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SALICYLATE GENERATES ANXIETY-LIKE BEHAVIOR AND TYPE 2 THETA OSCILLATION IN THE VENTRAL HIPPOCAMPUS OF MICE

DISSERTAÇÃO DE MESTRADO

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SALICYLATE GENERATES ANXIETY-LIKE BEHAVIOR AND TYPE 2 THETA OSCILLATION IN THE VENTRAL HIPPOCAMPUS OF MICE

Dissertação apresentada à Universidade Federal do Rio Grande do Norte em nome do Prorama de Pós Graduação em Neurociências como requisito parcial para obtenção do grau de Mestre.
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CONTENT

List of abreviations:

vHP – Ventral hippocampus

dHP – Dorsal hippocampus

IEG - Immediate-early genes

HF - Hippocampal formation

DG - Dentate gyrus

CA - Cornu Ammonis

PCs - Principal cells

PP - Perforanth Path

MF - Mossy fibers

SC - Schaeffer collateral

mPFC - Medial pre-frontal cortex

GC – Granger Causality

SR – Stratum radiatum

SP – Stratum piramidale

DCN – Dorsal Cochlear Nucleus

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RESUMO

Salicilato, o principal composto de diversos medicamentos, como a Aspirina, é conhecido por causar zumbido se consumido em altas doses ou de forma crônica (para o tratamento de osteoporose, por exemplo). Zumbido é o ouvir ou a percepção de um som quando nenhum estímulo físico está presente. O zumbido não é uma doença em si, mas um sintoma presente em diversas doenças, e está associado à ansiedade e outros distúrbios de humor. Apesar de estar diretamente ligado ao sistema auditivo, o zumbido não é gerado a partir de uma região específica do cérebro. Além disso, alguns estudos mostraram que o salicilato afeta várias regiões cerebrais além do sistema auditivo, como o estriado, amigdala e o hipocampo. Estudos iniciais atribuíram uma função unitária ao hipocampo: processamento de memorias declarativas. Entretanto, estudos mais recentes mostraram que o hipocampo não só possui outras funções, como processamento emocional, mas também pode ser dividido em ventral e dorsal, e a parte ventral desempenha um papel essencial no processamento emocional. A oscilação mais estudada do cérebro é o rítmo teta, e ela pode ser encontrada em todo o hipocampo. Dois tipos de teta podem ser distinguidos: o teta tipo 1, que é resistente a atropina, possui uma frequência mais alta (7 a 10 Hz) e está relacionado com comportamentos de padrão motor; e o teta tipo 2, que é sensível a atropina, possui uma frequência mais baixa (4 to 7 Hz) e ocorre durante anestesia, estado de imobilidade vigilante e situações de alta ansiedade. O presente estudo investigou os efeitos eletrofisiológicos do salicilato no hipocampo ventral de camundongos em estado de comportamento. Através da injeção de salicilato foi gerado teta tipo 2 no hipocampo ventral. Também foi encontrado que o salicilato leva a comportamentos de ansiedade.
Palavras chave: salicilato, hipocampo, teta do tipo 2, ansiedade

ABSTRACT

Salicylate, the main compound of many medications as Aspirin, is known to cause tinnitus if consumed in high doses or in a chronic way (for the treatment of osteoporosis, for example). Tinnitus is the hearing or perception of a sound when no physical stimulus is present. Tinnitus is not a disease itself, but a symptom present in some diseases, and is associated with anxiety and other mood disorders. Despite being directly related with auditory system, tinnitus is not generated from one specific region of the brain. Additionally, some studies showed that salicylate affects various brain regions besides the auditory system, as the striatum, amygdala and the hippocampus. Early studies have ascribed a unitary function to the hippocampus: declarative memory processing. However, more recent studies showed that the hippocampus not only has other functions, as emotional processing, but also can be divided into ventral and dorsal, and the ventral part plays an essential role in emotional processing. The most studied oscillation of the brain is the theta rhythm, and it can be found in the entire hippocampus. Two types of theta can be distinguished: the type 1, that is atropine resistant, has a higher frequency (7 to 10 Hz) and is related with motor pattern behaviors; and the type 2 theta, that is atropine sensitive, has a lower frequency (4 to 7 Hz) and occur during anesthesia, alert immobility and high arousal situations. The present study investigated the electrophysiological effects of salicylate in the ventral hippocampus of behaving mice. Through salicylate injection we generated type 2 theta in the ventral hippocampus. We also found that salicylate led to anxiety-like behavior.

Key words: salicylate, hippocampus, type 2 theta, anxiety

1. INTRODUCTION

1.1. Salicylate

Salicylates are agents found in many prescription drugs and in several of-the-counter medications. One of the most known and widespread used is the Aspirin, an analgesic, antipyretic and anti-inflammatory drug. Only in the USA, more than 10.000 tons of Aspirin are consumed each year (Muhammad Waseem et al, 2015). Aspirin can also be used chronically for treatment of some diseases, such as osteoporosis or vascular diseases (Yamaza et al., 2008).

Overconsumption of salicylate can lead to intoxication and even death. It's ingestion is also a common cause of poisoning in children. In 2005, more than 20.000 cases of overexposure to salicylate were registered in the USA, of which 64% required treatment in a health care facility and 50% were intentional overdoses (with 60 fatal cases). Doses ranging from 150 to 300 mg/kg are classified as mild to moderate toxicity. Doses from 300 to 500 mg/kg produce serious toxicity, and doses above 500 mg/kg are potentially lethal (Muhammad Waseem et al, 2015). Intoxication is measured by the serum salicylate level. Concentrations from 15 to 30 mg/dL are considered therapeutic. 30 to 50 mg/dL indicates mild intoxication and patients show nausea, hyperpnea, vomiting, dizziness or tinnitus, whereas severe intoxication appears from 50 to 70 mg/dL of serum salicylate levels and can lead to tachypnea, fever, sweating, dehydration, incoordination and listlessness. Doses higher than 75 mg/dL lead to coma, seizures, hallucinations, stupor, cerebral edema, dysrhythmias, cardiac failure, hypotension, coagulopathy, oliguria, renal failure and death. The medical approaches in the case of intoxication are restricted to limit the degree of absorption, enhance the elimination of salicylate and provide supportive care, since there is no antidote available for intoxication of salicylate (Pearlman & Gambhir, 2009).

When consumed orally, aspirin is rapidly absorbed by passive diffusion from the stomach (Hogden et al, 1957; Rowland et al, 1972). Once in the blood, aspirin is first hydrolyzed in salicylic acid in the liver. Both aspirin and salicylic acid, in the serum, bounds to serum albumin and are distributed through the body and the central nervous system. The half-life of aspirin in the stomach (absorption) range from 5 to 16 minutes and in the serum (before hydrolyzation) is approximately 20 minutes (Rowland et ai., 1972). Aspirin concentration in the blood reaches the peak at around 25 minutes after ingestion, for soluble preparations (Morgan and Truitt, 1965; Rowland et aI., 1972). Part of the salicylic acid is renally excreted and part is metabolized, producing salicyl acyl glucoronide, salicyl phenolic glucoronide, salicyluric acid and others (see Figure 1). In turn, the half-life of salicylic acid is dose dependent, so the large is

the dose ingested, the longer it will stay in the serum (Needs & Brooks, 1985). An interesting study with rats found that the pharmacokinetics of the salicylate is not linear, showing a rapid absorption that tents to decrease with time, both for intravenous injections and oral gavage (Wientjes & Levy, 1988). No studies were found using intra peritonial injections.

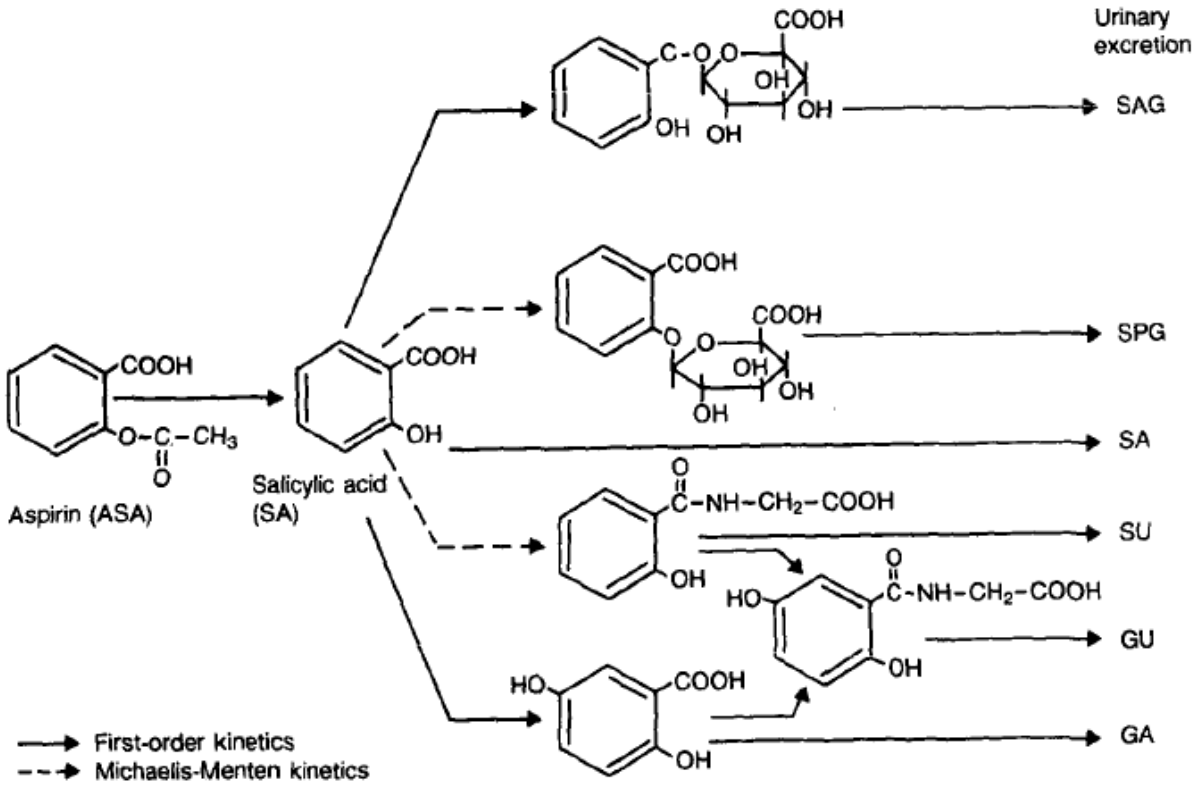

Figure 1. Salicylate metabolism.
Metalism route of aspirin once it reaches the serum. SAG = salicyl acyl glucoronide; SPG = salicyl phenolic glucoronide; SU = salicyluric acid; GA = gentistic acid; GU = gentisuric acid (retrieved from Needs & Brooks, 1985).

Mild salicylate intoxication may cause tinnitus, while severe intoxication can lead to tinnitus and hearing loss (Pearlman & Gambhir, 2009). Several studies tested salicylate effects on human hearing in controlled situations and has generated reversible dose-dependent hearing loss and tinnitus (McFadden et al, 1974). Hearing effects of salicylate were initially thought to be reversible; however, Chen et al. showed that rats treated chronically with a high dose of salicylate had suppressed neural output of the peripheral system (Chen et al., 2010). Furthermore, salicylate easily crosses the blood-brain barrier, making the brain susceptible to the effects of this drug (Chen et al, 2014).

Salicylate induced tinnitus has become a largely used animal model to study tinnitus and their alterations in the brain (Carol A. Bauer et al, 1999; Guitton et al., 2003; Jastreboff et al, 1988; Kaltenbach, 2011; Lobarinas et al, 2004; Puel & Guitton, 2007; Ruttiger et al, 2003;

Turner, 2007; Von Der Behrens, 2014). Most electrophysiological studies focus on the auditory system, while only a few have investigated the effects of the salicylate in other brain regions. Additionally, it has been shown that salicylate activates nonauditory regions involved with emotion and memory (Chen et al., 2014).

A study with positron emission tomography (PET) found that neural systems that mediate tinnitus may be linked with emotion controlling systems via the hippocampus (Lockwood et al., 1998). Landgrebe and colleagues found, through magnetic resonance imaging (MRI), that tinnitus patients have a significant decrease in grey matter concentration in the right inferior colliculus, in both sides of the hippocampus and in the cingulate cortex compared to controls (Landgrebe et al., 2009). This finding corroborates with a work showing that depression and anxiety are related with decreases in grey matter in the left posterior hippocampus (de Geus et al., 2007) .

Another recent study tested the effects of peripheral administration and direct injection of high doses of salicylate in CA1 of the dorsal hippocampus and found that both processes improved the consolidation of spatial memory in the Morris Water Maze, although there was no effects on the escape latency and swimming speed (Azimi et al, 2012). An eletrophysiological study in the striatum, hippocampus, lateral amygdala and cingulate investigated alterations of the excitability of neurons in a high salicylate dose condition in rats (Chen et al., 2014) and found suprathresholds hyperexcitability in striatum, hippocampus and lateral amygdala, suggesting hyperactivity that implicates in plastic changes in the nuclei.

