktadır. Türdeşlerden ya da tanıdık diğer kaynaklardan gelen yumuşak uyarım, tanıdık olmayan kaynaklardan gelene kıyasla daha olumlu işleniyor olabilir. Bundan hareketle, gelecek çalışmalar bu tanıdıklık etkisi üzerine odaklanıp CT- aferentlerinin uyarımını çalışırken türdeş temasını taklit edecek şekilde tasarlanabilir.

Anahtar Sözcükler: CT afferent, duygusal dokunma, sıçan vokalizasyonu, periaqueductal grey, somatosensoriyel korteks, insular korteks.

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LIST OF SYMBOLS

Aβ	Myelinated Aβ fibers
Aδ	Thinly myelinated Aδ fibers
μ_m	Micrometer
V	Fifth cranial nerve

LIST OF ABBREVIATIONS

AI	Anterior Insula
CGRP	Calcitonin Gene Related Peptide
CL	Contralateral
CT	C- Tactile
DCN	Dorsal Column Nucleus
DI	Dysgranular Insula
DLPAG	Dorsolateral Periaqueductal Gray
DMPAG	Dorsomedial Periaqueductal Gray
EIG	Early Immediate Gene
FA	Fast - Adapting
fMRI	Functional Magnetic Resonance Imaging
GDNF	Glial Cell line-derived Neurotropic Factor
Gfra2	GDNF family receptor alpha-2
GI	Granular Insula
IB4	Isolectin B4
IL	Ipsilateral
kHz	Kilohertz
M	Mean
MrgprB4	Mas Related G- Protein Coupled receptor B4
ms	Millisecond
L1	Cortical Layer 1 of Primary Somatosensory Cortex
L2	Cortical Layer 2 of Primary Somatosensory Cortex
L3	Cortical Layer 3 of Primary Somatosensory Cortex
L4	Cortical Layer 4 of Primary Somatosensory Cortex
L5	Cortical Layer 5 of Primary Somatosensory Cortex
L6	Cortical Layer 6 of Primary Somatosensory Cortex
LPAG	Lateral Periaqueductal Gray
LTMR	Low- Threshold Mechanoreceptor

PAG	Periaqueductal Gray
PAGP1	Periaqueductal Gray P1
PB	Phosphate Buffer
PIC	Posterior Insular Cortex
ROI	Region of Interest
Runx1	Runt-related transcription factor 1
S1	Primary Somatosensory Cortex
S1-Tr	Trunk region of Primary Somatosensory Cortex
SA	Slowly - Adapting
SEM	Standard Error of the Mean
SD	Standard Deviation
TH	Tyrosine Hydroxylase
TrkA	Tropomyosin receptor kinase A
trpV1	The transient receptor potential cation channel subfamily V member 1
USV	Ultrasonic Vocalization
VGLUT3	Vesicular Glutamate Transporter Type 3
VLPAG	Ventrolateral Periaqueductal Gray

1. INTRODUCTION

Skin is the largest organ of humans consisting of approximately 16% of total adult weight [6, 7]. It contains approximately 640.000 sensory receptors mediating different submodalities, 2 million sweat glands and 5 million hair follicles [8, 9]. Skin consists of two main layers, namely epidermis and dermis. Epidermis is the outer layer with stratified epithelial cells containing keratin and melanin, while dermis is the deeper layer that is rich with vessels, nerve endings, glands, fat cells, hair follicles, and mechanoreceptors. The skin on mammalian body is differentiated according to physical characteristics and the body locus. Hairless (glabrous) skin is found on the palm of the hands and the soles of the feet, and hairy skin covers remaining parts of the body [2, 10]. Generally, skin plays role in somatosensation, thermoregulation, protection of internal organs, and vitamin D synthesis [7].

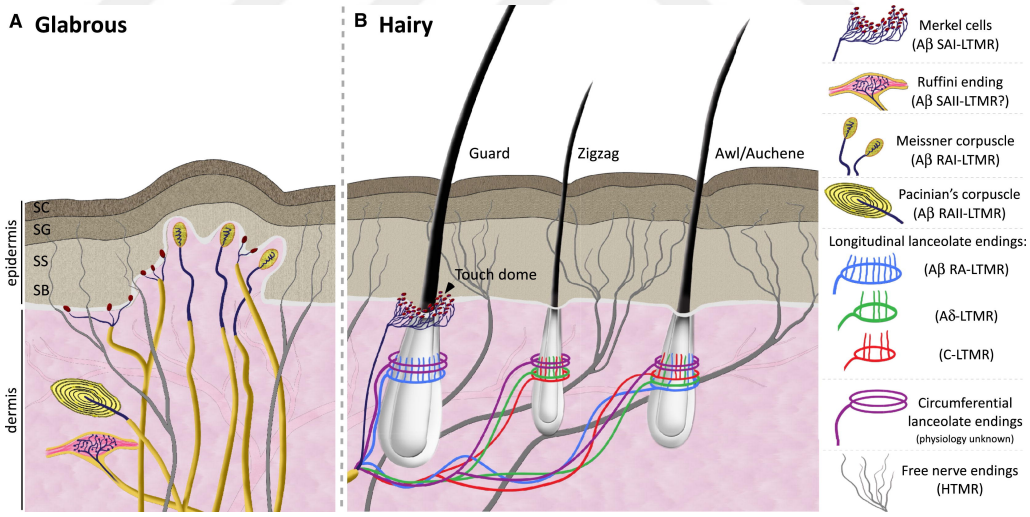


Figure 1.1 End organs and fibers for submodalities of somatosensation in (A) glabrous and (B) hairy skin (Adopted from [1]).

As the first developing and operating sensory organ, skin mediates 4 submodalities of somatosensation: touch, temperature, pain and itch [8, 11] (Figure1.1).Touch sensation occurs when mechanoreceptors are activated with contact stimuli, while thermal, nociceptive and pruritic senses occur by the activation of free nerve endings with related stimuli [9, 11].

1.1 Discriminative and Affective Touch

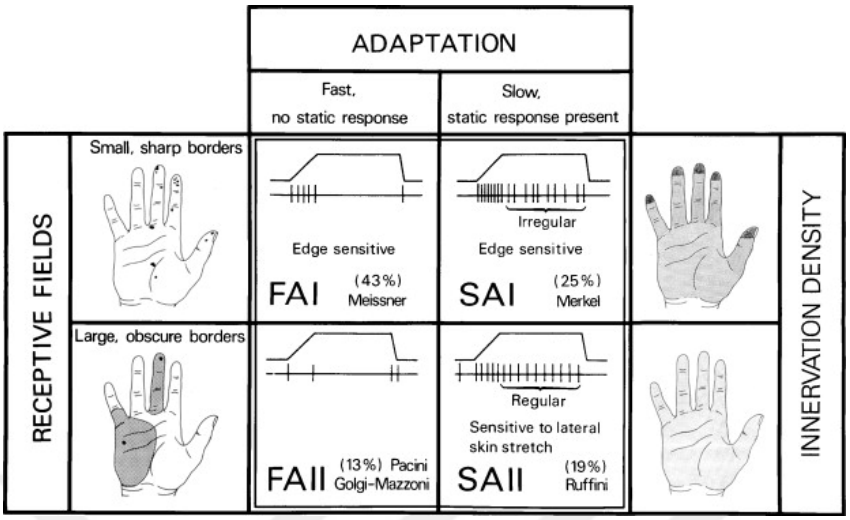


Figure 1.2 Mechanoreceptors in glabrous skin classified by their adaptation patterns and receptive field sizes (Adopted from [2]).

The touch sensation consists of two systems specialized to collect information about different aspects of a tactile stimulus [3]. First system has evolved to acquire data regarding discriminative properties of tactile stimuli such as indentation, stretch, movement and vibration. In glabrous skin, specific mechanoreceptors sensing these properties are known as Merkel cells, Ruffini endings, Meissner corpuscles and Pacinian corpuscles, respectively. On the tip of these mechanoreceptors, non- neuronal accessory structures selectively filter the mechanical stimuli and regulate the ionic environment around the nerve ending. The mechanical energy is transformed into an electrical signal, namely receptor potentials, by the alteration of ionic gradients in the unmyelinated end of the nerve. When receptor potentials exceed the threshold, action potential is fired and propagated along the myelinated nerve fibers [2, 10].

Each of these low-threshold mechanoreceptors and associated nerve fibers in glabrous skin are classified according to their receptive fields and adaptation patterns (Figure 1.2). Afferents that respond to the onset of sustained indentation are named as fast-adapting (FA), while ones that continue to respond during the stimulation are known as slowly-adapting (SA) afferents. Regardless of adaptation type and the size of the receptive field, all discriminative information about the tactile object is relayed

through myelinated and fast- conducting Aβ fibers whose cell bodies lie in the dorsal root ganglia.

In hairy skin, low-thresholds mechanoreceptors are found either in the touch dome (SA Merkel cells) or around the hair shaft forming longitudinal lanceolate endings (RA-LTMR). Information regarding discriminative properties of tactile stimuli are collected by the deflection of guard and awl/Auchene hair follicles and transmitted via myelinated Aβ fibers (Figure 1.3).

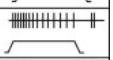
Physiological subtype	Associated fiber (conduction velocity[1])	Skin type	End organ/ending type	Location	Optimal Stimulus[4]	Response properties
SAI-LTMR	Aβ (16-96m/s)	Glabrous	Merkel cell	Basal Layer of epidermis	Indentation	
		Hairy	Merkel cell (touch dome)	Around Guard hair follicles		
SAII-LTMR	Aβ (20-100m/s)	Glabrous	Ruffini[2]	Dermis[3]	Stretch	
		Hairy	unclear	unclear		
RAI-LTMR	Aβ (26-91m/s)	Glabrous	Meissner corpuscle	Dermal papillae	Skin movement	
		Hairy	Longitudinal lanceolate ending	Guard/Awl-Auchene hair follicles	Hair follicle deflection	
RAII-LTMR	Aβ (30-90m/s)	Glabrous	Pacinian corpuscle	Deep dermis	Vibration	
Aδ-LTMR	Aδ (5-30m/s)	Hairy	Longitudinal lanceolate ending	Awl-Auchene/ Zigzag hair follicles	Hair follicle deflection	
C-LTMR	C (0.2-2m/s)	Hairy	Longitudinal lanceolate ending	Awl-Auchene/ Zigzag hair follicles	Hair follicle deflection	
HTMR	Aβ/Aδ/C (0.5-100m/s)	Glabrous Hairy	Free nerve ending	Epidermis/Dermis	Noxious mechanical	

Figure 1.3 Properties of low threshold mechanoreceptors and associated nerve fibers(Adopted from [1]).

Discriminative touch system enables organism to locate a tactile stimulus accurately in space and time. Information regarding the physical properties of stimuli is conveyed via large diameter, fast- conducting Aβ fibers to dorsal horn of spinal cord. Aβ fibers synapse on spinal Lamina III-IV and dorsal column nucleus (DCN) consecutively. Projections from DCN form dorsal column medial lemniscal system that terminates in ventral posterior nucleus of thalamus. The thalamic nucleus then delivers information to the primary somatosensory cortex projecting to secondary somatosensory cortex, and other multimodal association areas [3, 12].

The other system that contributes to the sensation of touch is related to the affective properties of tactile stimuli. In contrast to the discriminative touch system, receptors and fibers related to affective processing are only found in hairy skin. These receptors surround the shafts of awl/Auchene and zigzag hairs and form longitudinal

lanceolate endings. The deflection of hair stimulates these endings and electrical signal is transmitted via C-tactile afferents that have small axon diameters. Since they are unmyelinated, their conduction velocity is slower than myelinated Aβ fibers. Information relayed by C-tactile afferents enters the spinal cord from spinal Lamina I-II, and is transferred to ventromedial posterior nucleus of thalamus via spinothalamic tract. Thalamus then projects affective information regarding tactile stimuli to emotion related brain regions such as insula and orbitofrontal cortex [3, 12].

Both discriminative and affective properties of tactile stimuli to face are collected by trigeminal (V) nerve, and relayed to ventral posteromedial nucleus of thalamus via trigeminal lemniscus. Later, discriminative and affective information are thought to be delivered to the related brain structures [12]

Discriminative and affective touch pathways are displayed in Figure 1.4. McGlone, Wessberg and Olausson [3] state that discriminative and affective aspects of touch sensation are integrated in cortical networks responsible for the perception. Since perception is highly dependent on contextual factors and experience, both systems should be healthy and work cooperatively to evaluate the meaning of touch.

1.2 Gentle Touch and C- Tactile Afferents

The role of unmyelinated afferents in sensation of touch was discovered in 1939 by Zotterman who realized that light touch activated small- diameter, unmyelinated C-fibers in cat's hind limb [13]. These fibers were abundant in the hairy skin of cat and their conduction velocity was slower (1 m/s) compared to myelinated fibers [14]. Additionally, C-fibers were highly responsive to hair movements [15], and manipulation of stimulation velocity altered their impulse rate [16]. Since monkeys were shown to have less unmyelinated C-fibers than cats [17], scientists have concluded that these fibers progressively decreased in number throughout the evolution. However, advances in microneurography technique revived the interest to the function of unmyelinated C-tactile (CT) afferents in humans (For a review see [18]).

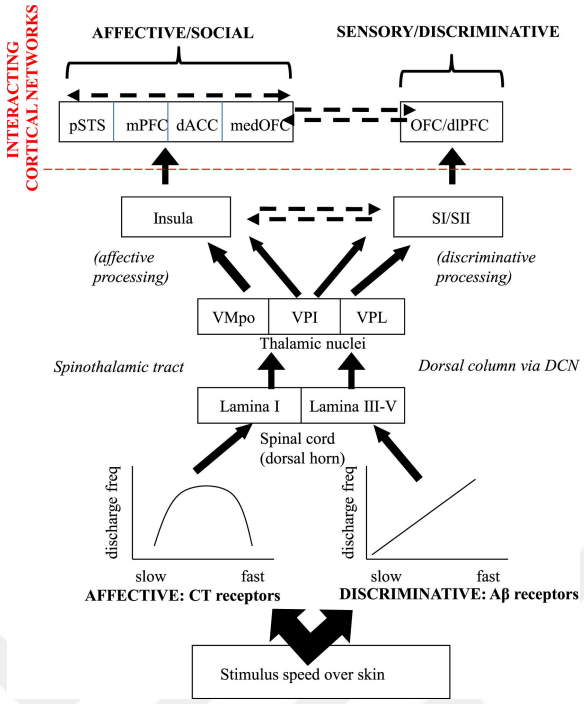


Figure 1.4 Affective and discriminative aspects of touch are processed in different systems to produce a complete perception of touch(Adopted from [3]).

In 1990, Nordin applied innocuous stimuli to faces of human participants and recorded the responses of unmyelinated CT-afferents [19]. Following studies using microneurography technique showed that humans also posses unmyelinated CT-afferents in hairy skin [20]. Researchers recorded the responses of single unit CT-afferents with tungsten electrodes placed in lateral antebrachial cutaneous nerve in the elbow and showed their high firing rate (50-100 impulses/s) to low indentation forces (0.3 – 2.5 mN). As with the non-human animal C-fibers, conduction velocity of CT-afferents is 0.6-1.3 m/s and they adapt to sustained stimuli intermediately [3, 9, 12, 20, 21]. Later studies revealed the velocity tuning of CT-afferents. Löken and colleagues [22] stimulated the forearm of human participants in different stroking velocities (0.1-30 cm/s) and recorded firing rate (impulses/s) of CT-afferents. They showed that neural discharge of CT afferents increased most to slow velocity stroking (3-10 cm/s) and decreased in response to faster or slower stimulation displaying an inverted U shaped pattern. Researchers also asked participants to rate how pleasant the stimulation was. Ratings corresponded to firing rate data and followed similar pattern as the 3-10 cm/s stimulation being the most pleasant, and other velocities being less pleasant [22].

One handicap of stimulation of hairy skin is that Aβ fibers are also activated together with CT afferents. This makes distinguishing the effect of CT afferent activation alone harder. There is a medical condition, called as sensory neuropathy, in which patients lack myelinated Aβ fibers that mediate discriminative touch. These patients are not able to feel tactile stimulations applied to glabrous skin, yet they can feel a " faint sensation" of touch when slow stroking is applied to hairy skin [23, 24]. Since these patients cannot feel vibrating stimuli on hairy skin but can feel gentle stroking, the role of CT-afferents in sensation of touch is supported. In another medical condition, patients lack unmyelinated C-fibers because of hereditary sensory and autonomic neuropathy. Patients lacking C-fibers have normal light touch perception, but their pleasantness ratings are significantly lower than healthy participants [25]. Dissociation of affective and discriminative systems is supported by different responses to gentle touch generated by patients with opposite pathologies.

Further evidence for the dissociation of affective and discriminative systems are obtained with the functional magnetic resonance imaging (fMRI) studies. Gentle touch to palm of hand activates contralateral somatosensory areas of healthy participants. On the other hand, gentle touch to forearm activates somatosensory areas, as well as emotion related areas like insular and orbitofrontal cortices in the same participants [23, 26]. fMRI studies with sensory neuropathy patients revealed that gentle touch to forearm activated contralateral insular cortex, but also inhibited the activity of somatosensory areas [23, 24]. Finally, relation of insular processing to CT-afferents involved affective touch is confirmed by the fact that lower pleasantness ratings of patients lacking C-fibers correspond to the lack of activity in insular cortices [25, 27].

Human insular cortices are covered by temporal and frontal lobes during the prenatal development (Figure 1.5) [28]. Morphologically, triangle shape of the insula is divided into two regions by sulcus centralis insulae. The anterior lobe of insula contains three gyri known as gyrus brevis primus, gyrus brevis secundus and gyrus brevis tertius, while posterior lobe contains two gyri, namely gyrus longus primus and secundus [29].

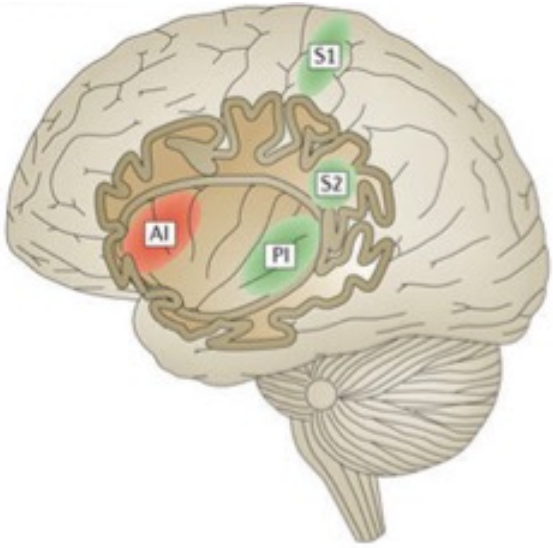


Figure 1.5 Human insula and primary somatosensory cortex(Adopted from [4]).

Another classification of insular region relies on cytoarchitectonic properties such as absence or presence of granular layer 4. The rostroventral agranular portion lacks granular layer 4 and is related to olfactory and gustatory processing. The caudal part shows more cortical lamination with a distinct granular layer. Granular insula is involved in somatosensory and auditory processing. The middle portion of insula does not display clear laminar differentiation and acts as a dysgranular transition zone between agranular and granular portions. This cytoarchitectonic formation determines the intra- insular information-processing pattern. Intra- insular connections are biased from anterior towards posterior portions. Anterior insula sends information to both mid- and posterior insula, but receives fewer connections. Similarly, posterior portion receives information from mid- and anterior insula with fewer afferents. Only middle portion have more balanced connections with two portions due to its polymodal integrative roles. Thus, there is a biased information flow from agranular to granular insula, which may be parallel to sophisticated laminar organization [28, 29].

In order to reveal somatotopic organization in insular cortex researchers gently stroked the forearm and thigh of participants. In both sensory neuropathy patients and healthy participants, contralateral posterior insular cortices were found to be somatotopically organized. In other words, distal parts of the body were represented in

more posterior parts of the insula compared to proximal parts of the body [30].

Even though the presence of two dissociated touch systems in humans was supported by psychophysical and imaging findings, there are still ongoing debates regarding the universality of these systems in other animals. In order to find biological underpinnings and evolutionary importance of having two distinct touch systems, researchers started to focus on animal models of CT-afferents using molecular, optical and biological approaches.

In recent years transgenic mice models have been abundantly used to reveal biological markers specific to C-afferents. The first study utilized a sensory neuron specific receptor subtype, namely Mas related G-protein coupled receptor B4 (MrgprB4). These receptors are expressed in non-nociceptive, small diameter and unmyelinated sensory fibers that projects to spinal Lamina 2. Similar to human CT-afferents, MrgprB4 positive fibers are found only in hairy skin and respond to innocuous, gentle stimuli. They have scattered nerve terminals branching extensively and found more frequently in proximal limbs than distal ones [31,32]. In addition to morphological similarities with human CT-afferents, stimulation of MrgprB4+ neurons is found to have an anxiolytic and positively reinforcing effect on animals. Pharmacological stimulation of MrgprB4+ neurons elicited conditioned place preference in mice indicating positive valence of activation of these neurons [32].

The other potential biomarker for CT-afferents is tyrosine hydroxylase (TH). TH expressing sensory neurons are shown to have small cell bodies, slow conduction velocities and intermediate adaptation to sustained stimuli. They are found more frequently in proximal parts of body rather than distal extremities such as hind and forepaws. Similar to MrgprB4+ neurons, TH expressing neurons are only found in hairy skin and terminate in spinal Lamina 2. Researchers also showed that TH positive neurons co-express VGLUT3 and form longitudinal lanceolate endings around zigzag and awl/Auchene hairs [33].

Commonly, none of the potential C-LTMRs express myelinated fiber markers

(NFH) or peptidergic nociceptor markers and receptors such as CGRP, TrkA, TrpV1. However, they seem to be classified into sub-types according to their protein and receptor expression patterns. MrgprB4 positive neurons co-express only IB4 and GDNF co-receptor c-Ret [31], while TH positive neurons co- express VGLUT3 and Gfra2 [33]. Other studies also show that TH and VGLUT3 positive neurons may express Runx1 [34] or TAFA4 [35].

There is no consensus about the sub-types of low threshold C mechanoreceptive fibers and their compatibility with human CT-afferents. Despite lack of experimental evidence in positive affective processing, TH+ and VGLUT3+ neurons are considered as more likely to be the non-human animal counterpart of CT-afferents. Even tough there are abundant similarities between MrgprB4+ neurons and CT-afferents, these neurons are considered as a different class of CT-fibers [36].

1.3 Communication of Affective States and Ultrasonic Vocalizations

In general terms, communication is defined as the process of transmission of any signal between a sender and a receiver for a useful purpose. Even tough it seems to evolved for conveying information to conspecifics, sender aims to express its emotional state to influence the other's behaviors rather than intentionally informing them [37–39]. By expressing an emotional state, sender induces a biologically well- adapted behavior compatible with the internal state of perceiver and this mutual adaptation increases the fitness of both parties [40, 41].

Animals use visual displays, chemical and tactile [42] signals as well as vocal signals that enable long- distance communication [43]. Conveying emotional state by vocal signals in mammals facilitates the mother- infant relationship, organization of social groups, mating and other reward seeking behaviors, defensive and avoidance behaviors [44]. Some vocal signals are in the ultrasonic range, which allows them not

to be detected by a predator. Rodents living in well-organized social colonies utilize ultrasonic signals as higher- order defensive systems [45]. They are more suitable for transmission of emotional state to conspecifics in the underground burrows, and augment the cooperative behavior within the colony [46].

Rats use three types of ultrasonic vocalizations (USV) to relay different emotional states depending on the occasion. Infant rats produce 40 kHz ultrasonic calls when they are separated from their mothers, especially during 4- 16 days of age [47,48]. Even though mean frequency is at 40-kHz, these calls are emitted in the range of 30-65 kHz for 80-140 milliseconds [49, 50]. Infant cries are indices of emotional distress and motivated to attract attention of the mother [51]. Dams display approach and inspection behavior towards the origin of calls supporting the idea of infant cries serve a communicative purpose with mother [52].