A recent work evaluated the expression of the immediate-early genes (IEG) Arc/Arg 3.1 and Erg-1, involved in transcription dependent plasticity, and NMDA subunit 2B (NR2B) in the hippocampal CA1 area of rats after chronic exposure to salicylate. They found that IEG expression was upregulated in the salicylate animals, indicating alterations to the synaptic structure of hippocampal CA1 neurons (Wu et al., 2015). Taken together, the findings discussed above suggests that salicylate affects many regions as well as some genes of the central and peripheral nervous system, both in acutely and chronically administration. This thesis will focus on the acute effects of salicylate on the hippocampus and in the behavior of mice.

1.2. Tinnitus and anxiety

Tinnitus is the hearing or perception of a sound when no physical stimulus is present. It is not a disease in itself, but a symptom present in many diseases. The cause of tinnitus varies, and it is generally related with hearing loss (about 90%). Many drugs like coffee, alcohol and aspirin, in their normal use or abuse, are known to cause or exacerbate tinnitus, just as some

anesthetics (National Research Council (US) Committee on Hearing, 1982; “Tinnitus | NIDCD,” access in 2016, september 15). To exemplify, in the United Kingdom, 1 in 10 people have some awareness of tinnitus and 1 in 200 are severely affected (“INFOGRAPHIC Tinnitus & Deafness Statistics | Visual.ly,” access in 2016, september 15) and in the United States about 20% of the population are affected by tinnitus (“Hearing Loss & Tinnitus Statistics,” access in 2016, september 15).

Despite the statistics in the UK, according to Eggermont and Roberts, in 1% to 3% of the general world population tinnitus constitutes a significant impairment of the life quality (Eggermont & Roberts, 2004). Severe mood problems, like anxiety or depression are present in 21% to 63% of the patients. Forty-five percent of tinnitus sufferers show anxiety, and some of this patients experience suicidal behavior (Andersson et al, 2002.; Holmes & Padgham, 2009; Stouffer, & Tyler, 1990; Tyler & Baker, 1983; Zöger et al, 2001).

Some studies have demonstrated the relation between tinnitus, anxiety and depression (Gül et al, 2015, McCormack et al., 2015). Application of a revised version of the Symptom Check List-90 concluded that chronic tinnitus patients are very prone to develop psychiatric conditions, like anxiety, depression and sensitivity, and suggested that a multidisciplinary approach could be good to lead with these patients (Gül et al, 2015). Examining the association between depression and anxiety and tinnitus in a large middle age United Kingdom population found that people with tinnitus (especially bothersome) report stronger symptoms of anxiety and depression compared with those without tinnitus (McCormack et al., 2015).

One of the major difficulties in treating Tinnitus is that it does not be generated from one specific brain regions (Han et al, 2009; Jastreboff, 1990; Knipper et al, 2010, Holt et al., 2010). For example, an interesting study investigated the effects of ablation of the dorsal cochlear nucleus (DCN), part of the cochlear nuclear, the first structure that processes the sound information that comes from the cochlea. Multiple hypotheses postulate that the DCN could serve as a source of tinnitus (Levine, 1999; Potashner et al, 2000). However, this study showed that bilateral ablation of the DCN after the generation of tinnitus had no effects on tinnitus, while ipsilateral ablation enhanced the evidence of tinnitus. Through this, Brozoski and Bauer hypothesize that tinnitus generated by acoustic trauma may trigger persistent changes in more then one level of the auditory system (Brozoski & Bauer, 2005). However, the DCN is important on the generation of tinnitus, once bilateral ablation of the DCN before acoustic trauma prevents the development of tinnitus (Brozoski et al, 2012). Together, this results show that the DCN is necessary for the generation of tinnitus but for it's maintenance. Furthermore, it has been demonstrated that the limbic system is involved in the auditory processing, playing an important

role in the generation and suppression of tinnitus, what indicates that the limbic system is important for the treatment of tinnitus (Kraus, 2012). Despite the promising researches showed above, there is still no treatment for tinnitus, and a multidisciplinary approach seems to be the best approach in the search for a treatment for tinnitus. Therefore, studies of different brain areas, not just of the auditory system, are still necessary to understand the neurobiology of tinnitus for the development of treatments.

1.3. Hippocampus: from anatomy to internal function differentiation

The hippocampus is one of the major structures of the mammalian's brain. In primates, the hippocampus is located in the medial temporal lobe and the structure is also known as Cornu Ammonis (CA). The CA region was subdivided into CA1, CA2, CA3 and CA4 by (Lorente De Nó, 1934). Later, Theodor Blackstad and David Amaral classified the CA4 as the "deep, polymorphic layer of the dentate gyrus" (Blackstad, 1956). The hippocampus proper (CA regions) is part of the hippocampal formation (HF), which also comprises the dentate gyrus (DG) and the subiculum. The HF is divided into three layers. The first and deepest layer comprises interneurons and a mixture of afferent and efferent fibers. This layer is referred as stratum oriens in CA regions and hilus in the DG. The second layer, or the cell layer, is called pyramidal cell layer (stratum pyramidale) in CA regions and granule layer in the DG and is composed of interneurons and principal cells, or pyramidal cells (PCs). The third and most superficial layer is called molecular layer in the DG and subiculum and is subdivided into three in the CA regions: the stratum lucidum, a layer that receives inputs from the DG and exist just in CA3; the stratum radiatum, where are located the apical dendrites of the principal cells; and the stratum lacunosum-moleculare, composed of the apical tufts of the apical dendrites (van Strien et al, 2009).

The hippocampus is very interconnected. With main input from the entorhinal cortex (EC) via perforanth path (PP) to the DG. The granule cells of the DG projects to pyramidal cells of CA3 via mossy fibers (MF), which in turn projects to the pyramidal cells in CA1 via schaeffer collateral (SC). CA3 also connects to the contralateral side of the hippocampus via commissural fibers (Andersen et al, 1971; Neves, Cooke & Bliss, 2008) (See Figure 2).

The organizational rules of the hippocampus discussed above are preserved along the dorso-ventral axis, in rodents, and along the antero-posterior axis, in humans. The long and curved, C-shape, form of the hippocampus is maintained in all mammals (Strange et al, 2014). Due to technical advantages like easy accessibility and the well defined anatomy, most hippocampal studies has focused on the dorsal part. Initially, it was ascribed a unitary function

to the hippocampus: declarative memory processing (Squire, 1992). Recent studies demonstrated that the hippocampus has other functions involving memory, cognition and emotion. Furthermore, there are strong differences between the dorsal and the ventral parts, that are functionally distinct (Fanselow & Dong, 2010) (See Figure 3B). There is a recent rise in interest to separate dorsal and ventral hippocampus as distinct structure but the classical hippocampus literature already indicate a dichotomy. For example, Hughes showed that whereas a lesion on the dorsal hippocampus (dHP) impairs the spatial learning, a lesion on the ventral hippocampus (vHP) has no such effect (Hughes, 1965). Other publication demonstrated that lesions restricted to the vHP or dHP led to different effects on behavior in three tests: spontaneous alternation task; probability learning task; and lever alternation task (Stevens & Cowey, 1973).

Moser and colleagues conducted a lesion study in hippocampus and proposed a functional dissociation along the dorso-ventral axis (Moser et al, 1993). There are also differences in the external inputs to vHP and dHP (Swanson & Cowan, 1977). The same holds for the outputs. While the main outputs of the vHP goes to amygdala, pre-frontal cortex, hypothalamus and lateral septum, structures involved in affective-motivational behavior, the dHP mainly connects to visual, auditory and somatosensory cortices and to an area involved in spatial navigation and memory, the medial entorhinal cortex (Ciocchi et al, 2015; Grigoryan & Segal, 2016; Strange et al., 2014; Swanson & Cowan, 1977) (See Figure 3). More, outputs from the dHP and vHP are overlapped, but have different axonal patterns (Cenquizca & Swanson, 2006, Cenquizca & Swanson, 2007). Strange also demonstrated a higher density of aminergic terminals in the vHP and Dougherty showed that dendritic branching pattern is significantly different between vHP and dHP neurons (Dougherty et al, 2012; Strange et al., 2014). Additionally, electrophysiological properties of the dHP and the vHP are different. The two parts of the hippocampus differs in distribution of receptors, leading to different sensitivity and making reactions to the same stimulus sometimes opposite (Grigoryan & Segal, 2016). Density of the NMDA subunits NR2A and NR2B is higher in the dHP than in vHP, and the expression of mRNA for GluRA, GluRB and BluRC subunits of the AMPA receptors is also higher in the dHP (Grigoryan & Segal, 2016).. For example, LTP induced by high frequency stimulation was greater in the dHP than in the vHP when NMDA receptors are blocked (Moschovos & Papatheodoropoulos, 2016). Some studies demonstrated that seizures are more likely to initiate in the vHP rather than in the dHP and these seizures tend to start in the vHP before spreading to other areas. Moreover, the vHP plays an important role in the development of temporal lobe epilepsy (Dougherty et al., 2012). CA1 pyramidal cells (PCs) in the ventral hippocampus fire

significantly more than PCs in the dorsal hippocampus when somatic current is injected through whole-cell current-clamp patch clamp (Dougherty et al., 2012). Together, these studies suggest a labor division within the hippocampus. Thereby, it is important to separate studies of the hippocampus into focus on the dorsal or the ventral part, to better understand its role in neuronal activity and animal behavior.

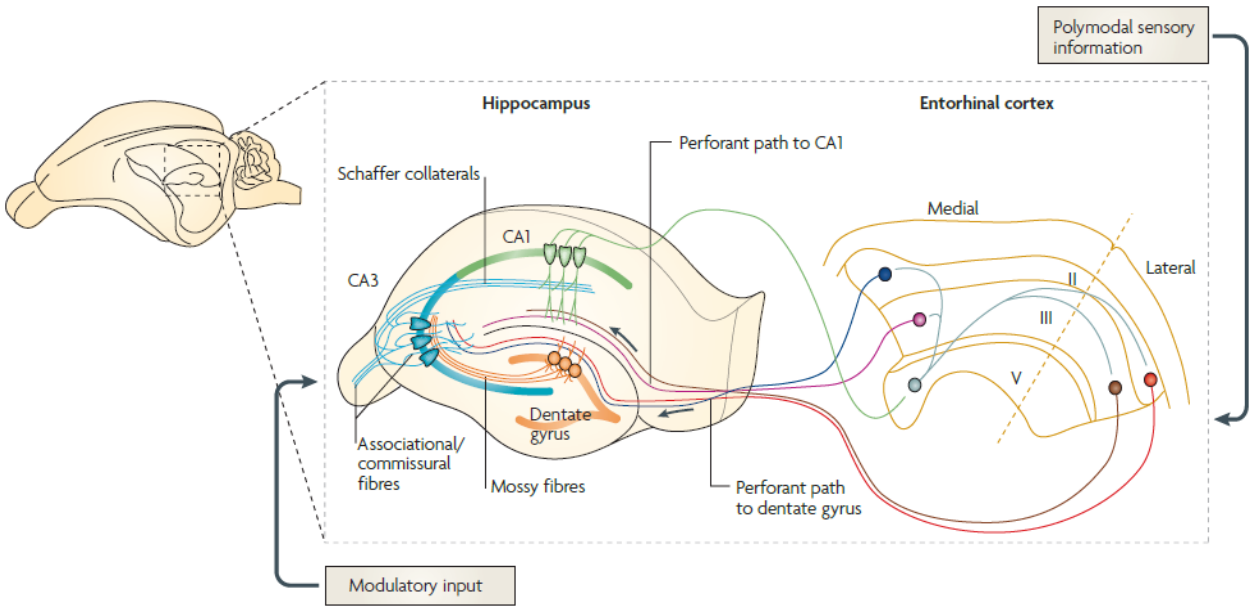

Figure 2. Basic anatomy of the hippocampus.
The wiring diagram of the hippocampus is traditionally presented as a trisynaptic loop. The major input is carried by axons of the perforant path, which convey polymodal sensory information from neurons in layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory synaptic contact with the dendrites of granule cells: axons from the lateral and medial entorhinal cortices innervate the outer and middle third of the dendritic tree, respectively. Granule cells project, through their axons (the mossy fibres), to the proximal apical dendrites of CA3 pyramidal cells which, in turn, project to ipsilateral CA1 pyramidal cells through Schaffer collaterals and to contralateral CA3 and CA1 pyramidal cells through commissural connections. In addition to the sequential trisynaptic circuit, there is also a dense associative network interconnecting CA3 cells on the same side. CA3 pyramidal cells are also innervated by a direct input from layer II cells of the entorhinal cortex (not shown). The distal apical dendrites of CA1 pyramidal neurons receive a direct input from layer III cells of the entorhinal cortex. There is also substantial modulatory input to hippocampal neurons. The three major subfields have an elegant laminar organization in which the cell bodies are tightly packed in an interlocking C-shaped arrangement, with afferent fibres terminating on selective regions of the dendritic tree. The hippocampus is also home to a rich diversity of inhibitory neurons that are not shown in the figure (adapted from Neves et al., 2008).