Adult and juvenile rats use primarily two kinds of ultrasonic vocalizations. Rewarding and hedonic experiences trigger the production of 50-kHz ultrasonic signals associated with joy and happiness [5, 53]. This positive affective state manifests itself with increased locomotor activity, rearing and exploration [54]. These appetitive calls are produced in frequencies between 35-70 kHz with mean frequency of 50-kHz and last 20-80 ms [54]. Social approach by conspecifics, juvenile rough- and- tumble play, male- female social exploration and copulation are some of the social stimuli that elicit 50-kHz USVs. Additionally, pharmacological stimulation of mesolimbic dopaminergic system initiates positive affective state accompanied by 50-kHz calls (for a review see [5]). Peripheral or central administration of drugs like amphetamine and cocaine elicit 50-khz USV response via the activation of dopaminergic pathways in nucleus accumbens and ventral tegmental area [55] (Figure 1.6). 50-kHz USVs are considered as the homolog for human laughter and claimed to aim establish and maintain social proximity between rats [5, 56].

Encountering with aversive stimuli or anticipation of it causes adult and juvenile rats to produce low frequency (18-32 kHz) and long (300-3000 ms) 22-kHz alarm calls. These types of calls are associated with anxiety and fear, and correlates with freezing,

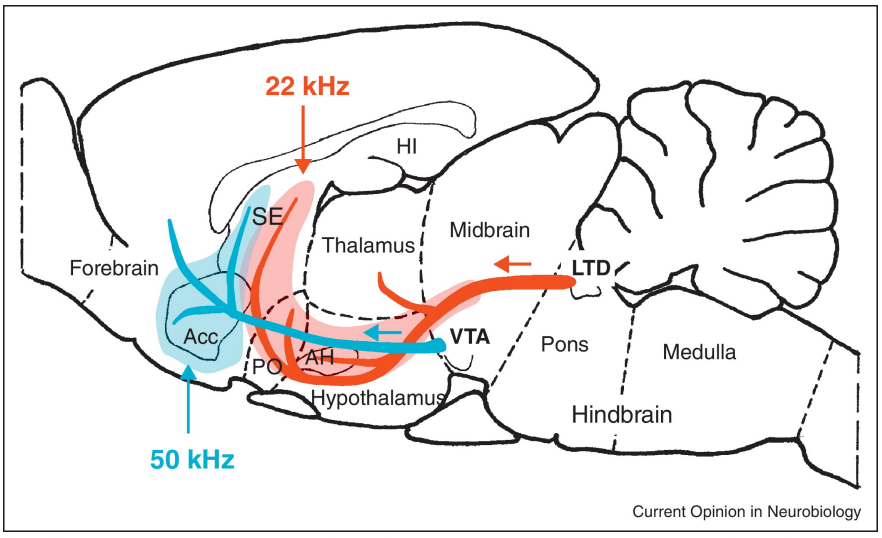


Figure 1.6 Different systems mediate the production of 50-kHz and 22-kHz ultrasonic vocalizations (Adopted from [5]).

motionless posture and strong expiratory movements. [53, 57]. Exposure to predators and intruders, fighting with conspecifics, painful and startling stimuli like footshock or air puff are shown to trigger 22- kHz USV response citeknut:02- [5]. Studies administering cholinergic agents showed that 22-kHz USVs and related anxiety behavior is mediated by the midbrain cholinergic system. Emission of 22-kHz alarm calls is associated with social transmission of fear and expression of affective distress [5].

Juvenile and adult rats are shown to react to tactile stimulation from familiar humans in a positive manner. When tickled by an experimenter, play-experienced rats show increased locomotor activity and 50-kHz USV emission. However, tactile contact with an unfamiliar experimenter evokes avoidance behavior, freezing and 22-kHz USV emission. Repeated exposure to tickling procedure ameliorates the affective state indicated by decreasing 22-kHz calls and increasing 50- kHz calls [58]. Other acute tactile stimulation procedures are also perceived as aversive by naïve rats, but 22-kHz USVs decrease in number by repeated stimulation paradigms [59].

1.4 Neural Activity and c-Fos Expression

In animals, neuronal activation can be detected indirectly by using immunohistochemistry methods. One of these methods is based on the staining of the early immediate genes (EIG) such as c-Fos, c-Jun, zif 268 and nur/77. Since the expression of EIGs depends on the calcium influx triggered by action potentials, c-Fos expressing cells are considered to be activated by external or internal stimuli [60].

These genes act as a rapid response to stimulation within minutes and control the expression of late response genes. Calcium influx caused by action potential regulates c- Fos expression via cAMP and CREB cascades. The product of c-Fos gene interacts with products of other IEGs and forms a heterodimer. This heterodimer binds with DNA elements and initiates transcription of late response genes. Activation of IEGs can be triggered by the convulsant drugs, electrical stimulation of the neuron, and peripheral sensory stimulations [61].

Tactile stimulations are shown to increase c-Fos expression in related areas of somatosensory cortex. Mechanical stimulation of whiskers has been shown to increase c-Fos expression in barrel field of contralateral somatosensory cortex [62,63]. Similarly, playback of 22-kHz and 50-kHz ultrasonic vocalizations trigger c-Fos expressions in related brain regions. Rats exposed to conspecifics' positive affective state calls express more c-Fos protein in frontal association cortex and nucleus accumbens, while playback of 22-kHz negative affective state calls increased c-Fos expressions in amygdala and periaqueductal gray, especially rostral dorsolateral, dorsomedial and lateral sub-regions [64–67].

1.5 Aim of the Study

Despite the discovery of possible molecular markers for low-threshold C mechanoreceptive fibers in non-human animals, evidence regarding direct modulation of affective

state in response to gentle touch is lacking. One study investigated the direct effect of gentle touch on affective state in rats. Okabe and colleagues [68] stimulated the dorsal body of rats with a cotton glove manually once in a day for a week. They also recorded ultrasonic vocalizations as indices of affective state and at the end of the last session they measured neural activity in oxytocin related brain regions with c-Fos staining. Rats that received gentle touch produced more 50-kHz USVs compared to the control group, yet there was no difference in c-Fos expressions [68].

In the light of previous findings, the present study aimed to investigate the effect of gentle touch on immediate affective state as indicated by USV emissions and neural activity as measured by c-Fos activation in related brain regions. Particularly, it is attempted to replicate tuning patterns of human CT-afferents in response to different stimulation velocities. In that sense, it is the first study that controls the effect of gentle touch delivered with different velocities in rats. Even though USV recordings and c-Fos expressions were utilized in the study cited above, the current study is the first to investigate neural activity in the USV and affective-touch-related regions at the same time.

Studies with human participants highlight the affective consequences of CT-afferent stimulation. In parallel with these findings, it is hypothesized that the current stimulation paradigm will succeed in altering affective state of rats. Considering the inverted U shaped response pattern of CT-afferents, it is further hypothesized that the stroking with moderate velocity would be perceived as the most pleasant by rats rather than the slower and faster velocities. Therefore, 50-kHz USV emissions are expected to increase, while 22-kHz USVs are expected to decrease during tactile stimulation with moderate velocity. Also, c-Fos expressions in USV related brain regions are expected to change correspondingly.

Due to the contralateral processing of sensory stimuli, unilateral stimulation is expected to evoke more c-Fos expression in brain regions of contralateral hemisphere than ipsilateral counterpart. Since the gentle touch also activates Aβ afferents, contralateral trunk regions of contralateral primary somatosensory (S1) cortex is expected

to contain more cells expressing c-Fos protein. This difference is expected to be the most salient in input and output layers of S1.

Stimulation of CT-afferents in human participants is correlated with the activity of contralateral posterior insular cortices. Accordingly, it is hypothesized that the current gentle touch paradigm will activate the corresponding part of the insula in rats. In rodents, insular cortex is found on the lateral surface of the cerebral hemispheres, just dorsal to the rhinal sulcus [69]. Similar to human insula, it consists of three cytoarchitectonically distinct areas according to laminar differentiation. Granular, dysgranular and granular portions of insula are placed in ventro- dorsal order. The most ventro-rostral insula starts with agranular portion that is involved with nociceptive and autonomic processes. The most caudal part consists of granular insula specialized for visceral and somatosensory information processing. The dysgranular area is located on the dorsal side of agranular and ventral side of granular portions, and is associated with visceral and gustatory processing [70]. Therefore, granular part of the contralateral posterior insular cortex (PIC) is expected to express more c-Fos in response to unilateral gentle touch stimuli.

In terms of velocity modulation, moderate speed stroking is hypothesized to be the most pleasant. Therefore, it is expected to alter neural activity in contralateral PIC compared to slow and fast velocities, while preserving approximately same level of activity in S1.

Lastly, c-Fos expressions in touch related areas (S1 and PIC) are expected to correlate with vocalizations emitted during the stimulation, as well as with USV related brain regions (e.g. PAG, ventral tegmental area etc.).

2. MATERIALS AND METHODS

2.1 Subjects

Twenty- four experimentally naive male albino Wistar rats weighting between 300-400 gr, were used in the experiments. They were maintained in a 12:12 light/dark cycle and were provided ad libitum water and food. All procedures of the experiment were approved by Boğaziçi University Institutional Ethic Committee for the Local Use of Animals in Experiments (BÜHADYEK).

2.2 Tactile Stimulation

Rats were randomly assigned to three different stimulation velocities in the first experimental session and were exposed to other velocities in following sessions. The order of velocities was counterbalanced and each experimental session was separated by a week. Rats were taken from their home cages and placed in the experiment room in which they were allowed to habituate for 10 minutes. Then, rats were exposed to stimulation for 5 minutes and remained in the post-stimulation episode for another 5 minutes. Tactile stimulation was applied manually by the same experimenter with an artist's brush. Bristles were 25 mm long, and the width of the brush was 25 mm. They received slow (3 cm/s), moderate (9 cm/s), or fast (18 cm/s) velocity stimulation. The right side of the dorsal trunk of the free behaving rat was marked in 9 cm length. Assigned stroking velocity was adjusted according to the time required to complete one single rostro-caudal stroke. In order to deliver slow, moderate and fast velocity 3 seconds, 1 second and 500 milliseconds were required, respectively, to cover the area of stimulation. During the stimulation rats were allowed to move freely and explore the brush. Following the stimulation, rats remained in stimulation area for 5 more minutes, and then they were returned to their home cages.

2.3 USV Recording

USV emissions were recorded by the system developed by Professor Burak Güçlü in the Institute of Biomedical Engineering. The range of recording frequency was 13 kHz-100 kHz. The dynamic range was 35-108 dB SPL. The sampling rate of the data acquisition was 400 kHz and resolution was 16 bits. In order to identify vocalizations, sound data was visualized in a custom made MATLAB program. Vocalizations in sound spectrograms were visually classified based on their frequency, duration and intensity properties. Only 22-kHz vocalizations were detected throughout the experiment. Those with durations longer than 300 milliseconds were considered as a bout, and the number and duration of bouts were counted as indicators of affective state. In accordance with the previous literature signals with durations less than 300 ms were not analyzed.

2.4 Slice Preparation

Ninety- minutes after the last tactile stimulation, rats were anesthetized with intraperitoneal ketamine (100mg /kg) and xylazine (10mg/kg) injections, and were intracardially perfused with 0.9 % saline and 4 % paraformaldehyde 0.1 M phosphate buffer, consecutively. The brains were sectioned in 50 μm thickness in 0.1 PB via a vibrating- blade microtome (Leica, VT 1000 S, Germany).

2.5 c-Fos Staining

On first day, free- floating sections were blocked by 3 % hydrogen peroxide (H_2O_2) and 10 % methanol in 0.1 M phosphate buffer (PB) between two rinsing (PB). Rabbit anti-fos primary antibody (polyclonal, sc-52, Santa Cruz Biotechnology) was used in 1:2500 dilutions and dissolved in 0.1 M PB containing triton-x and gelatin. Sections were incubated in primary antibody for 48 hours at +4 °C. Afterwards sections were rinsed in 0.1 M PB. The secondary antibody, biotinylated goat anti-rabbit

IgG (BA-1000, Vector Laboratories) was used with a dilution of 1:400 in 0.1 M PB containing triton-x and gelatin. Sections were incubated in secondary antibody for 1 hour at room temperature that was followed by another rinsing step. Next, sections were exposed in 1:800 dilution to ABC (PK-6100, Vector Laboratories) in 0.1 M PB containing triton-x and gelatin for 1 hour. After the last rinsing step, DAB (SK-4100, Vector Laboratories) was applied and sections were mounted on gelatinated slides. Following day, mounted sections were exposed to alcohol series for dehydration and xylene for clearing. Slides were sealed with mounting medium (Vecta Mount). The details of immunohistochemistry procedure for c-Fos are explained in Appendix A.

2.6 Imaging and Cell Counting

The Rat Stereotaxic Atlas [71] was used to determine the regions of interest (ROI) in the brain with respect to the bregma. Natural boundaries of regions were determined according to the atlas. Table 2.1 shows the coordinates for regions of interest. Regions of interest were imaged via Leica camera and imaging system (Figure 2.1). Cells expressing c-Fos were counted with ImageJ software manually.

Table 2.1
Stereotaxic coordinates for regions of interest.

	Coordinates	
Region of Interest	**Start**	**End**
Primary Somatosensory Cortex	-2.40	-3.00
Posterior Insular Cortex	-2.40	-2.92
Rostral Periaqueductal Gray		
P1	-4.20	
DM	-5.28	
L	-5.28	-7.00
DL	-5.88	
VL	-6.60	

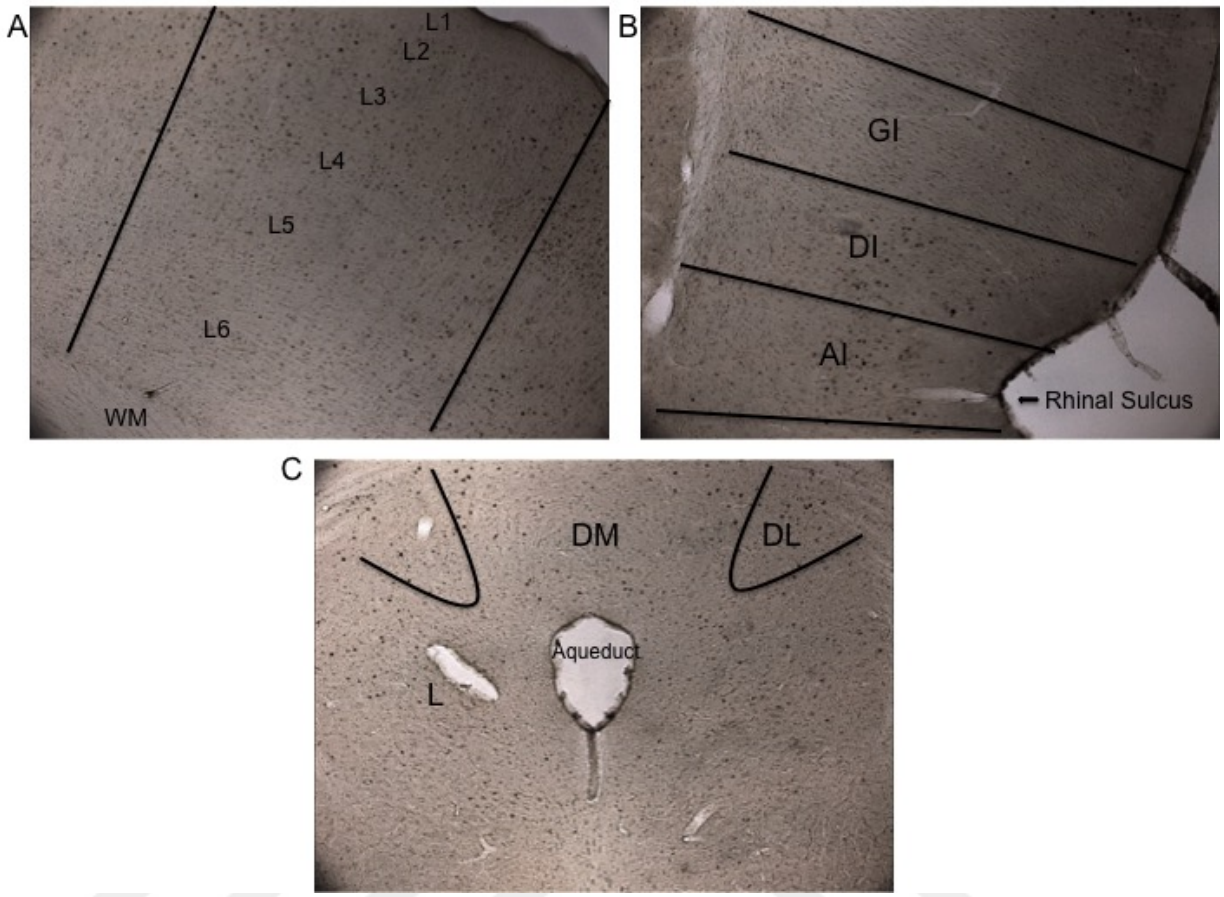


Figure 2.1 Region of Interests. A) Trunk region of primary somatosensory cortex (Bregma -2.76) B) Posterior insular cortex (Bregma -2.76) C) Periaqueductal gray (Bregma -6.00).

2.7 Statistical Analysis

Experimental procedure is as in Figure 2.2. The number and duration of ultrasonic vocalizations measured for all animals (n=24) were analyzed with repeated subjects ANOVA to reveal the effect of velocity modulation. The hemispheric differences in c-Fos expressions in response to unilateral stimulation, regardless of the stimulation speed received in the last stimulation session were tested for each animal. A repeated measures t-test (n=18) was conducted comparing the c-Fos expressions in contralateral and ipsilateral somatosensory and insular cortices. Next, the effect of stimulation velocity received in the last experimental session on the c-Fos expression was investigated. Due to low number of subjects per group (n=6), a non-parametric Kruskal-Wallis test was conducted.

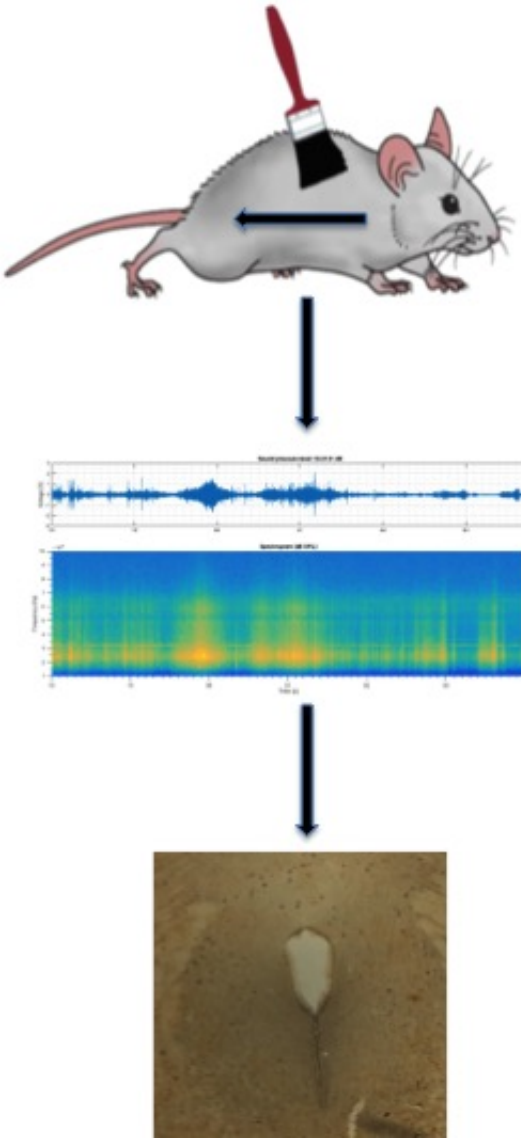


Figure 2.2 Experimental procedure.

3. RESULTS

3.1 Ultrasonic Vocalizations

Rats were subjected to three experimental sessions and allowed to rest between sessions for one week. Each session consisted of a ten-minute long habituation episode, five-minute long stimulation and five-minute long post-stimulation episode. Ultrasonic vocalizations were recorded during whole session. Rats received different velocity of stimulation in each week, and the order of the velocities was counterbalanced.

As a first step, ultrasonic vocalizations were visualized and classified according to their frequency and duration range by MATLAB. Results showed that rats emitted negative affect indicating 22- kHz, but not positive affect indicating 50 kHz vocalizations. Calls longer than 300 ms were considered as a bout and the number and duration of bouts were counted. Therefore, further analyses were conducted with respect to negative affective state.

In order to test whether the stimulation paradigm alters the affective state, a 3x 4 repeated measures ANOVA was conducted for number and duration of USVs emitted throughout the three experimental sessions. Means and standard deviations are displayed in Table 3.1. Regardless of stimulation speed, all rats emitted more USVs in their first experimental session compared to the following ones ($F(2,46) = 25.16$, $p < 0.01$). Similarly, they emitted longer calls in first session than second and third sessions ($F(2,46) = 18.18$, $p < 0.01$).

During the first five minutes of habituation rats emitted more alarm calls compared to the second half of habituation indicating rats became familiarized to the room and their affective state was stabilized. As seen in Figure 3.1a, initiation of the tactile stimulation increased the number of 22-kHz USV emissions and with the termination of stimulus emission was decreased to pre-stimulus level ($p < .01$). Number of USV

Table 3.1
Means and standard deviations for number and duration of ultrasonic vocalizations.

		Ultrasonic Vocalizations			
		Number		Duration (s)	
Session	**Episode**	**Mean**	**Std Dev**	**Mean**	**Std Dev**
1	Habituation (0- 5 min)	40.5	17.6	42.4	19.3
	Habituation (6- 10 min)	12.9	11.1	12.9	11.1
	Stimulation	33.1	13.4	46.3	19.9
	Post- Stimulation	10.5	6.9	10.0	6.8
2	Habituation (0- 5 min)	24.5	12.0	26.2	12.8
	Habituation (6- 10 min)	9.5	8.7	9.3	8.6
	Stimulation	21.5	8.1	33.0	14.7
	Post- Stimulation	5.1	4.0	5.5	4.6
3	Habituation (0- 5 min)	19.7	8.9	20.8	9.3
	Habituation (6- 10 min)	7.0	6.6	5.6	6.1
	Stimulation	27.3	15.5	36.9	24.1
	Post- Stimulation	9.6	5.3	9.6	5.7

emissions during stimulation was significantly greater than the pre-stimulus and post-stimulus episodes ($F(3, 69) = 67.61$, $p < 0.01$).

Correspondingly, rats produced longer calls during the first 5 min of habituation and stimulation than the second half of the habituation and post- stimulation episodes regardless of stimulation speed delivered (Figure 3.1b), ($F(3,69) = 75.67$, $p < 0.01$). Additionally, pairwise comparisons showed that duration of USVs were greater during stimulation than the pre-stimulus and post- stimulus episodes ($p < 0.01$). Thus, current stimulation paradigm was shown to sufficient to alter affective state of rats.