1.4. The emotional hippocampus, its connectivity and theta rhythm

As mentioned above, the hippocampus has an important role in emotional processing and its ventral part is the main player in this functionality. Lesions on the vHP influenced the neuroendocrine stress response and the emotional behaviour of rats in an elevated plus maze

test, but such changes were not observed when the dHP was lesioned (Kjelstrup et al., 2002). The same anxiolytic effects were observed in social interaction and hyponeophagia (a measurement of anxiety in mouse) tests with cytotoxic lesions on the vHP (Deacon et al, 2002). Brain tissue oxygenation, a measure of brain activity, increased during anxiety tasks in the vHP of rats, while tissue oxygenation in the dHP increases during spatial processing (McHugh et al, 2011) (See Figure 3B). Local infusions of the GABAa agonist muscimol led to anxiolytic effects when injected in the vHP and to anxiogenic effects when injected in the dHP (Zhang et al, 2014). Human studies have found that trait anxiety is related to activity in the posterior hippocampus, but not anterior, and that anxiety and depression are related to decreases in grey matter in the posterior hippocampus (de Geus et al., 2007; Satpute et al, 2012).

There is a triad of neural circuits involved in anxiety-like behaviors: ventral hippocampus, medial pre-frontal cortex (mPFC) and amygdala. It has been shown that vHP projects directly to the mPFC, both in mice and rats (Parent et al, 2010; Verwer et al, 1997). In turn, the mPFC projects to the central, medial, basolateral and cortical nuclei of amygdala (Vertes, 2004). For a detailed connectivity map of the hippocampus, see Figure 3A. Additionally, Shah and Treit demonstrated through a lesion study that the mPFC has an important role in anxiety-like behaviors (Shah & Treit, 2003) and, later, that blocking GABAa receptors in the mPFC leads to anxiolytic effects (Shah et al, 2004). Adhikari and colleagues suggested that the theta-frequency synchronization between vHP and mPFC can play a role in anxiety (Adhikari et al, 2010).

Theta, an oscillation that varies from 4 to 10 Hertz, is the most studied rhythm of the brain and can be found in the entire hippocampus. Some authors consider theta ranging from 5 to 12 Hertz (Lisman & Idiart, 1995). The first discovery of slow hippocampal oscillations was made by Jung and Kornmuller in rabbits (Jung & Kornmüller, 1938), but Green and Arduini were the first ones to perform an extensive study in hippocampal electric activity, describing some of the main characteristics of the theta rhythm (Green & Arduini, 1954). Later, theta was demonstrated to appear during the rapid eye movement (REM) sleep (Jouvet, 1969), during voluntary locomotion and exploratory behaviors (Vanderwolf, 1969) and in mnemonic processes (Lisman & Idiart, 1995; Miller, 1989; Raghavachari et al., 2001). Two types of theta can be distinguished: the type 1, that is atropine resistant, and type 2, that is atropine sensitive. The next session will focus on differences between theta type 1 and type 2 oscillations, where theta type 1 is the classic theta, while theta 2 is the slightly slower oscillation, often ignored in current literature.

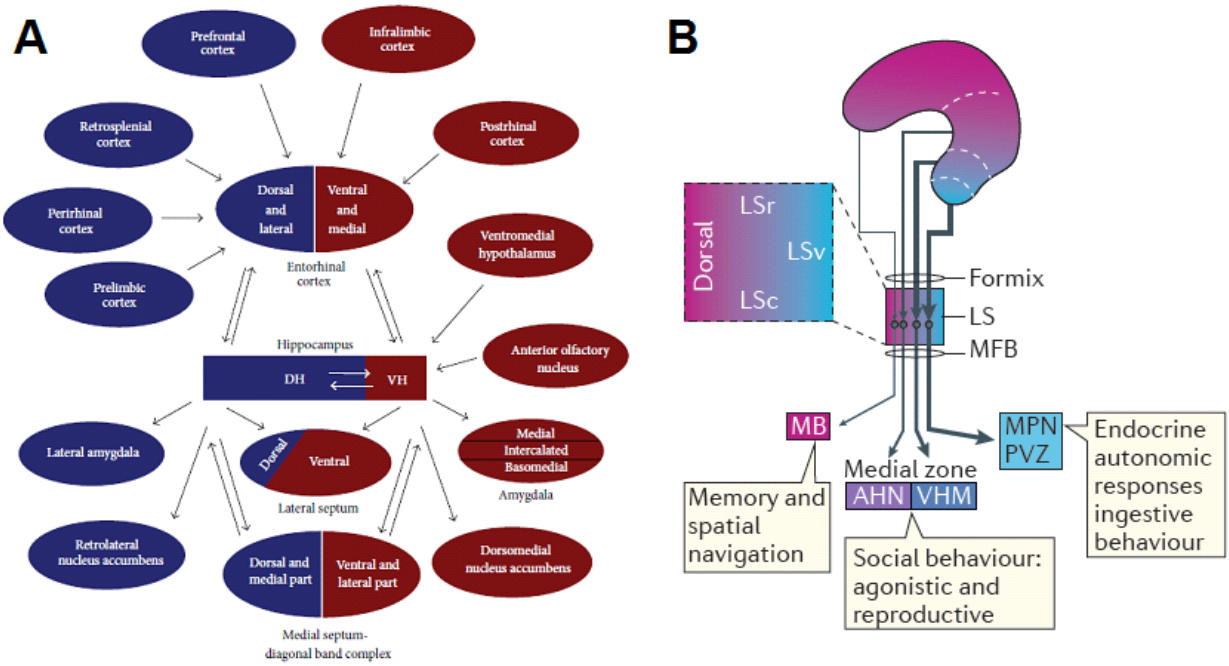

Figure 3: Connectivity and functional differentiation between ventral and dorsal hippocampus.
(A) A schematic diagram of the major connections of the dorsal (dHP) and ventral (vHP) sectors of hippocampus (adapted from 2016 - Lasting differential). (B) The hippocampal output to the lateral septum (LS) and hypothalamus. The LS can be divided into rostral (LSr), caudal (LSc) and ventral (LSv) parts. The most ventral tip of the CA1–subiculum (blue) projects to LSv, which projects to the medial preoptic nucleus (MPN) and hypothalamic periventricular zone (PVZ). More dorsal parts of the CA1–subiculum field project to the LSr, which in turn projects to hypothalamic medial zone nuclei, including the anterior hypothalamic nucleus (AHN) and the ventromedial hypothalamic nucleus (VMH). The dorsal subiculum sends a small projection to the dorsal LS, which is relayed to the mammillary body (MB). The thickness of the arrows indicates the projection density (Strange et al., 2014).

1.5. Type 1 and type 2 theta oscillations

Vanderwolf identified two different frequencies of theta in behaving rats, one around 6 Hertz and other around 7 Hertz, and clamed that "different behaviors which occur will be accompanied by different patterns of hippocampal activity". They found, for example, that slower theta activity (similar to type 2 theta) appears just before the jump avoidance response of the animals in an aversive situation, arguing that theta organize voluntary motor acts (Vanderwolf, 1969). However, Kramis recorded theta activity during anesthesia and alert immobility, demonstrating that theta can appear not only during motor acts. Specifically, he observed two types of theta: the first, of a lower frequency and ranging from 4 to 7 Hertz, occurred during behavioral immobility, under ether or urethane anesthesia and with electrical stimulation of the hypothalamic ascending cholinergic fibers, but was abolished with injection of 25 to 50mg/kg of atropine sulfate intraperitoneally. Additionally, injections of atropine methyl

nitrate, that does not cross the blood-brain barrier, did not affect the slower theta, demonstrating that the effects of atropine on this oscillations is on the central nervous system. The second theta, ranging from 7 to 12 Hertz, appeared only when the animals were behaving with repeated motor patterns as head movements or walking and was insensible to atropine sulfate, but was abolished under ether or urethane anesthesia (Kramis et al, 1975). The faster theta was, later, defined as type 1 (movement related) theta and the slower theta was defined as type 2 (immobility related) theta, and the characteristics described above were confirmed (Bland, 1986).

As briefly mentioned above, type 1 theta appears in, according to Vanderwolf classification, type 1 behaviors, that includes walking, jumping, swimming, head movement, changes in posture and manipulation (Kramis et al., 1975). Type 1 theta is not sensitive to muscarinic antagonists as atropine sulfate, but is sensitive to most anesthetics (Bland, 1986). The frequency of type 1 theta changes as a function of animals' speed (Kuo et al, 2011a) and speed modulation is correlated with spatial memory performance (Richard et al., 2013). On the other hand, the appearance of type 2 theta in the rodent hippocampus is dependent of a high arousal situation, like in innate anxiety tests with predator smell (Sainsbury et al, 1987), and is also linked with sensory processing during moments of high arousal (Pitkänen et al, 1995). Interestingly, some theories postulate the coexistence of the type 1 and type 2 thetas (Lai-Wo, 1984).

The mechanisms of generation of the two types of theta are also different. Stimulation of glutamatergic neurons in the medial septum led to the appearance of type 1 theta oscillations in the hippocampus, showing dependence of the glutamatergic rather than the cholinergic system (Fuhrmann et al., 2015). The rules are different for the type 2 theta. A series of studies provided more evidences for the cholinergic nature of type 2 theta oscillations (Bland, 1986). For example, injections of the choline transport system blocker hemicholineum-3, causing a drastic reduction of acetylcholine available in the terminals of cholinergic fibers, in the ventricle led to severe attenuation of type 2 theta, while systemic injections of choline chloride restored the this oscillation (Robinson & Green, 1980). In fact, older publications showed that major cholinergic inputs to the hippocampus comes from the medial septum (Lewis & Shute, 1967), and stimulation of the fimbria fornix enhances the population spikes in stratum pyramidale layer of CA1 hippocampal region (Krnjević & Ropert, 1982).

2. OBJECTIVE

Acess the anxiogenic effets of salicylate using behavioral and electrophysiological recordings.

3. METHODS

3.1. Animals

Three to five weeks old male wild-type C57BL/6 mice with 17 to 25g were used for the electrophysiological procedures and recordings. The mice were individually housed on a 12h/12h day/night cycle to maintain their normal biorhythms and had free access to food and water. All protocols were approved by the Ethics Committee of the Universidade Federal do Rio Grande do Norte, numbered as 052/2015. Every effort was made to minimize the suffering and discomfort of the mice and to reduce the number of the animals used.

3.2. Custom made arrays

Tungsten insulated electrodes (50 micrometers, impedance between 80Kohms and 300Kohms or 35 micrometers, impedance between 200Kohms and 1Mohm) from California Wires were used to manufacture the arrays and bundles that were assembled to a 16 channel custom made PCB with Omnetics connector (serie number 1434). Two different configurations were used: Type 1, with 15 electrodes (3x5, electrode spacing of 200 micrometers) for the ventral hippocampus or type 2, with 10 electrodes (2x5, electrode spacing of 200 micrometers) for the ventral hippocampus and 6 electrodes (bundle) for the dorsal hippocampus (See Figure 5B).

3.3. Surgery Procedure

Animals were deeply anesthetized with a mixture of ketamine (150mg/kg) and xilazine (7mg/kg) diluted in saline. Supplementation was done with ketamine diluted in saline. Mice temperature were maintained at 37º to 38º by a Supertech TPM-5h digital biological temperature controller (See Figure 4). Mice were head fixed by a Supertech stereotaxic and anterior-posterior and medium-lateral coordinates were calculated using bregma as zero, while dorso-ventral was measured from the brain surface. Electrodes were implanted through burr holes using a MP-225 micromanipulator (from ButterInstrument) and targeted the ventral portion of the hippocampus (centered at 3,2mm anterior, 3mm lateral, 3,5mm depth) for the type 1 and type 2 array configuration and the dorsal portion of the hippocampus (centered at 2,2mm anterior, 1,8mm lateral, 2mm depth) for the type 2 array configuration (See Figure 5A). A screw placed over the cerebellum served as ground and three additional screws served as anchors. The electrodes and the screws were cemented directly to the skull with dental acrylic (from Jet). Following the surgery, animals were housed individually and were monitored during the week after to prevent

suffering. The recovery was allowed for at least 10 days.