Next, the effect of velocity modulation on vocalizations produced during stimulation was tested. Repeated measures ANOVA revealed that velocity manipulation had a statistically significant effect on affective state measured by 22- kHz USV emissions, ($F(2,46) = 7.48$, $p = 0.002$). The amount of emissions did not differ between slow ($M = 21.42$, $SD = 13.42$) and moderate ($M = 26.25$, $SD = 12.68$) velocities. Fast velocity ($M = 34.88$, $SD = 12$) stimulation caused more 22- kHz USV emissions than slow and

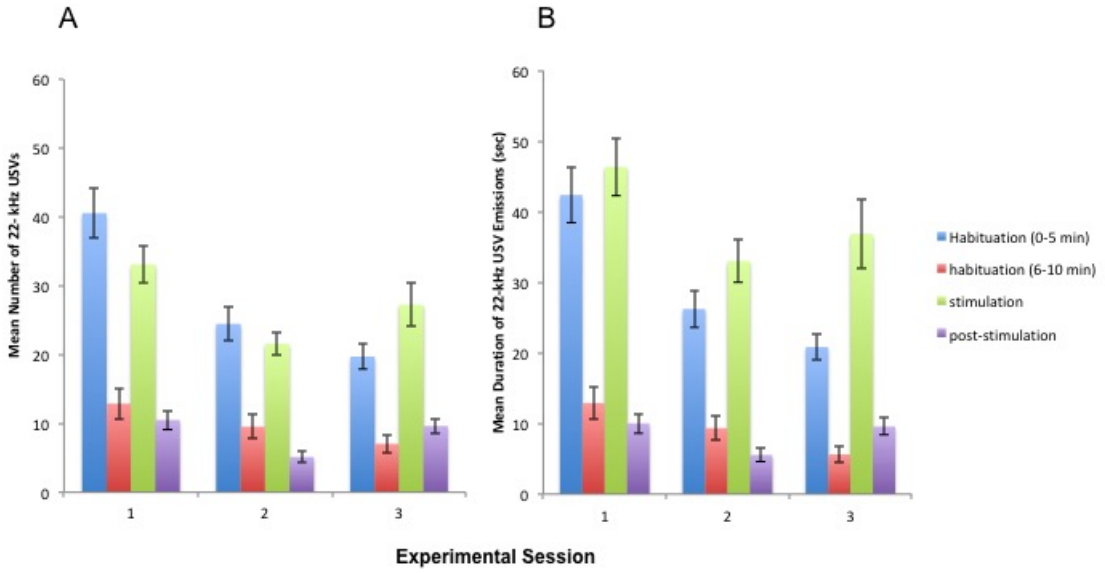


Figure 3.1 A) Mean number and B) duration of 22-kHz USVs through out the experimental sessions.

moderate velocities (p= 0.001 and p= 0.011, respectively). (Figure 3.2a)

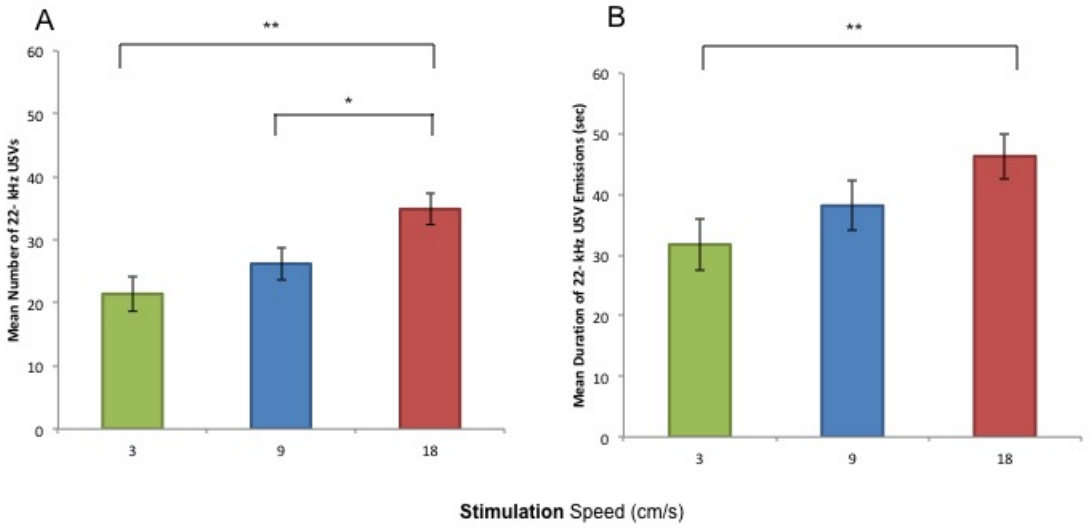


Figure 3.2 A) Mean number and B) duration of 22- kHz USVs during stimulation.

Similarly, the duration of 22- kHz USVs are altered by velocity modulation (F (2,46) = 4.71, p= 0.014). The duration of vocalizations in response to slow (M= 31.75. 83, SD= 21) and moderate (M= 38.1, SD= 20.1) velocities was not different from each other. In the case of fast velocity (M=46.3, SD= 18.3) stimulation, rats produced significantly longer calls than slow stimulation (p = 0.004), but difference with moder-

ate velocity stimulation remained marginal (p= 0.079) (Figure 3.2b). Further analysis was conducted to reveal how stimulation velocity modulates USV responses minute by minute during stimulation. Rats emitted more and longer calls during the first minute of stimulation episode compared to the following minutes, yet vocalizations did not differ in response to velocity modulation. These supplementary results are presented in Appendix B.

3.2 c-Fos Expressions

Ninety minutes after the last stimulation session, rats were anesthetized and perfused. Fixated brain sections were stained for c-Fos, and positive cells in periaqueductal gray, trunk area of primary somatosensory cortex and posterior insular cortices in contralateral and ipsilateral hemispheres were counted.

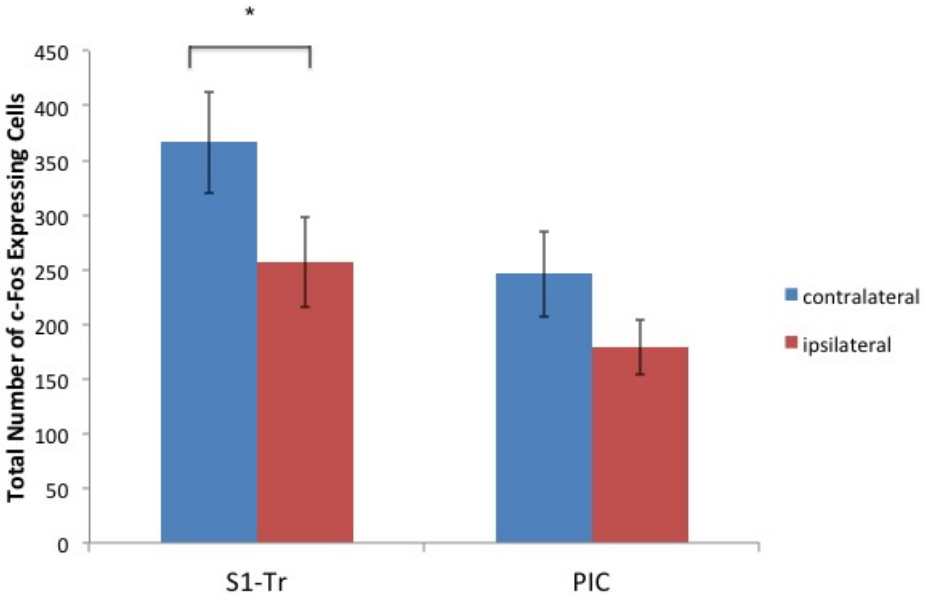


Figure 3.3 Hemispheric differences in c- Fos expressions in response to unilateral stimulation.

Regardless of speed, tactile stimulation was applied unilaterally (right dorsal trunk) and expected to be processed more in contralateral hemisphere. A paired samples t- test was conducted to reveal any hemispheric asymmetries in c-Fos expression. Contralateral somatosensory cortex (M= 366.44, SD= 197.03) was found to express

more c-Fos protein than the ipsilateral hemisphere (M= 256.78, SD= 176.14); (t (17)= 3.56, p= .002). (Figure 3.3)

Further analysis for differences in cortical layers reveals that except layer 1, all layers in contralateral somatosensory cortex contain significantly more cells expressing c-Fos protein (Figure 3.4). Means and standard deviations regarding the c- Fos expressions in layers were given in Table 3.2.

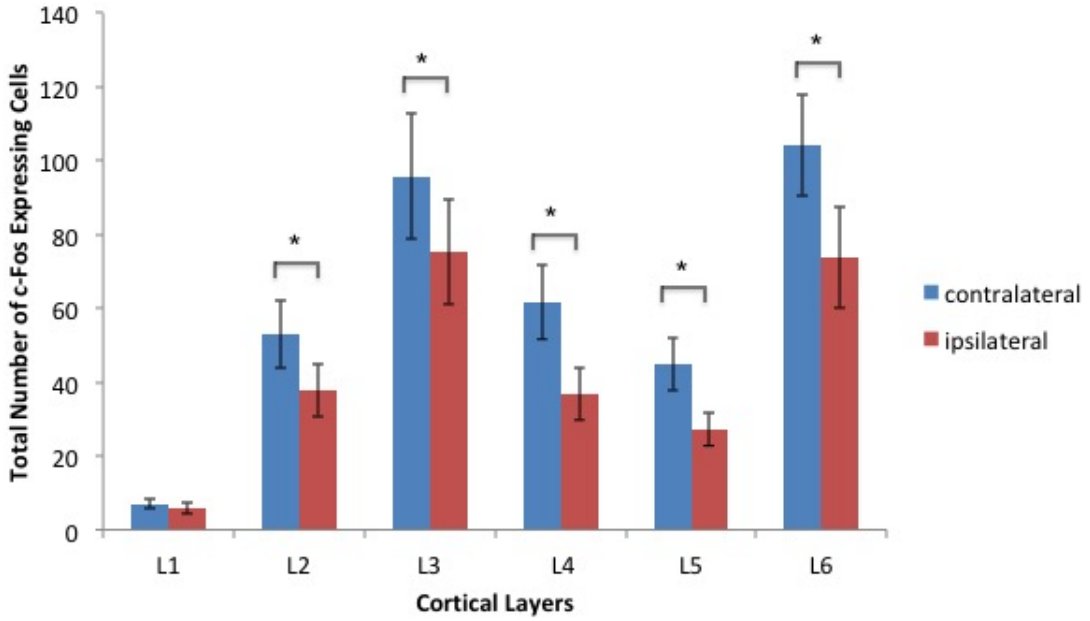


Figure 3.4 c- Fos expressions in contralateral and ipsilateral somatosensory cortices.

Another area thought to be related to c-tactile processing is posterior insular cortex. Paired samples t- test results showed that cells in the contralateral PIC expressed marginally more c-Fos protein (M= 245.8, SD= 167.6) than ones in ipsilateral PIC (M= 179.1, SD= 106.6); t (17)= 2.101, p = .051. Sub-regions of PIC were also tested for any significant differences in c-Fos expression. Contralateral granular insular cortex (M= 63.1, SD= 47.4) was found to contain more cells expressing c-Fos than ipsilateral counterpart (M= 36, SD= 26.4); t (17)= 2.262, p= .037. Dysgranular and agranular insular cortices did not differ in c-Fos expression between hemispheres (Figure 3.5).

In order to reveal the effect of stimulation speed on the number of c-Fos ex-

Table 3.2
Means and standard deviations for c-Fos expressions in layers of contralateral and ipsilateral somatosensory cortices.

	Contralateral		Ipsilateral		
Layer	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**t (n=18)**
1	7.0	5.6	5.9	1.3	.849
2	59.9	38.7	37.6	30.2	2.532*
3	95.7	72.3	75.4	60.5	2.120*
4	61.8	43.1	36.8	29.9	3.255**
5	44.8	30.9	27.2	19.0	3.033**
6	104.2	58.2	73.9	58.4	2.293*

* p <.05

** p <.01

pressing cells, a non-parametric Kruskal-Wallis test was run. Stimulation speed was shown to have no effect on c-Fos expressions in PAG, S1 and PIC (Fig 13). Means and standard deviations are given in table 4. Further analysis was conducted indicating that sub-regions of these areas also did not differ in terms of c-Fos expression with respect to stimulation speed. Tables showing means and standard deviations of sub-regions of PAG, S1- trunk and PIC as well as related figures are presented in Appendix C.

Table 3.3
Means and standard deviations for c-Fos expressions in PAG, S1 and PIC.

ROI	**Mean**	**Std. Dev**
PAG	691.6	398.1
Contralateral S1	366.4	197.0
Ipsilateral S1	256.8	176.1
Contralateral PIC	245.8	167.6
Ipsilateral PIC	179.1	106.6

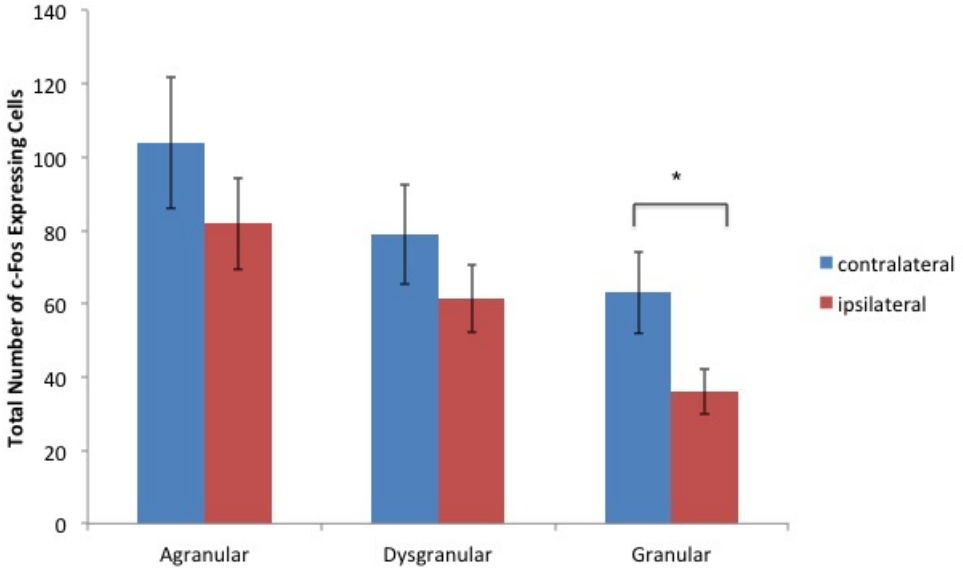


Figure 3.5 Hemispheric differences in c-Fos expressions of PIC.

3.3 Correlations Between Vocalization and c-Fos Expression

First of all, regardless of velocity received correlations between brain regions and sub- regions were investigated. Correlations (n=18) between PAG, S1 –trunk and PIC in both hemispheres are presented in Table 3.4.

Table 3.4
Correlation coefficients for PAG, S1 and PIC.

	Variable	1	2	3	4	5
1	PAG	-				
2	Contralateral S1	.74**	-			
3	Ipsilateral S1	.71**	.76**	-		
4	Contralateral PIC	.68**	.52*	.49*	-	
5	Ipsilateral PIC	.66**	.67**	.78**	.60**	-

* p<.05

** p<.01

Next, the number and duration of 22- kHz vocalizations emitted in the course of stimulation and c-Fos expressions were analyzed for possible correlations. There were no significant correlations, but further analysis with minutes of stimulation was conducted. Results showed that number of emissions in the last minute of stimulation

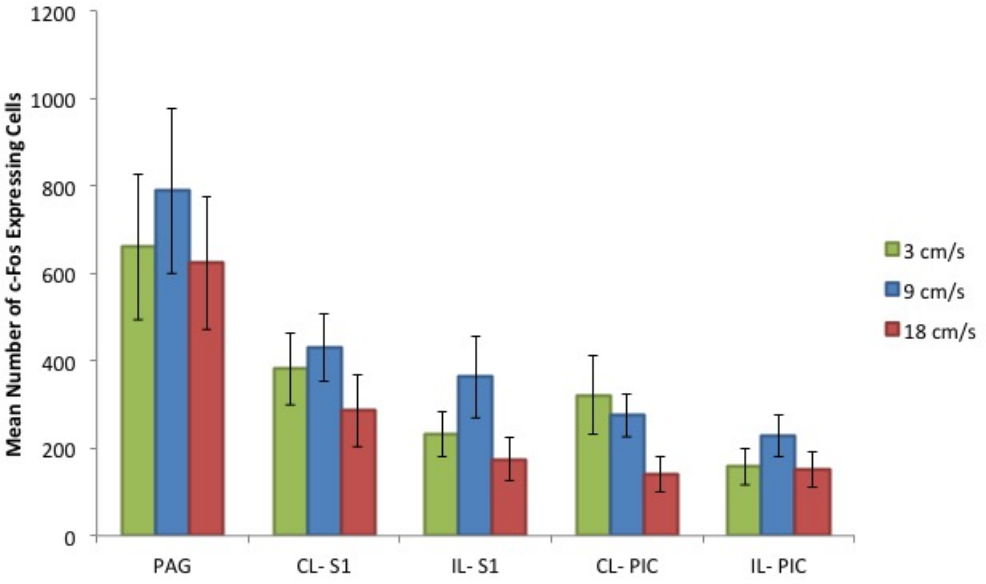


Figure 3.6 Mean number of c-Fos expressing cells in PAG, S1 and PIC.

was correlated with c-Fos expressions in PAG (r= .60) and contralateral S1 (r=. 54). Table 3.5 shows the coefficients for the correlations between c- Fos expressions and number of USV emissions in minutes of stimulation.

Similarly, duration of emissions in the last minute of stimulation correlated with c –Fos expression in PAG (r= .51) and contralateral S1 (r= .55). Coefficients for sub-regions of ROIs are presented in Table 3.6.

Table 3.5
Coefficients for correlation between number of USVs during stimulation and c-Fos expression in ROIs.

	Minutes of Stimulation				
ROI	**1**	**2**	**3**	**4**	**5**
PAG (total)	.12	.18	-.08	-.02	.60**
P1	-.06	-.05	-.13	-.06	.32
DM	.09	.21	.02	-.06	.61**
L	.20	.21	-.09	.05	.58*
DL	.17	.22	-.14	-.04	.60**
VL	-.15	-.01	.06	-.17	.40
Contralateral S1 (total)	-.16	-.05	-.18	-.26	.54*
L1	.02	-.28	.05	-.02	.07
L2	-.11	-.04	-.03	.01	.32
L3	-.10	-.05	-.17	-.20	.46
L4	-.15	-.12	-.21	-.28	.53*
L5	-.23	-.10	-.13	-.25	.43
L6	-.13	.09	-.15	-.31	.40
Ipsilateral S1 (total)	.00	.15	.22	.02	.33
L1	-.32	-.51*	-.22	-.20	.07
L2	.01	.23	.20	.04	.34
L3	-.10	.09	.15	-.04	.34
L4	-.12	-.02	.25	-.01	.21
L5	.11	.217	.41	-.04	.24
L6	.16	.22	.18	.11	.29
Contralateral PIC (total)	-.10	.02	-.20	-.28	.33
AI	-.22	-.08	-.16	-.31	.20
DI	-.04	.03	-.24	-.29	.35
GI	.03	.18	-.16	-.14	.42
Ipsilateral PIC (total)	.12	.30	.13	-.00	.42
AI	.23	.28	.19	-.03	.51*
DI	.05	.15	.04	-.12	.38
GI	-.05	.43	.07	.22	.12

* $p<.05$

** $p<.01$

Table 3.6
Coefficients for correlation between duration of USVs during stimulation and c-Fos expression.

ROI	Minutes of Stimulation				
	1	**2**	**3**	**4**	**5**
PAG (total)	.05	.28	.10	.13	.51*
P1	-.02	.06	-.04	.14	.30
DM	.04	.33	.17	.10	.56*
L	.12	.29	.11	.19	.48*
DL	.03	.29	.03	-.02	.49*
VL	-.25	.09	.16	.02	.34
Contralateral S1 (total)	-.16	.10	-.08	-.02	.55*
L1	.20	-.11	.11	.12	.07
L2	-.05	.00	.12	.29	.30
L3	-.11	.03	-.07	-.00	.47*
L4	-.19	.03	-.10	-.09	.50*
L5	-.20	.08	-.12	-.06	.47*
L6	-.17	.23	-.13	-.17	.44
Ipsilateral S1 (total)	.09	.29	.29	.31	.38
L1	-.24	-.45	-.22	-.11	.03
L2	.08	.34	.31	.30	.41
L3	-.03	.19	.21	.23	.39
L4	-.04	.11	.28	.26	.24
L5	.19	.43	.39	.19	.31
L6	.24	.37	.24	.35	.29
Contralateral PIC (total)	-.11	.10	-.13	-.09	.36
AI	-.21	-.02	-.10	-.11	.21
DI	-.09	.08	-.17	-.16	.37
GI	.04	.29	-.09	.05	.47*
Ipsilateral PIC (total)	.18	.42	.28	.31	.47
AI	.27	.44	.34	.26	.54*
DI	.08	.24	.17	.16	.40
GI	.07	.46	.18	.51*	.20

* $p<.05$

** $p<.01$

4. DISCUSSION

4.1 General Discussion

In the present study, the effect of gentle touch on affective state and neural activity was investigated. Studies with human participants showed that slow stroking (3-10 cm/s) on hairy skin elicits pleasantness correlating with the activity in contralateral insula [22, 23]. Findings on animals partially supported the relationship between gentle touch and affective state. Stimulation of low-threshold C-mechanoreceptive fibers elicited conditioned place preference in mice [32] and gentle stroking facilitated the production of 50-kHz appetitive calls in rats [68]. However, to our knowledge, the present study is the first one controlling the velocity of gentle touch in rats and measure direct indicators of affective state as ultrasonic vocalizations. Moreover, it is the first study that investigates the relation between gentle touch and activity in insular cortices. Results showed that the tactile stimulation paradigm was able to alter affective state, albeit in a negative manner. Rats emitted more and longer 22-kHz USVs in response to fast stimulation, yet c-Fos expression did not change in response to velocity modulation [72].

4.2 Implications of the Results

The present findings regarding the negative affective state induced by tactile stimulation paradigm is consistent with the previous literature [58, 59]. Possibly, 5-minute long stimulation with a brush once in a week was not sufficient for rat to become familiarized with and perceive it as pleasant. Even though their level of negative affect was ameliorated in the last experimental session compared to the first one, rats continued to emit 22-kHz USVs during the stimulation episode. Since the familiarization to stimulation paradigm would cancel out the effect of velocity modulation, rats were exposed to only one stimulation session per week. Otherwise, it would be

difficult to differentiate whether rats perceive stimulation as appetitive because they are familiarized or their affective touch system is activated.

Due to the inverted U shaped response patterns of CT-afferents to increasing stimulation speed [22], it was expected to reveal similar pattern in rats. However, increase of the speed of gentle stroking aggravated the negative affective state indicated by increasing amount and duration of 22-kHz USVs. This increasing trend may be partly because of the activation of guard hairs together with the zigzag and awl/Auchene hairs. Guard hairs are sparsely distributed, but connected to myelinated and fast-conducting Aβ fibers transmitting the discriminative properties of tactile stimuli. On the other hand, zigzag and awl/Auchene hairs are more frequent and transmit affective aspect of touch via unmyelinated and slow- conducting C-fibers that form lanceolate endings in the hair shafts [33]. Another reason for the increasing behavioral trend in response to increasing velocities may be the activation of other low-threshold fibers engaging in the perception of itch and pain, rather than low-threshold ones. Despite the differences in adaptation properties, Aδ fibers are known to respond similar stimuli as CT-afferents [1].

Since the gentle touch also activates Aβ fibers in healthy rats, activation in contralateral primary somatosensory cortices was expected. This activation is especially salient in layer 4 receiving input from thalamus and layer 5 sending output to thalamus and other cortical regions. Additionally, layer 2 and 3 were activated, indicating the information transfer between two hemispheres. This insight is supported by the correlation between contralateral and ipsilateral hemispheres. The number of cells containing c-Fos protein in Layer 2 of contralateral S1 is found to be significantly correlated with ipsilateral layer 2 and 3, r= .75 and r= .70 respectively. Similarly, c-Fos expression in contralateral Layer 3 is correlated with expressions in ipsilateral Layer 2 (r= .75) and Layer 3 (r= .83). Further correlations between layers are given in Appendix D.