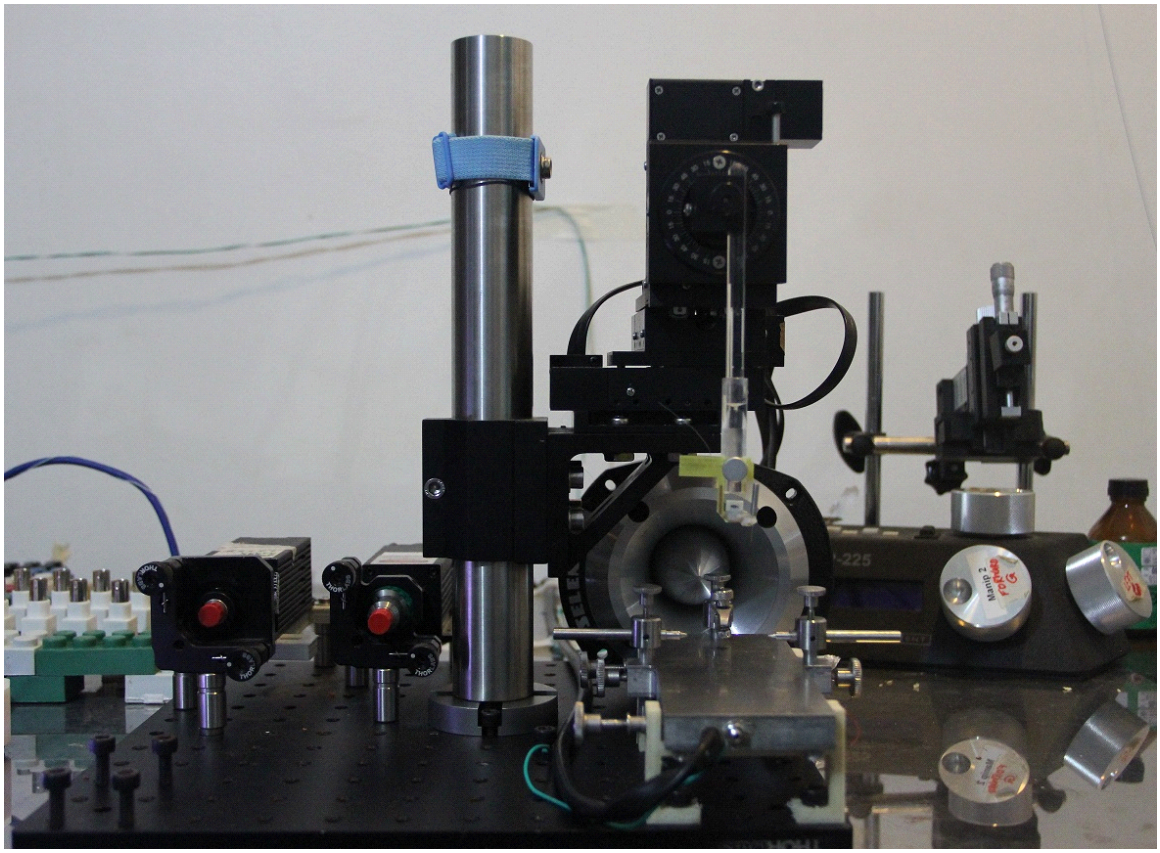

Figure 4. Setup used for surgery.
The MP-225 micromanipulator (from ButterInstrument) is attached to a ThorLabs adapter plate by a stainless steel bar, and the Supertech TPM-5h heatpad is attached to the same adapter plate by a custom made support.

3.4. Data acquisition

Electrophysiological data were acquired using a digital amplifier system (Intan RHA2000 Evaluation board with a Intan RHA2116 Headstage) at 25kHz and stored as raw signal for post-processing. Video recordings were done with a Sony XCD-SX910 firewire camera. Intan board and camera were triggered by a National Instruments board (NI USB-6351) using a custom made Matlab routine.

3.5. Experimental protocol.

For the first test, animals were left for two hours in the experimental room before the start of the recordings. A maximum of three mice were recorded per day and the recordings were done during the night (waking cycle). Each mouse received a injection of Sodium Salicylate (300mg/kg, pure Sodium Salicylate diluted at the left in saline at 50mg/mL) or saline intra peritonealy. After sixty minutes individual mouse was placed in a rectangular open field arena

(40cm X 32cm X 15cm) made of opaque white plastic for electrophysiological and video recordings. Two sessions of ten minutes were recorded. Mice were then put back to the animal house and rested for seven days until the next step of the protocol.

After the resting period (seven days), mice were brought to the experimental room and were left for two hours. Animals that were injected with Sodium Salicylate in the first test received injection of saline in the second test and animals that received saline in the first test were injected with Sodium Salicylate in the second test (300mg/kg of Sodium Salicylate, diluted in saline at 50mg/mL). After sixty minutes individual mouse was placed in a rectangular open field arena (40cm X 32cm X 15cm) made of opaque white plastic for electrophysiological and video recordings. Two sessions of ten minutes were recorded. Mice were then put back to the animal house and rested for seven days until the next recording session. By doing this, we divided the animals in two groups: group 1, that first received injection of saline; and group 2, that first received injection of Sodium Salicylate.

For a third test, after the resting period mice were brought to the experimental room and were left for two hours. All animals received the same treatment: injection of Sodium Salicylate (300mg/kg of Sodium Salicylate, diluted in saline at 50mg/mL) followed by an injection of atropine (40mg/kg) (See Figure 5C). The same protocol of the first test was repeated. Open field arena was cleaned with a 70% ethanol and allowed to dry completely after each recording.

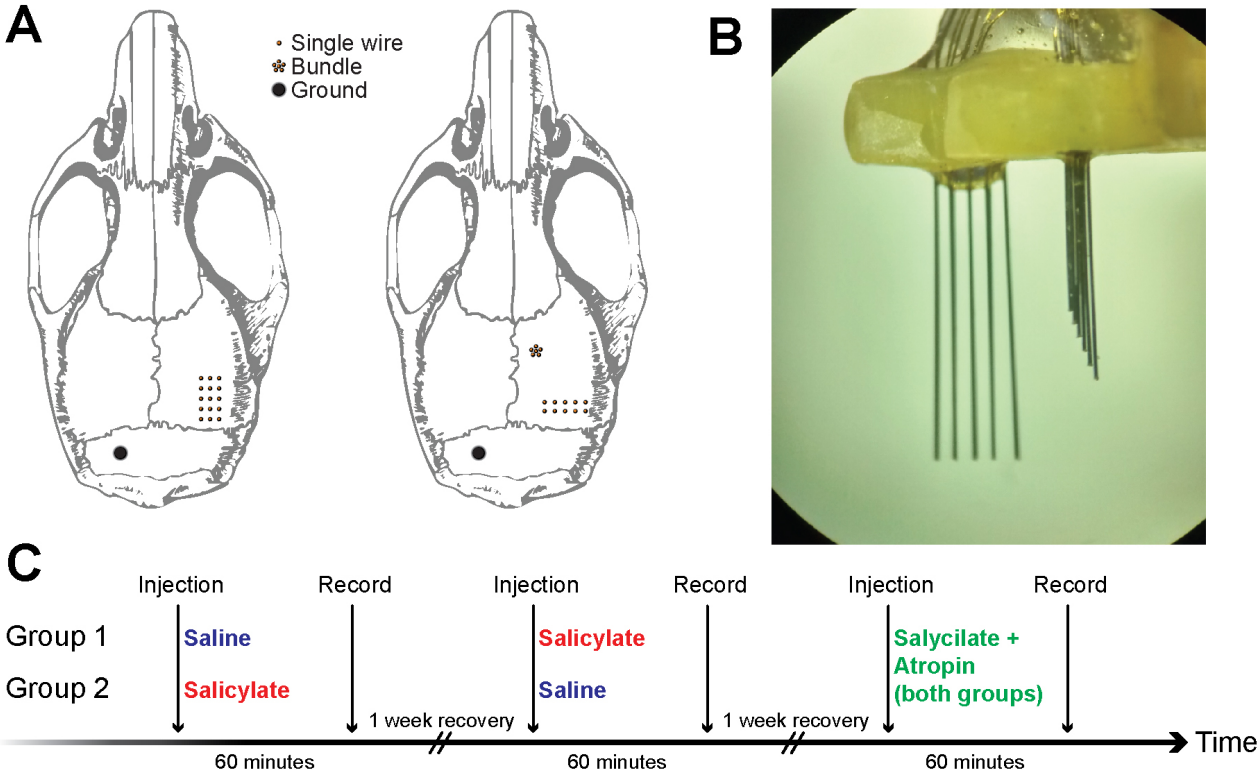

Figure 5. Methods description.

(A) Illustrative skull schematic with arrays disposition. On the left, representation of the 15 electrodes array centered at 3,2 anterior, 3 lateral and 3,5 depth. On the right, representation of the 16 electrodes array, with 10 for the vHP centered at 3,2 anterior, 3 lateral and 3,5 depth and 6 for the dHP centered at 2,2 anterior, 1,8 lateral and 2 depth. (B) Photography of a 16 electrodes array for vHP and dDP. (C) Timeline of the experimental protocol. Mice were divided into two groups. Group 1 received saline in the first test while group 2 received Salicylate in the first test. For the second test injections were inverted. Both groups received injection of Salicylate and Atropine for the third test.

3.6. Histology

Animals were deeply anesthetized with Thiopental and electrolytic lesions were made in the main electrodes to determine the position of the electrode tip. A positive square pulse of 500 microamperes was injected during 5 seconds to lesion the tissue. After the lesion, mice were perfused by transcardial perfusion with phosphate buffered solution (pH 7,4) and a fixative solution (paraformaldeyde at 4%). Brains were stored overnight in paraformaldeyde (PFA) and then put on saccharose at 30% until the brain sink. Brains were sectioned in 50 micrometers slices using a cryostat and the slices were stained using cresyl violet.

3.7. Signal processing and data analysis

Extracellular recordings were collected from freely moving animals implanted with a 16-wire array. Recordings were performed using a modified Intan RHA software (Intantech) piped with a custom made Matlab program. Power spectral densities (PSD) for all channels were computed using the *Welch* method (*pwelch* Matlab command). To allow merging of data from different animals, PSD values were normalised (PSDs were divided by the total power). In the open field experiments, we also acquired video that was synchronised with the local field potential (LFP) recordings by TTL pulses produced by an arduino board. A custom Matlab software (imaging processing toolbox) was used for tracking the animal in the field by thresholding the animal compared with the background. In some experiments, we correlate theta power with animal speed by downsampling total theta power vs. time obtained from a spectrogram (Matlab *spectrogram* command) of a recording channel placed at the stratum radiatum (SR) of both ventral and dorsal hippocampus. In the experiments involving dorsal and ventral hippocampus recordings, we calculated the coherence between dorsal and ventral SR channels using the Matlab command *mscohere.* Data is presented by mean ±standard error of the mean (SEM). Unless otherwise noted, statistical significance was calculated using paired *t* test for electrophysiological data and *rank sum* test for the behavioral analysis.

4. RESULTS

In this study, we used 9 mice in behavior and electrophysiology experiments, 5 mice implanted with arrays in the ventral hippocampus and 4 mice with wire arrays targetting the dorsal and ventral hippocampus. Mice was placed in an open field arena while both imaging and LFP were acquired using a custom made Matlab software and Intan Acquisition Software.