Considering the neural activity in contralateral posterior insular cortices in humans, c-Fos expressions in PIC were measured. Findings revealed the activation of

contralateral PIC in rats in response to gentle touch. Hemispheric differences between PIC were prominent especially in granular regions that are related to somatosensory processing [70]. This further supports the notion of corresponding activity in PIC in response to gentle touch. Moreover, high inter- insular correlations may be considered as an indication of intra- insular connectivity and information processing. (See Appendix D for correlation table)

Production of 22- kHz USVs is mediated by the mesolimbic cholinergic system including PAG and septum. PAG is a midbrain structure involved in pain processing, vocalization, autonomic regulation, lordosis, fear and anxiety [73]. PAG cooperates with insular cortex for pain processing, amygdala for fear and anxiety. Specifically, dorsal and lateral parts of rostral PAG are shown to be involved with vocalizations [73]. Correspondingly, the current results showed more c-Fos expression in lateral and dorsomedial parts of PAG, regardless of stimulation speed (Appendix C). The number of c-Fos expressing cells in sub-regions of PAG is found to correlate highly with each other (Appendix D). Especially, high correlations between DAMPAG, LPAG and DLPAG may be responsible for negative affective calls.

In the literature, PAG is mostly associated with negative affective state calls and defensive behaviors [74], while insula is considered as a place for positive emotions [75]. In contradiction to this, the current study revealed high positive correlation between these two regions. This may suggest that these regions participate in evaluation of emotional valence of gentle touch, but not decide whether it is positive or negative. This co-activation may also be interpreted as the activation of other fibers that signal itch and pain.

The correlations between the number and duration of 22-kHz USVs emitted during the last minute of stimulation session and c-Fos expressions in PAG and contralateral S1 indicate that the present gentle touch paradigm targets related brain regions. The reason for correlation with only the last minute of stimulation may be a consequence of slow amplification of c-Fos signals. Cumulatively enhanced c-Fos protein may become salient enough later than the stimulation.

4.3 Clinical Implications

Overall, CT- afferents are thought to be related with mother-infant attachment and social communication [3,12]. Tactile contact with mother is necessary for an infant mammal for healthy development. Lack of sufficient tactile contact with mom either because of separation or nurturing style of mother distresses infant and cause physiological and psychological problems (for a review, see [76]). In the case of autism in which infants show sensory abnormalities, disturbed social and communicative behaviors, CT-afferents and related affective touch system is hypothesized to be disrupted [3, 12]. Children with autism have sensory abnormalities and display sensory defensiveness [77]. They may avoid tactile contact with their mothers and peers, yet they may show unusual interest in other tactile stimuli such as repetitively touching to certain textures or intense hugging and squeezing [78]. These touch related abnormalities are thought to support the role of CT-afferents in socio-emotional processing [79]. Studies in children with autism revealed no or partial changes in the sensation of mechanical touch [80–82], but altered sensitivity and emotional processing for tactile modality [83–85]. Severity of autistic traits is shown to aggravate the feeling of pleasantness and brain activity in CT-targeted and emotion-related regions such as insula, superior temporal sulcus and prefrontal cortex [86, 87]. Therefore, studying the relation between CT-afferents and socio-affective processing may suggest that improving conditions of individuals with psychiatric conditions involving sensory abnormalities. It may also provide an opportunity to develop new behavioral therapies for the rehabilitation of abnormal behaviors.

4.4 Limitations

In general, the current gentle touch paradigm was able to alter affective state, albeit in a negative manner. This may indicate the importance of familiarity factor in the perception of gentle touch. Affective outcomes of touch are more likely to be observed between conspecifics rather than cross- species contact. In order to perceive

contact from different species as pleasant, organism may need to be familiarized both to contact and its source [68].

One limitation of the study is a direct consequence of co-activation of Aβ fibers with CT-afferents. This makes it difficult to differentiate the effect of CT-afferent stimulation alone. Additionally, applied velocity range was limited due to the size of stimulation area. Our preliminary data indicated the difficulty of stimulation with speeds slower than 3 cm/s in rats. Thus, our limited velocity range may prevent us to reveal the whole range of affective outcomes. Another limitation of the study is the unilateral stimulation. Due to the fact that stimulation was delivered manually, rats received tactile stimulation to the right side of the dorsal trunk. Even tough receiving to the other side of the body would not make any difference in terms of processing in somatosensory cortex, previous literature shows hemispheric differences in insular cortex when processing emotions [75].

4.5 Future Work

Future research may be designed to rule out the familiarity factor and isolate the effect of gentle touch alone. Prior to the experimental manipulation, rats may be habituated to only one stimulation velocity and their response to other velocities may be measured. Moreover, velocity modulation may be controlled mechanically, ruling out the confounding effect of experimenter. In addition, the co-activation of myelinated fibers should be prevented either using animal models mimicking sensory neuropathy or block the information transmission with pharmacological agents. Denervation of Aβ fibers may facilitate studying the effect of stimulation of CT-afferents in socio-affective contexts. In addition to sensory neuropathy models, animal models of autism may be also utilized to study the impact on CT-afferents in altered neural circuitries. Information regarding the exact pathway of affective touch should also be studied in detail by using tracing techniques. By utilizing in vivo recordings, it may be possible to identify low- threshold C-mechanoreceptive fibers that involved in gentle touch, and measure responses to different kinds of innocuous stimuli as in the human studies.

Additionally, effects of direct stimulation of CT-targeted neurons in PIC may be studied to reveal behavioral responses of awake animals. Lastly, in vivo recordings and imaging techniques such as fMRI and PET during the gentle touch may be utilized to identify the exact brain regions involved in the processing of gentle touch.

4.6 List of publications produced from the thesis

1. Ultrasonic vocalization and c-Fos expression in rats stimulated by 'affective' touch, E. Tunçkol, R.S. Canbeyli, B. Güçlü, *Society for Neuroscience, SfN, 48th Annual Meeting*, (abstract submitted), November 3 - 7, 2018.

APPENDIX A. c-Fos Immunocytochemistry

Solutions

1. PB : 0.1 M Phosphate Buffer (pH 7.6)
2. Blocking solution : Super Mix (100 ml PB + 500 μl triton-x + 0.25 gr gelatin)
3. Primary Antibody : Rabbit anti-fos (polyclonal, sc-52, Santa Cruz Biotechnology)
4. Secondary Antibody : Biotinylated anti-rabbit IgG (BA- 1000, Vector Laboratories)
5. ABC (PK- 6100, Vector Laboratories)
6. DAB (SK- 4100, Vector Laboratories)
7. Mounting medium: Vecta Mount

Table A.1
c-Fos Staining Protocol

Purpose	Duration and Temperature	Solution
	First Day	
Wash	3 x 10 min @ RT	PB
Block	10 min @ RT	10 % Methanol and 3 % H_2O_2 in PB
Wash	3 x 10 min @ RT	PB
Incubation in primary ab	48 hours @ +4°C	anti-fos 1:2500 in super mix
	Second Day	
Wash	3 x 10 min @ RT	
Incubation in secondary ab	1 hour @ RT	Goat anti-rabbit 1:400 in super mix
Wash	3 x 10 min @ RT	PB
ABC	1 hour @ RT	1:500 in super mix
Wash	3 x 10 min @ RT	PB
DAB	3 min on ice	2 drops buffer + 4 drops DAB + 1 drop Nickel + 2 drops of H_2O_2 in 5 ml of dH_2O
Mount		PB 0.1 M, on gelatinated slides. Allow slides to dry for overnight
	Third Day	
Dehydration	1 min	50 % EtoH
	2 min	70 % EtoH
	2 min	95 % EtoH
	A few dips	95 % EtoH
	1 min	50 % EtoH
Histoclear	5 min	Xylene
Cover Slipping		Vecta Mount- Mounting Medium

APPENDIX B. Ultrasonic Vocalizations in Minutes of Stimulation

Average data regarding the ultrasonic vocalizations during the minutes of stimulation are presented

Table B.1
Means and standard deviations for 22- kHz vocalizations during the stimulation.

Descriptive Statistics for 22- kHz USVs During Stimulation					
		Number		**Duration (s)**	
Stimulation Velocity	**Minutes**	**Mean**	**Std Dev**	**Mean**	**Std Dev**
3 cm/s	1	6.0	3.9	10.1	6.8
	2	4.3	3.6	6.8	7.1
	3	4.3	4.4	6.1	6.8
	4	2.1	3.2	2.7	4.4
	5	4.0	4.2	6.1	6.6
9 cm/s	1	9.3	5.2	13.9	7.4
	2	6.1	4.6	9.1	7.7
	3	3.9	3.6	6.0	5.9
	4	3.1	3.1	4.9	5.4
	5	3.8	3.5	4.9	5.4
18 cm/s	1	12.0	5.1	17.5	9.3
	2	7.2	5.8	9.2	7.5
	3	5.8	4.7	7.5	6.9
	4	5.2	5.1	6.0	5.9
	5	4.8	4.4	6.1	6.8

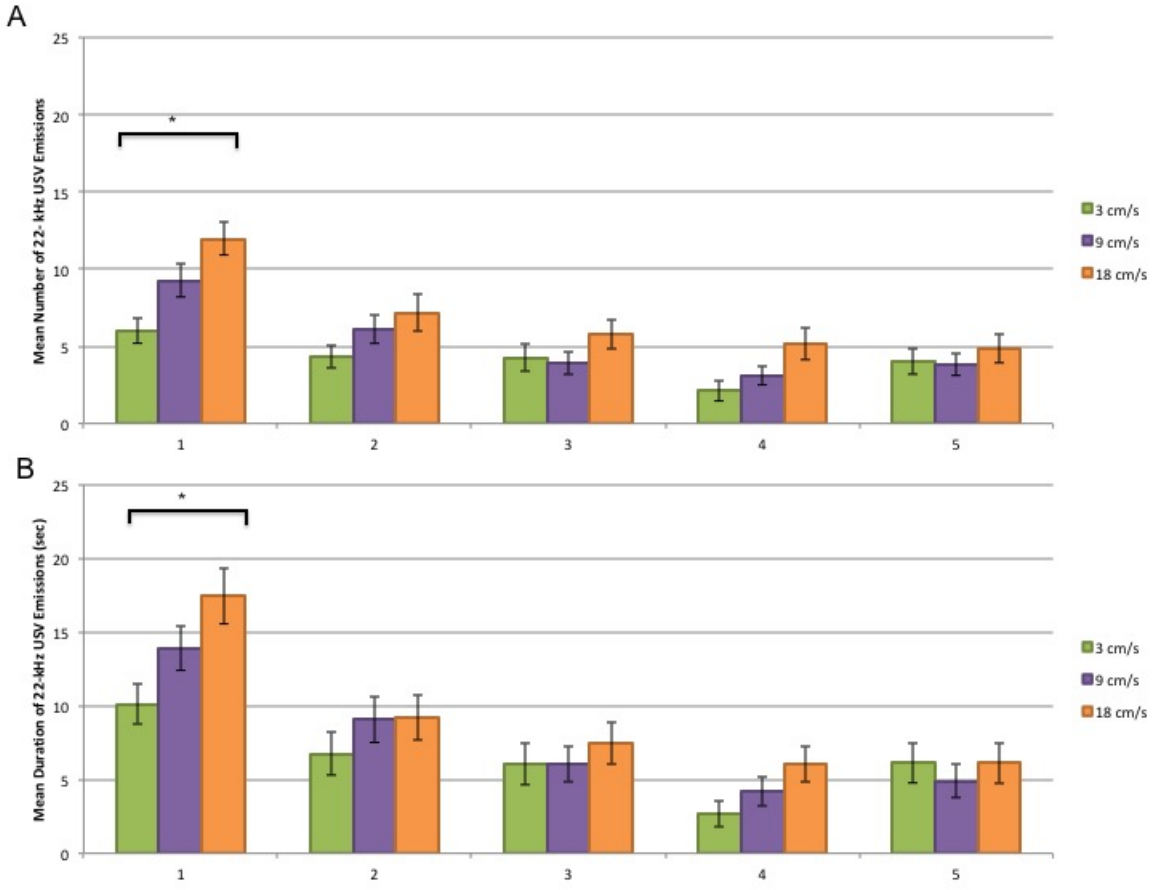


Figure B.1 A) Mean number and B) mean duration of 22-kHz USVs are displayed with respect to stimulation velocities.