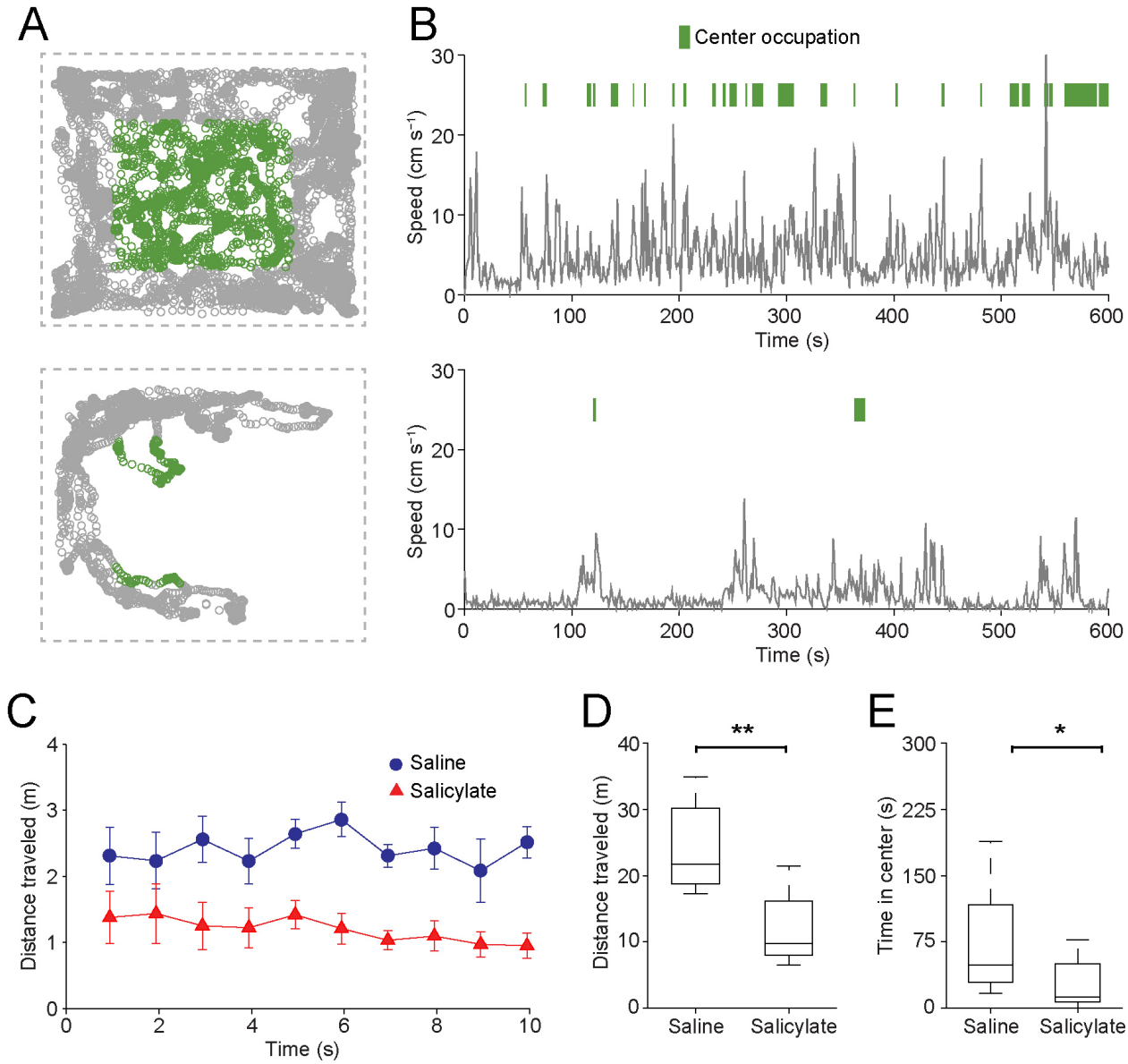

Figure 6. Salicylate induced anxiety-like behavior in open-field arena.
(A) Video tracking data for an example mouse during open field exploration. Panels show the same mouse in saline (top) and salicylate (bottom) conditions. Green dots represents moments in the center. (B) Speed of locomotion through time (same experiment as in A) superimposed with the raster plot of center occupation (green bars) during saline (top) and salicylate (bottom) conditions. (C) Distance traveled through time for all animals calculated for each minute (n = 8 animals). (D) Total distance traveled as a function of injection (n=8 animals, p=0,0047, rank sum test). (E) Time spent in center as a function of injection (n=8 animals, p=0,049, rank sum test).

4.1. Salicylate induces anxiety-like behaviour in open-field

We first tested whether salicylate injection induces anxiety-like behaviour. Total travelled distance assess mice locomotion activity while the time spent in the centre is associated to anxiety-like behaviour (Carola et al, 2002; Walsh & Cummins, 1976). Salicylate severely decreased both distance travelled and time spent in the centre (Figure 6). In control conditions (saline), mice had a mean travelled distance of 24,18m±5,66m but 60 minutes after salicylate injection, total travelled distance of 11,93m±4,47m (rank sum test, p=0,0047, n=8, Figure 6D). Time spent in the centre for saline injection was equal to 74,43s±49,77s and 27,13s±24,01s for salicylate injection (rank sum test, p=0,049, n=8, Figure 6E). Taken together, these results indicate that salicylate lowers locomotion and increase anxiety-like behaviour.

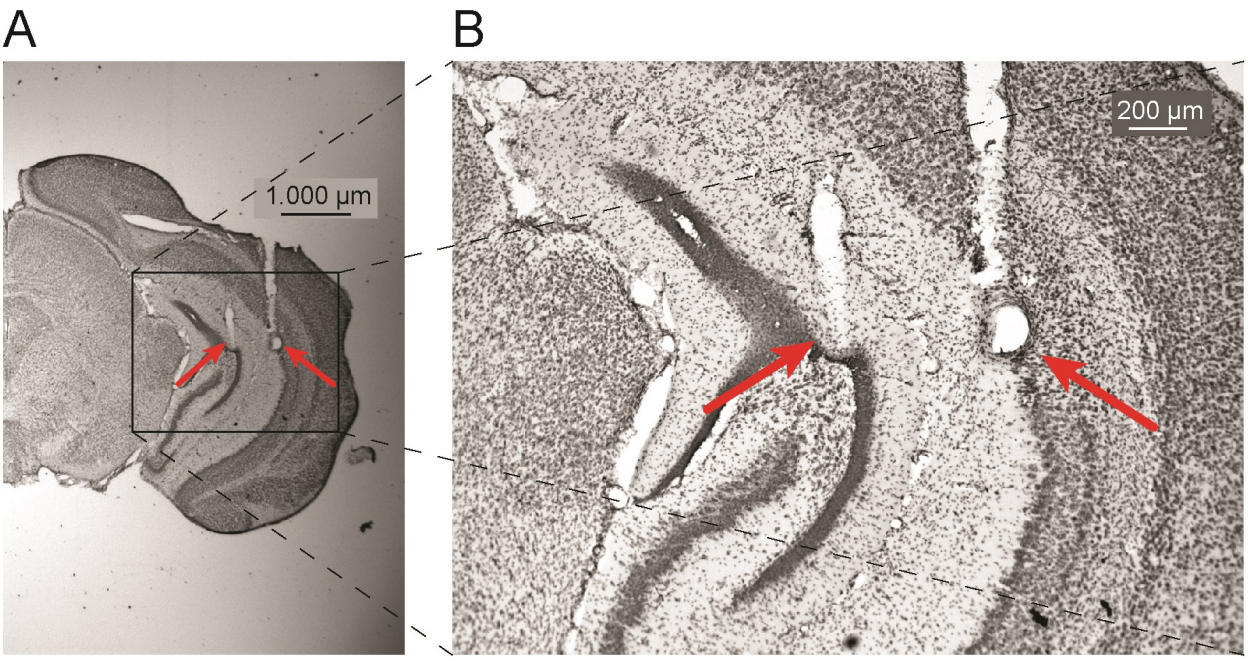

Figure 7. Histology of the electrodes implanted on the ventral/intermediate hippocampus. (A) Top-down view of coronal brain section showing electrode position in the intermediate/ventral hippocampus. Electrodes trace were made by current injection (See Methods > Histology) and are pointed by the red arrows. Only 2 electrodes were selected to receive current injection (B) Zoomed top-down view of coronal brain section showing electrode position.

4.2. Salicylate increase low-frequency theta oscillation

Next, we assess local field potential (LFP) recordings from the intermediate/ventral hippocampus (Figure 7) associated to the open-field test. Individual power spectral densities were normalised by the sum of the total PSD. Salicylate injection produced a large increase in power of oscillations ranging from 4-7Hz (example in Figure 8). It's important to mention that

the 4Hz observed has no relation with a state of drowsiness. During most of the recording time animals kept a state of alert. We first tested differences in 7-10Hz theta oscillation as this rhythm is associated with locomotion (Bland, 1986; Kramis et al., 1975; Kuo et al, 2011b; Vanderwolf, 1969). As expected, mean 7-10Hz theta power was lower in salicylate-treated sessions than in saline-treated controls (Figure 7). Mean normalised 7-10Hz theta power was equal to 0.08±0.012 (μV^2/Hz) in the saline condition vs. 0.04±0.008 (μV^2/Hz) after salicylate injection (paired *t* test, p=0.02, n=9, Figure 9). On the other hand, the injection of salicylate increased the power of 4-7Hz oscillation from 0.06±0.006 (μV^2/Hz) to 0.10±0.010 (μV^2/Hz) (paired *t* test, p=0.007, n=9, Figure 9). Taken together, these results show that salicylate injection causes an increase in 4-7Hz oscillation and a decrease in movement related theta oscillation.

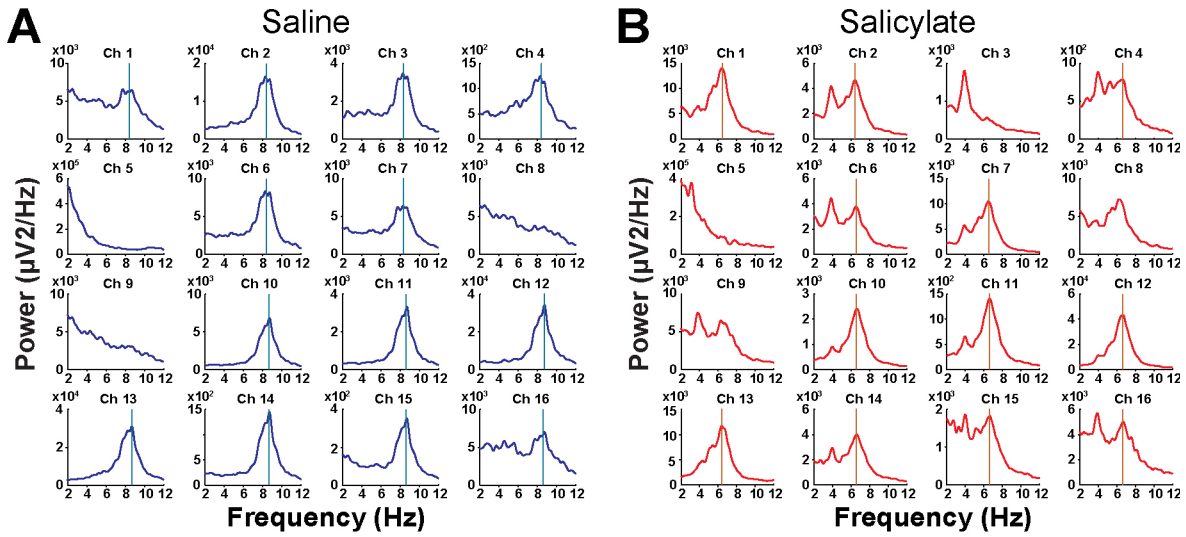

Figure 8. Frequency changes equally in all channels for salicylate-treated animals.
(A) PSD analysis for all 16 channels of one animal in saline condition during 10 minutes of recording. Blue vertical lines mark the peak of the frequency. Some channels are outside the hippocampus and for this reason doesn't have hippocampal theta oscillations. (B) PSD analysis of all channels of the same animal as in A, but for the salicylate condition. Red vertical lines mark the peak of the frequency.

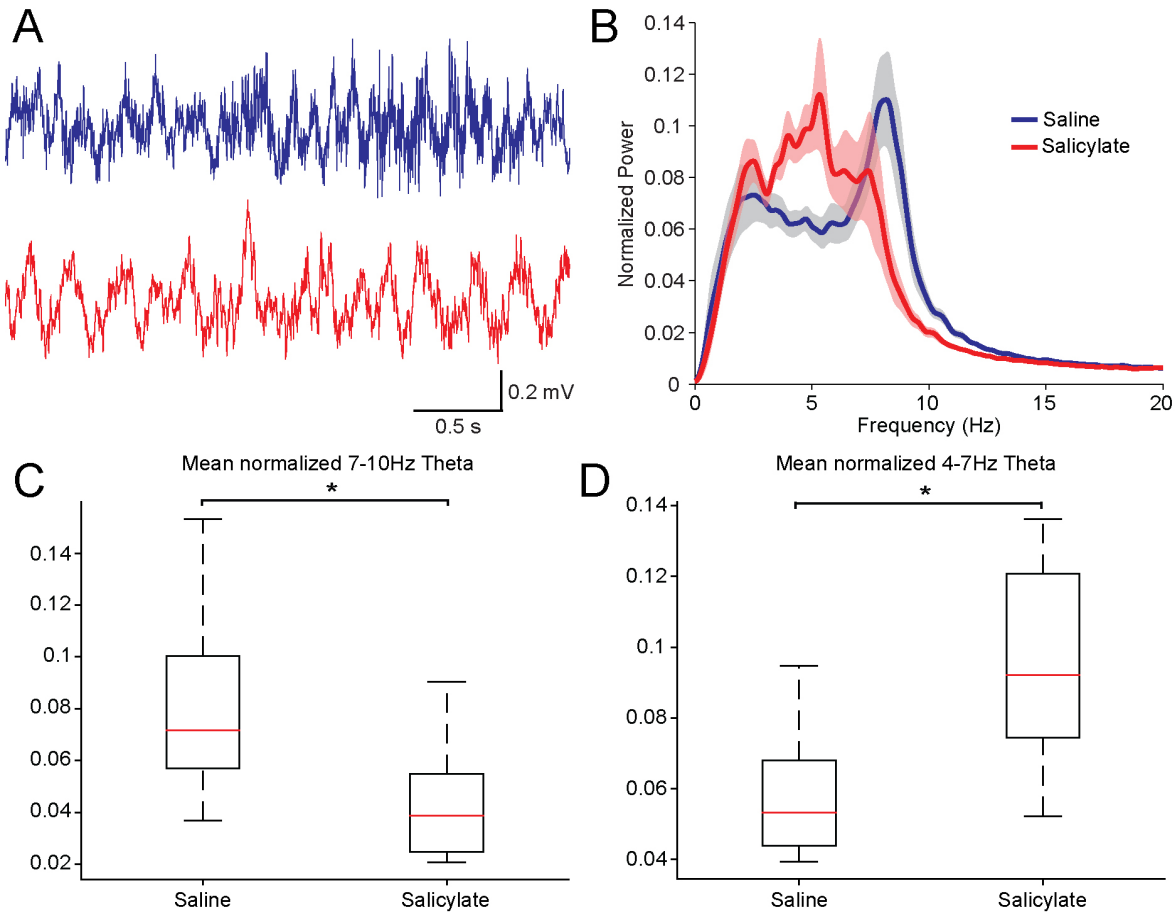

Figure 9. Salicylate incleases lower frequency theta oscillation.
(A) Raw trace example of one animal, same electrode, in saline condicion (blue) and salicylate condition (red). (B) Administration of salicylate increased the 4-7Hz theta oscillation in the ventral hippocampus (n=9). (C) Significant difference was found for the peak of 7-10Hz theta (paired t test, p=0.02, n=9). (D) Significant difference was found for the peak of 4-7Hz theta (paired t test, p=0.007, n=9).

As mentioned above, in the dorsal hippocampus, theta type 1 oscillation (7-10Hz) in mice is strongly associated with movement (Bland, 1986; Kramis et al., 1975; Kuo et al, 2011b; Vanderwolf, 1969). In the ventral hippocampus however, this correlation, while positive, is significantly weaker than in the dorsal counterpart. We then analysed the relation between theta type 1 (movement associated – 7-10Hz) and the 4-7Hz oscillation with movement (Figure 10). We used the *spectrogram* Matlab function to obtain an array of J x I power values where J were frequencies ranging from 0 to 20 Hz and I were time points from 0 to 600s (Figure 10A, only 0 to 10Hz is shown). Hence, 4-7Hz power vector was the mean I for 4Hz < J < 7Hz and theta type 1 power vector was the mean I for 7Hz < J < 10Hz. During salicylate injections, the mean correlation coefficient (r) for theta type one was equal to 0.26±0.062 while the mean r for 4-7Hz oscillation was equal to -0.17±0.08 (paired t test, p=0.0006, n=9, Figure 10D). These results show that the increase in 4-7Hz oscillation is not related to increase in velocity like type 1 theta oscillation.

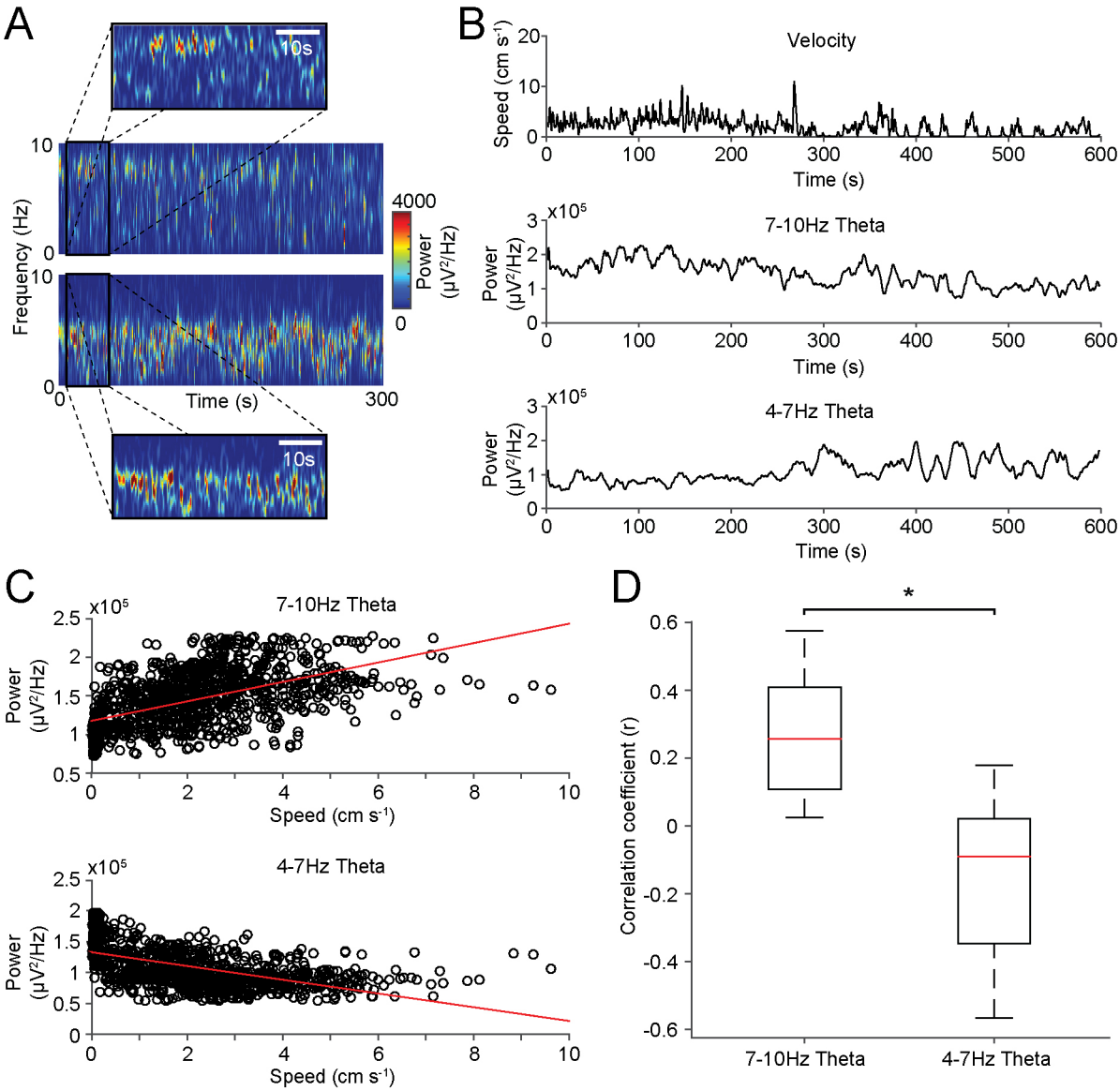

Figure 10. Different frequencies of theta changes differently with locomotion.
(A) Time-frequency spectrogram of one animal, same channel, for saline condition (top) and salicylate condition (down) (B) One animal example showing the relation between velocity and the two different frequencies of theta (salicylate condition). (C, D) Power of theta incleases as a function of locomotion speed for the 7-10Hz theta, but decreases for the 4-7Hz theta (paired *t* test, n=9, p=0,0006).

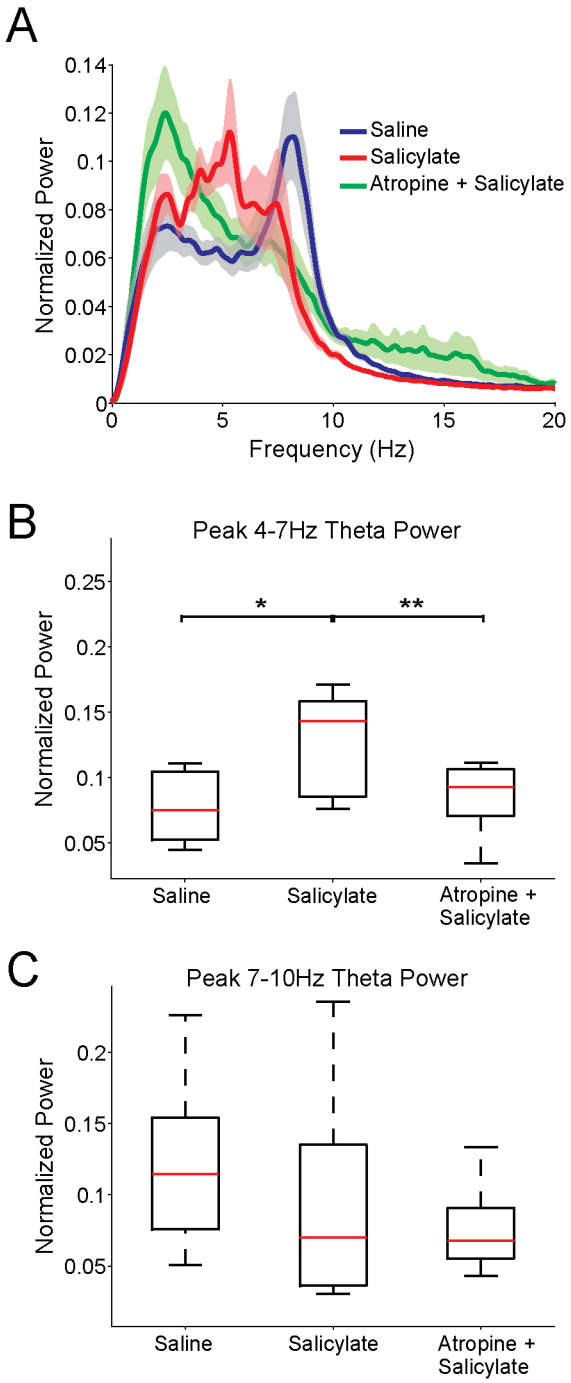

Figure 11. 4-7Hz theta generated by salicylate administration is atropine sensitive.
(A) Administration of salicylate increased the 4-7Hz theta oscillation in the ventral hippocampus, but conjugated administration of salicylate with atropine didn't inclease the 4-7Hz theta (*n*=9). (B) Salicylate treated animals shows a significantly higher peak for 4-7Hz, while no difference was found between saline treated mice and salicylate + atropine treated animals (*n*=9). (C) Salicylate didn't significantly affect 7-10Hz theta (*n*=9).

Classically, theta oscillation has been divided in two types: a higher frequency type 1 oscillation that is associated with movement (showing a strong correlation between movement speed and theta power) and insensitive to atropine (cholinergic blocker) (Bland, 1986; Kramis et al., 1975; Kuo et al, 2011b; Vanderwolf, 1969) and a type 2 theta, that is not correlated with speed but sensitive to atropine (Vanderwolf, 1969; Bland, 1986). To test whether the 4-7Hz oscillation induced by salicylate could be classified as theta type 2, we also injected atropine in salicylate-treated animals (Figure 11). Atropine decreased 4-7Hz oscillation without affecting 7-10Hz type 1 theta oscillation (Figure 11). Hence, these results indicate that the salicylate induced 4-7Hz oscillation could be classified as type 2 theta.

In the dorsal hippocampus, theta type one modulates the amplitude of higher frequency gamma oscillations (Colgin, 2015; Lisman & Jensen, 2013; Tort et al, 2007). We then tested whether salicylate-induced type 2 theta oscillation modulate higher frequency oscillations in the ventral hippocampus. One of the most commonly used methods to assess whether theta modulates other frequency is to compute the modulation index obtained by a distribution of amplitude values of higher frequencies over the phase values of the lower frequencies obtained by Hilbert transform (Figure 12A). Salicylate-induced theta type 2 oscillation strongly modulated 60-120Hz gamma phase oscillation. Maximum modulation index in control condition (of theta type 2 phase) was equal to 0.0003±0.0001 and equal to 0.0010±0.0002 (paired *t* test, p=0.02, n=9, Figure 12). Although differences have been found in the modulation index, no differences were found in gamma power after salicylate injection compared to saline injection (data not shown). These data shows that salicylate-induced type 2 theta can modulate gamma oscillations in the hippocampus.

We next tested whether salicylate induced theta oscillation was also present at the dorsal hippocampus. For these sets of experiments a ‘bundle’/array electrode was used (see Methods). The wire bundle (6 electrodes) was placed at the dorsal hippocampus and a 10-wire array aimed at the Stratum Radiatum (SR) of ventral/intermadiate hippocampus (Figure 13). We have selected two electrodes for the analysis (based on histology): one at the Stratum piramidale (SP) of dorsal hippocampus and one at the SP of the ventral/intermediate hippocampus (Figure 13). As expected, in control conditions (saline), we observed a strong theta type 1 oscillation in the dorsal hippocampus (Figure 14A and B) and this oscillation was masked by a slower rhythm after salicylate injection (Figure 14A and B). Peak coherence between the dorsal and ventral hippocampus LFP showed a shift in peak frequency (Figure 14C, n=5). These results indicate that salicylate generates type 2 theta oscillation also in the dorsal hippocampus and this oscillation prevails in the whole hippocampus axis after salicylate injection.