APPENDIX C. The Effect of Velocity Modulation on c-Fos Expression

Data regarding c-Fos expressions in sub-regions of PAG, PIC and S1 is presented.

Table C.1
Means and standard deviations for c-Fos expressions in sub-regions of PAG, S1 and PIC.

		Descriptive Statistics	
Regios	**Sub-regions**	**Mean**	**Std. Dev**
PAG	P1	115.8	70.1
	DM	173.7	110.0
	L	289.7	161.9
	DL	89.8	75.7
	VL	22.6	21.2
Contralateral Primary Somatosensory Cortex-Trunk	L1	7.0	5.6
	L2	52.9	38.7
	L3	95.7	72.3
	L4	61.8	43.1
	L5	44.8	30.9
	L6	104.2	58.2
Ipsilateral Primary Somatosensory Cortex-Trunk	L1	5.9	6.4
	L2	37.6	30.2
	L3	75.4	60.5
	L4	36.8	29.9
	L5	27.2	19.0
	L6	73.9	58.4
Contralateral Posterior Insular Cortex	Agranular	103.9	75.9
	Granular	63.1	47.4
	Dysgranular	78.9	57.9
Ipsilateral Posterior Insular Cortex	Agranular	81.9	53.2
	Granular	36	26.4
	Dysgranular	61.2	39.0

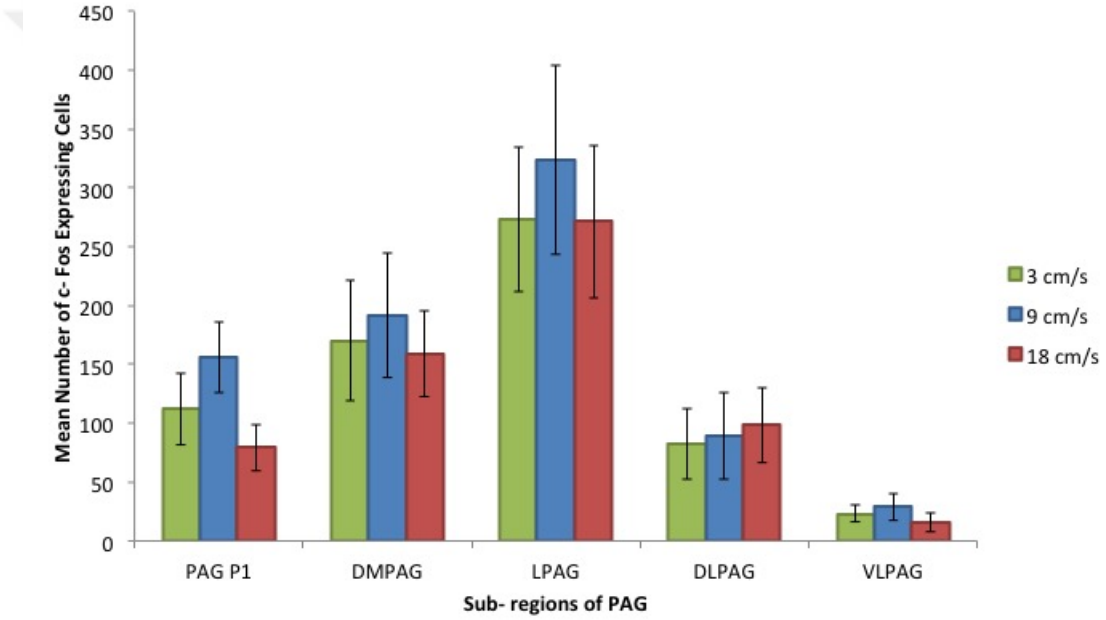


Figure C.1 Mean number of c-Fos expressing cells in P1, DM, L, DL and VL sub-regions of PAG is displayed with respect to stimulation velocities.

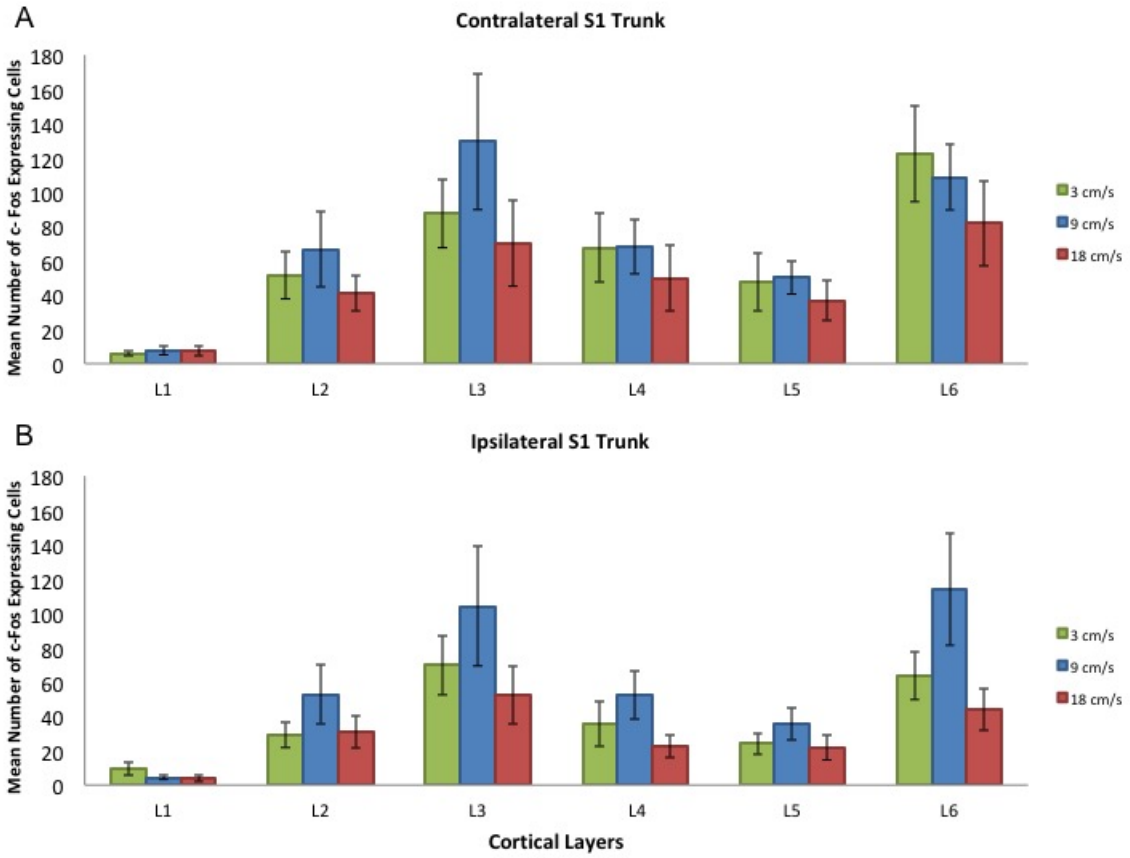


Figure C.2 Mean number of c-Fos expressing cells in cortical layers of A) contralateral and B) ipsilateral somatosensory cortices (trunk) is displayed with respect to stimulation velocities.

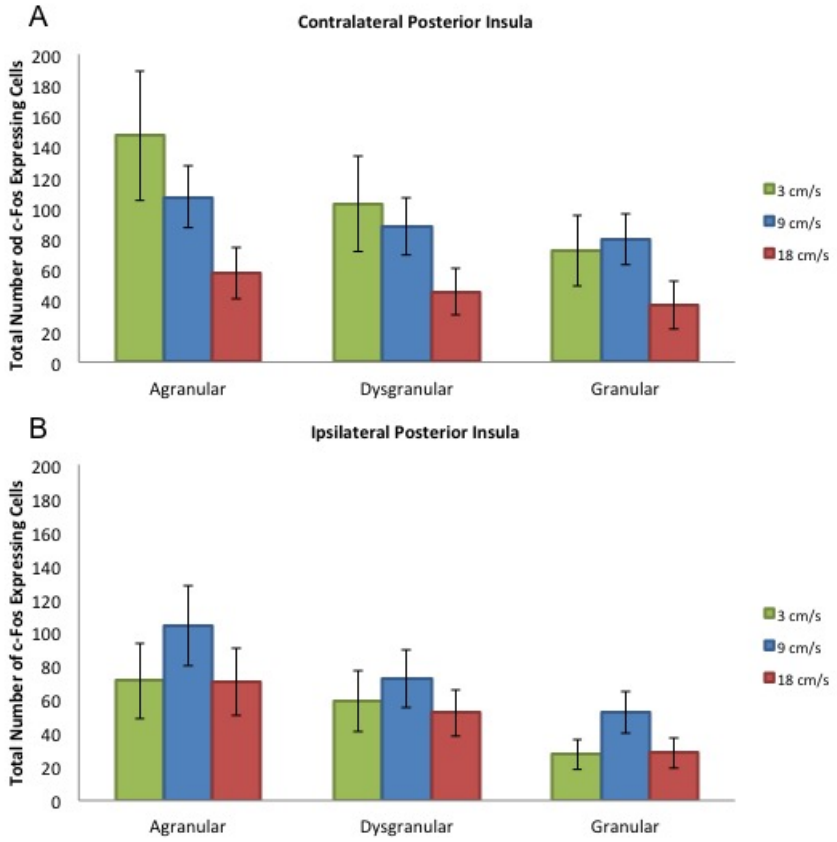


Figure C.3 Mean number of c-Fos expressing cells in sub-regions of A) contralateral and B) ipsilateral posterior insular cortices is displayed with respect to stimulation velocities.

APPENDIX D. Intra-regional Correlation

Data regarding intra- regional correlations of c-Fos expressions in PAG, S1 and PIC is presented.

Table D.1
Correlation coefficients c-Fos expressions in sub-regions of PAG.

	Variables	**1**	**2**	**3**	**4**	**5**
1	PAGP1	-				
2	DMPAG	.54*	-			
3	LPAG	.66**	.89**	-		
4	DLPAG	.46	.91**	.91**	-	
5	VLPAG	.37	.77**	.72**	.77**	-

* p<.05

** p<.01

Table D.2
Correlation coefficients c-Fos expressions in sub-regions of PIC.

Variables			**1**	**2**	**3**	**4**	**5**	**6**
1		Agranular	-					
2	Contralateral	Granular	.67**	-				
3		Dysgranular	.82**	.87**	-			
4		Agranular	.52*	.67**	.69**	-		
5	Ipsilateral	Granular	.30	.15	.11	.53*	-	
6		Dysgranular	.58*	.40	.60*	.86**	.63**	-

* p <.05

** p <.01

Table D.3
Correlation coefficients c-Fos expressions in cortical layers of S1-trunk.

	Variables		1	2	3	4	5	6	7	8	9	10	11	12
1	Contralateral	Layer 1	-											
2		Layer 2	.13	-										
3		Layer 3	.20	.73**	-									
4		Layer 4	.23	.44	.79**	-								
5		Layer 5	.52*	.14	.56*	.79**	-							
6		Layer 6	.19	-.02	.39	.72**	.83**	-						
7	Ipsilateral	Layer 1	.58*	.15	.21	.49*	.61**	.50*	-					
8		Layer 2	.18	.75**	.75**	.41	.30	.21	.01	-				
9		Layer 3	.19	.70**	.83**	.62**	.48*	.37	.25	.92**	-			
10		Layer 4	.29	.42	.62**	.66**	.60**	.53*	.46	.65**	.86**	-		
11		Layer 5	.33	.11	.41	.50*	.60**	.64**	.23	.55*	.63**	.77**	-	
12		Layer 6	.09	.35	.48*	.50*	.36	.54*	.13	.59*	.63**	.70**	.78**	-

* p <.05

** p <.01

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