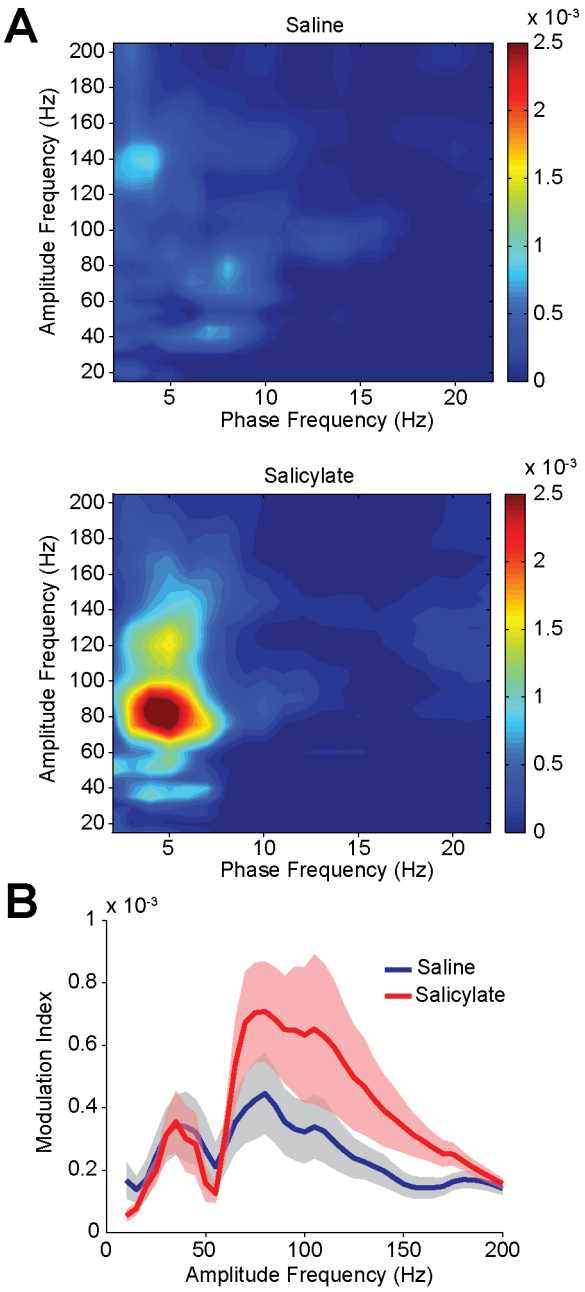

Figure 12. Salicylate incleases theta-gamma coupling in the frequency of 4-7Hz.
(A) Spectrogram showing theta-gamma coupling for one channel example in saline condition (top) and salicylate condition (down). (B) High gamma oscillation couple strongly with theta in salicylate treated animals (paired *t* test, p=0.02, n=9).

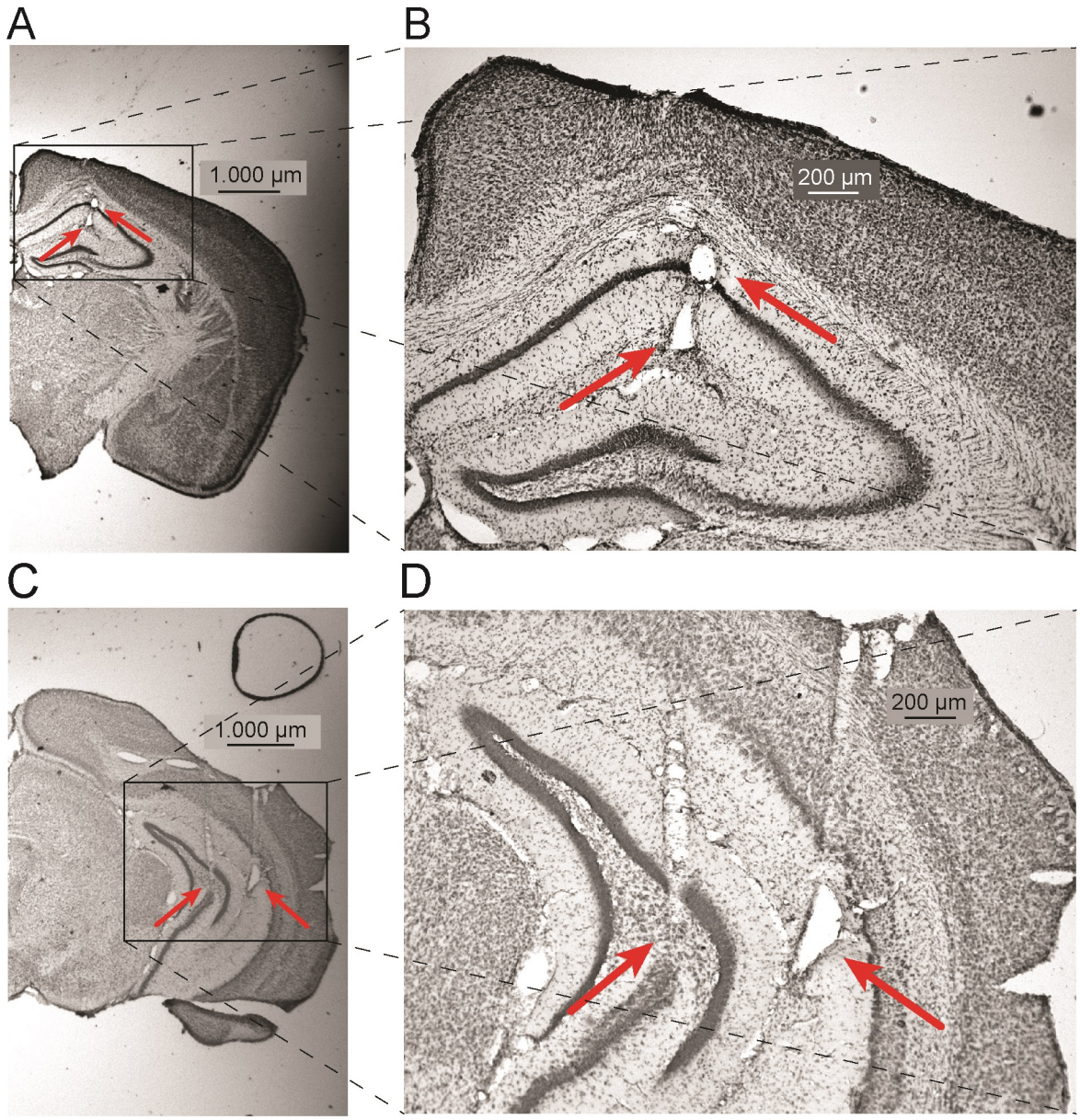

Figure 13. Histology of the electrodes implanted on the ventral/intermediate and dorsal hippocampus.
(A) Top-down view of coronal brain section showing electrode position in the dorsal hippocampus. Electrodes trace were made by current injection (See Methods > Histology) and are pointed by the red arrows. (B) Zoomed top-down view of coronal brain section showing electrode position. (C) Top-down view of coronal brain section showing electrode position in the ventral hippocampus. Electrodes trace were made by current injection (See Methods > Histology) and are pointed by the red arrows. (B) Zoomed top-down view of coronal brain section showing electrode position.

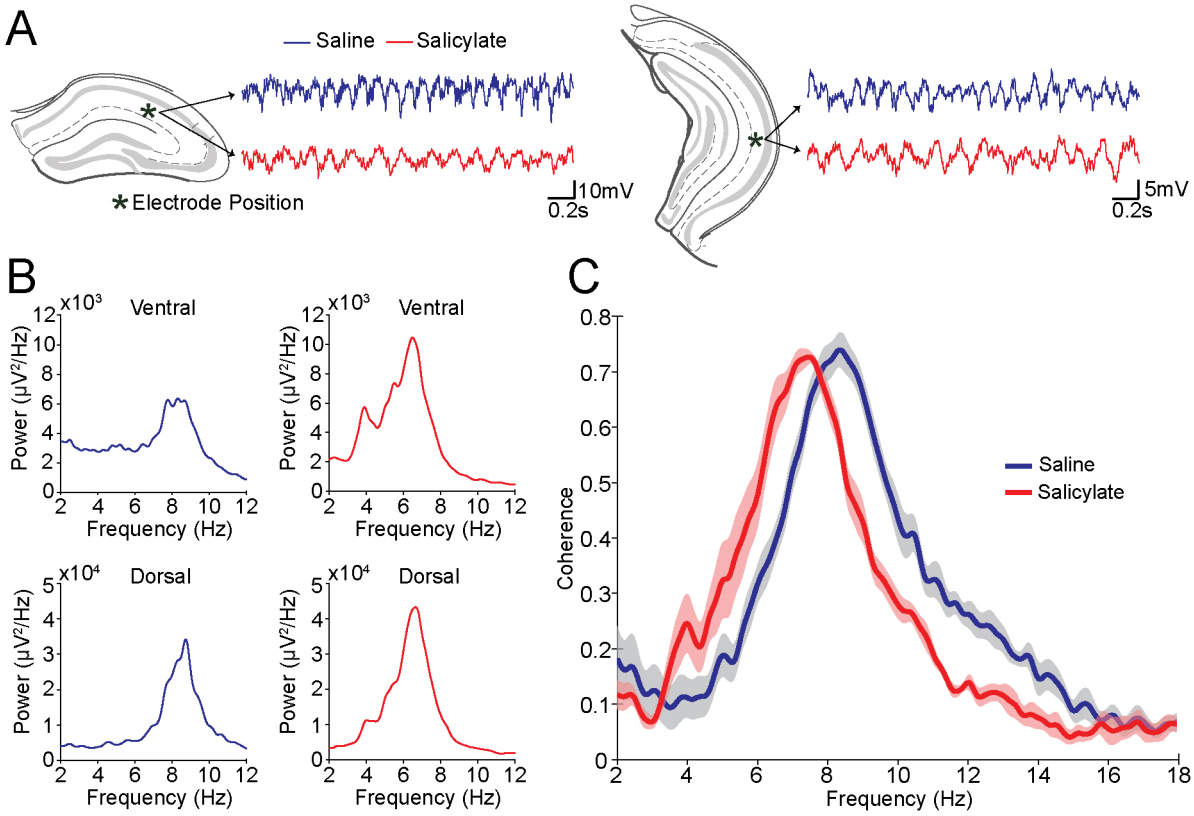

Figure 14. Theta coherence between vHP and dHP changes with salicylate.
(A) Schematic of the position of electrodes used for the analysis with respective raw trace for salicylate condition and saline condition. (B) PSD of the same channels showed in A. (C) Salicylate does't affect the coherence index between ventral and dorsal hippocampus, but causes a change in the center frequency (*n*=5).

In order to assess whether type 2 theta oscillation is generated in the ventral hippocampus, we have computed the Granger causality for the dorsal and ventral hippocampus LFP. In both saline and salicylate conditions, the ventral hippocampus Granger causes the 4-7Hz oscillation (Figure 15). However, these results should be interpreted with caution as the type 1 theta oscillation was not Granger caused by the dorsal hippocampus as described in the literature (Lubenov & Siapas, 2009). Hence, while the Granger analysis indicate that the ventral hippocampus 'leads' the generation of type 2 theta oscillation, the lack of type 1 theta peak in the control condition indicate that either the analysis is producing spurious results or that type 1 theta is generated simultaneously in the dorsal and ventral hippocampus. In any case, as mentioned previously, these results need further assessment.

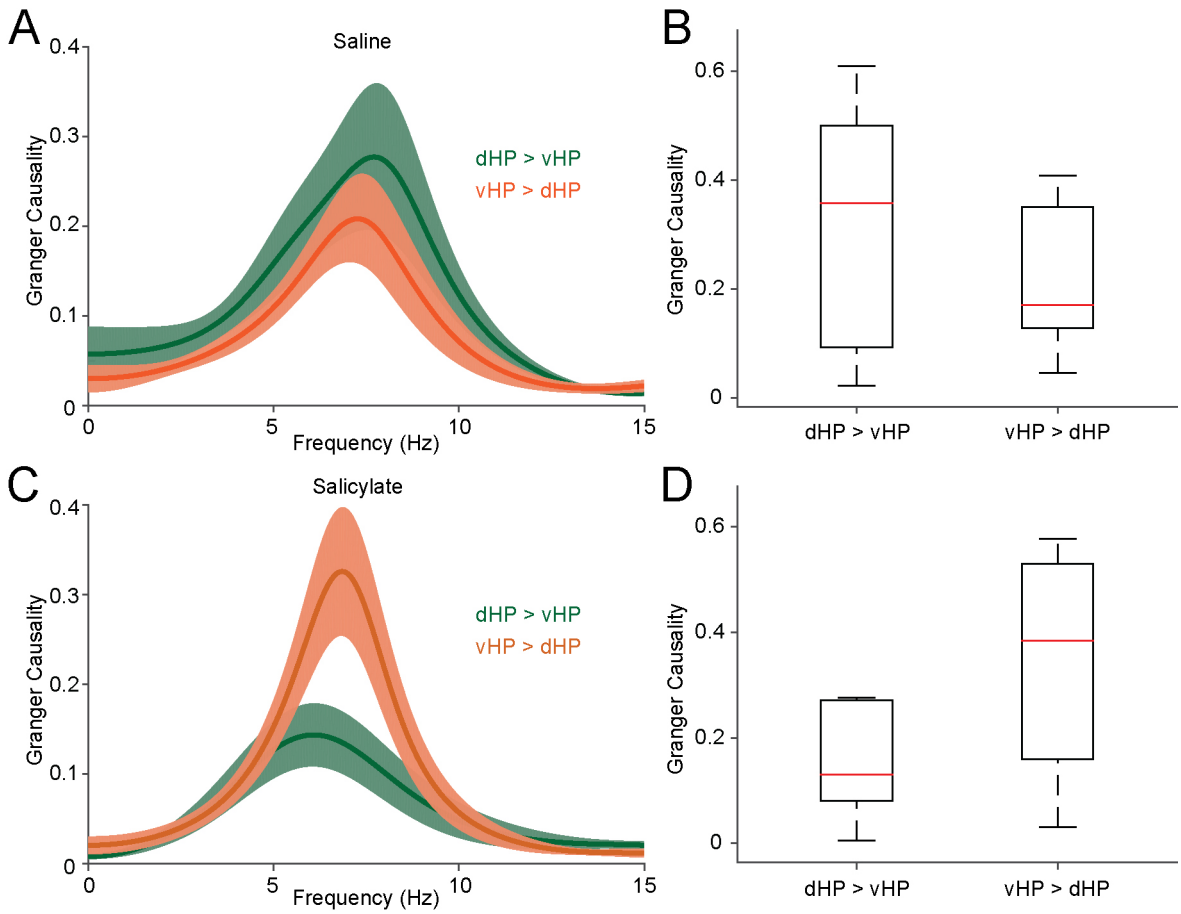

Figure 15. Directionality of theta oscillations changes in salicylate condition.
(A, C) Granger causality analysis shows a higher index for the direction of dHP to vHP in saline treated animals, but a higher index for vHP to dHP in salicylate treated animals (*n*=5).

5. DISCUSSION

Various assays have been used to acess anxiety in animal models. The most used assay for rodents, developed by Calvin Hall in 1932, is the open field. This model is based on the tendency that rodents have to avoid illuminated open spaces, what is a natural way to avoid predation, and to stay in contact with the walls (Thompson, Grabowski-Boase, & Tarantino, 2015). Initially, Hall examined urination, defecation and locomotion activity only. However, other behavioral aspects were later considered to the analysis (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002; Walsh & Cummins, 1976). In the present study we find differences in locomotion activity in mice treated with salicylate. Distance traveled was significantly lower in the salicylate group compared with the saline group and salicylate led to linear decrease in locomotion activity over recording time. Also, the time spent in center was lower in the salycilate group compared to the control group. However, two animals spent more time in centre in the salicylate case compared with saline. It possibly happened due to the position of the headstage's cable that made it difficult for the mice to move across the borders of the open field. Additionally, little of the time spent on center was spent with exploratory behaviors as animals were in state of immobility. One animal was considered outlier and was excluded from the analysis due to a restraining headstage cable that impaired the movement of the animal in the boarders, causing the time spent on center to be very high. Nevertheless, most of the locomotor inactivity time during the recording session of this animal was of a freezing state of immobility, indicating a possible high anxiety level. Together, this data suggests an incleased level of anxiety in mice treated with salicylate injections when compared with saline injections. Future directions point to need of investigating the behavior of mice in other assays to acess anxiety, as the elevated plus maze.

Salicylate administration led to a increase in type 2 theta oscillation in the ventral hippocampus. We found a sligtly decrease, but not significant, in type 1 theta that is in accordance with the differences in locomotion activity verified in behavioral analysis. With administration of salicylate associated with atropine, we confirmed the cholinergic dependence of the slower theta oscillation recorded in our experiments. Further evidences that the oscillation recorded and classified as type 2 theta, we investigated the correlation between theta and locomotion. The results showed a positive correlation between type 1 theta and speed of the animal, what is in accordance with the whole literature of this classic theta related to movement, but a negative correlation between type 2 theta and speed, confirming the literature reports about this oscillation. However, there is an aspect often ignored to consider: some injectable drugs can

cause pain due to a different pH from the body. For exemple, lidocaine, altough be used as anesthetic, cause pain because of its acidic pH and one way to avoid this problem consists in buffer the solution to be injected (Cepeda et al., 2010; Skarsvåg et al., 2015). Regarding salicylate there is no reports on the literature relating its pH with pain. On the other hand, it is known that type 2 theta is related with fear and anxiety (Vanderwolf, 1969; Bland, 1986), and it's not known whether pain contribute to the inclease in type 2 theta.

	Frequency	Behavior	Atropine	Salicylate
Type 1 Theta	7-10 Hz	Locomotion, exploration	Resistant	Doesn't affect
Type 2 Theta	4-7 Hz	Alert, immobility	Sensitive	Affect

Despite the hippocampus be composed mostly by pyramidal cells (PCs), a population that are relatively homogeneous, the interneurons, that comprises only twenty percent of the cells, are the most diverse population (Freund & Buzsáki, 1996; Klausberger & Somogyi, 2008). Differences in position, molecular and functional properties, extrinsic inputs and targeting makes internerons population so diverse (See Figure D1) (Freund & Buzsáki, 1996; Klausberger & Somogyi, 2008) (See Figure 16). Interneurons in the hippocampus plays a crucian role in synchronizing network activity due to it's positioning. The existence of oscillations, like theta, depends on the interactions between the interneurons and PCs (Allen & Monyer, 2015). Different types of interneurons have different fire patterns, and phase-lock differently to hippocampal oscillations: for example, bistratified cells shows a strong phase-lock to gamma oscillation, while O-LM cells have no coupling to gamma, but fire strongly phase-locked to theta rhythm (See Figure D1) (Klausberger et al., 2003). An interesting study conducted by Yan-Yan Su and colleagues investigated the effects of salicylate in PCs and interneurons of the auditory cortex and found that salicylate depresses firing in fast-spiking interneurons, but not in pyramidal cells, suggesting that salicylate acts via interneurons leading to raised excitability in the central auditory system. Through this findings, we hypothesize that salicylate changes theta oscillations in the hippocampus, specifically increasing type 2 theta, by acting via interneurons. Going deeper, we found an increase in Theta- Fast Gamma coupling, in the frequency range of type 2 theta, in the salicylate group. The function of the Theta-Gamma coupling is not well understood yet, but some publications elucidate aspects related to this frequency coupling (Colgin, 2015; Lisman & Jensen, 2013). Specially, a study conducted by Craig and McBain found that specific types of interneurons, as axo-axonic and bistratified cells, are involved in the generation of intrinsic fast gamma in CA1 without the involvement of pyramidal cells (Craig &

McBain, 2015). Our hypothesis is that salicylate acts in this specific types of interneurons.

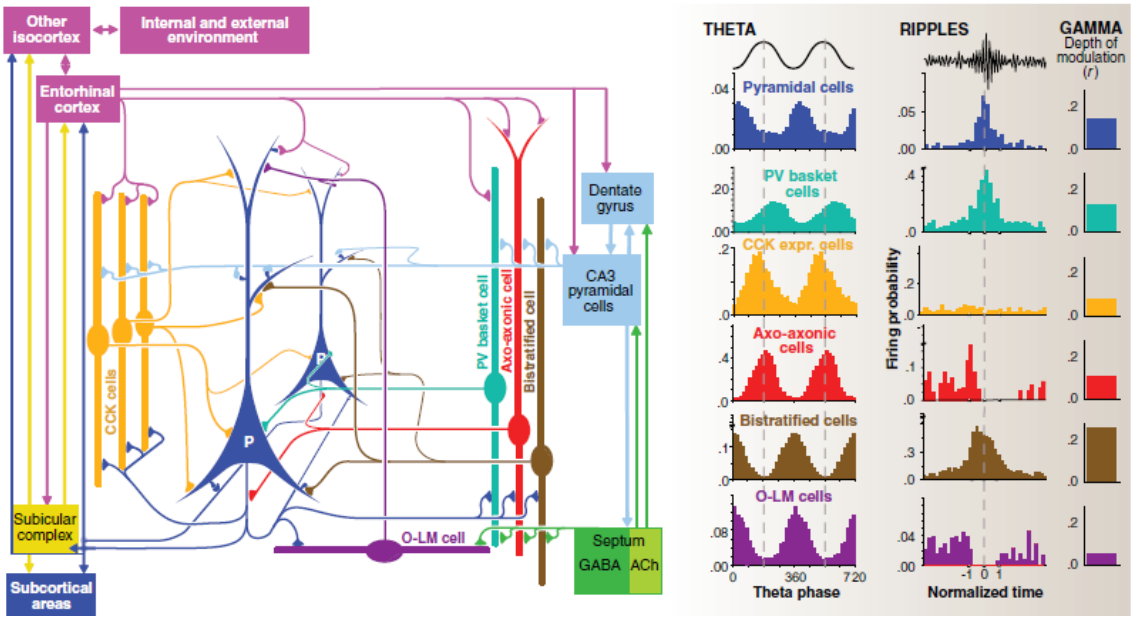

Figure 16. Position and electrophysiological characteristics of interneurons in hippocampus.
Spatiotemporal interaction between pyramidal cells and several classes of interneuron during network oscillations, shown as a schematic summary of the main synaptic connections of pyramidal cells (P), PVexpressing basket, axo-axonic, bistratified, O-LM, and three classes of CCKexpressing interneurons. The firing probability histograms show that interneurons innervating different domains of pyramidal cells fire with distinct temporal patterns during theta and ripple oscillations, and their spike timing is coupled to field gamma oscillations to differing degrees. The same somatic and dendritic domains receive differentially timed input from several types of GABAergic interneuron (adapted from Klausberger & Somogyi, 2008).

Theta oscillations are known to pattern hippocampal activity over time. Despite little is known on how theta occur in space an interesting study by Lubenov and Siapas demonstrated that theta is an oscillation that have a pattern activity trought anatomical space, suggesting a topografic organization of the hippocampal outputs (Lubenov & Siapas, 2009). Later, another study has shown the same theta oscillatory behavior, but with a different phase (Patel, Fujisawa, Berényi, Royer, & Buzsáki, 2012). With this in mind, we investigated the coherence of theta between the ventral and the dorsal hippocampus and found a high coherence both in saline group and salicylate, but with difference in frequency. We found a high coherence in the range of type 1 theta for the saline group and in the range of the type 2 theta for the salicylate group. Since we did not recorded a laminar profile of the hippocampus, it was not possible to analyze the spread of theta across the hippocampal axis. However, we conducted a Granger Causaity (GC) analysis iin order to evince the traveling caracteristic of theta. We found a higher GC index

for the directionality of theta from the dHP to the vHP in the saline group, which is in accordance with the previous literature, but a higher GC index for the directionality of theta from the vHP to the dHP in the salicylate group. This finding could suggest that the type 2 theta can be generated in the ventral hippocampus and thavels to the dorsal hippocampus as a travelling wave, in contrast to the type 1 theta. However, the lack of causality of stronger dHP type 1 theta oscillation may suggest that the analysis may not be robust enough to determine causal relationships of different brain in the generation of LFP.

Our initial hypothesis was that tinnitus, induced by salicylate, would generate anxiety-like behavior. However, we cannot conclude from our data whether the increase in type 2 theta was caused by tinnitus perception or it was a direct effect of salicylate. Further studies using gap detection for tinnitus assessment may help to elucidate this question. Preliminary data show that animals with substantial increase in theta 2 also show a 'positive' tinnitus sign in the gap detection test. However, we have yet to record from more subjects.

In summary, we found that salicylate application generates anxiety-like behaviors and type 2 theta oscillation. To our knowledge, this is the first model of type 2 theta in rodents. Different from type 1 theta, type 2 theta is poorly study and the development of an animal model will throw light on the cellular mechanisms of type 2 theta generation. The association of type 2 theta oscillation with anxiety was demonstrated more than 30 years ago but type 2 theta has been neglected by recent studies in the hippocampus. With the salicylate model, it will be possible to re-examine the relationship between anxiety/depression with hippocampus oscillations. Moreover, it has been shown that slower theta oscillation in the ventral hippocampus is synchronous with oscillations in the medial prefrontal cortex during anxiety (Adhikari, Topiwala, & Gordon, 2010); hence, future studies may also show that salicylate synchronizes the vHP and the medial prefrontal cortex.

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