

## **Windup of Nociceptive Flexion Reflex Depends on Synaptic and Intrinsic Properties of Dorsal Horn Neurons in Adult Rat**

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**Abstract :**

Windup, a progressive increase in spinal response to repetitive stimulations of nociceptive peripheral fibres, is a useful model to study central sensitization to pain. Windup is expressed by neurons in of both dorsal and ventral horn of the spinal cord. In juvenile rats, it has been demonstrated both *in vivo* and *in vitro* that windup depends on calcium-dependent intrinsic properties and their modulation by synaptic components. However, the involvement of these two components in the adult remain controversial. In the present study, by means of electromyographic and extracellular recordings, we show that windup in adult, *in vivo*, depends on a synaptic balance between excitatory NMDA receptors and inhibitory glycinergic receptors. We also demonstrate the involvement of L-type calcium channels in both the dorsal and ventral horn of the spinal cord. These results indicate that windup in adults is similar to juveniles rats and that windup properties are the same regardless spinal network, i.e. sensory or motor.

**Keywords:** l-type calcium channels; nociception; spinal cord; central sensitization; windup

## Introduction

The dorsal horn of the spinal cord is the first relay of nociceptive inputs raising from the periphery. Dorsal horn neuronal networks integrate nociceptive information and output neurons project both to supraspinal areas and motoneurons. Within this network, deep dorsal horn neurons (DHNs) receive convergent inputs from tactile and nociceptive fibers and express a form of short term activity-dependent plasticity, so-called windup. Windup is a form of early onset spinal sensitization [1, 2] and is considered as a simple tool to study pain processing plasticity in the spinal cord (for reviews see [3, 4]. In clinics, windup protocol serves to evaluate temporal summation of pain, and is considered as a good predictor for clinical pain intensity in idiopathic pain syndromes such as fibromyalgia [5, 6]. Windup is also expressed in motor network and repetitive nociceptive stimulations of the hindpaw elicit windup of segmental reflexes [7]. Moreover, repetitive stimulations of the sciatic nerve elicit windup of ventral roots [4]. Finally, motor neurons express windup in different animal models such as rats or turtle [8, 9].

At the level of the dorsal horn, windup is expressed by wide dynamic range neurons and has been extensively studied. In adult rats, it has been shown that windup depends on excitatory synaptic properties as NMDA glutamate receptors and neurokinin receptors. [10]. Windup also depends on DHNs intrinsic properties since windup is blocked by L-type calcium channels (LTCs) antagonists and increase by agonist of these channels [11]. Other studies performed in spinal slices from juvenile rats confirmed the role of LTCs in this phenomenon and also show the involvement of another cationic non specific conductance (CAN) [12]. DHNs project to the ventral horn network, activating motor neurons eliciting muscular contraction necessary for

nociceptive flexion reflex [7]. Repetitive nociceptive stimulations of the ipsilateral paw elicit a windup of nociceptive flexion. It has been shown that windup of the flexion reflex in juvenile rats depends on a synaptic balance between excitation and inhibition that allow expression of intrinsic properties [11, 13, 14]. However, the flexion reflex is the output of a neural circuit that comprise both DHNs and motor neurons and the latter ones also exhibit a windup of their discharge that present the same molecular sensitivity as windup of DHNs [8, 9, 15]. Because of the different model used (juvenile versus adult, turtles), the site of recordings (DHNs, motor neurons, flexion reflex), our understanding of windup remains partial. For instance, it is still unknown if the balance between excitation and inhibition also control windup at the level of the dorsal horn of the spinal cord. The involvement of intrinsic properties in the windup of the flexion reflex in adult rats is still controversial [4]. Finally, previous studies on reflex have been performed in juvenile rats and we cannot exclude developmental modifications altering windup molecular sensitivity.

We propose in this study to analyze in anesthetized rats, the windup for DHNs by means of extracellular recordings and the windup of flexion reflex with electromyographic recordings in the biceps femoris muscle of the ipsilateral hindpaw and compared its molecular sensitivity between adult and juvenile rats.

## Methods

### *Experimental procedures*

We used both juvenile (14 to 21 days old (55g)) and adult wistar rats in this study (250-350g). All experimental procedures were approved by the local ethic (agreement number : APA3650) committee according to the International Association for the Study of Pain (IASP) ethical guidelines for experimental research were followed. At the end of each experiment, rats were killed with an overdose of anaesthetics.

### *Drug administration*

Rats were anaesthetized with intraperitoneal urethane (1.1 mg/kg). A catheter (PE-10; Phymep, France) was inserted into the sub-arachnoid space, and the tip was pushed forward to the L4-L5 region. During recordings, drugs were injected using the catheter connected to a Hamilton syringe and flushed with 10  $\mu$ l saline (dead volume of the catheter). Nicardipine and verapamil (Sigma Aldrich, France) were freshly dissolved in saline. AP-5 and strychnine (Sigma Aldrich, France) came from 10 mM stock solutions stored at -20°C. Flufenamic acid (FFA) were freshly dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich), then diluted with saline. For dose response curve, the lower dose was first intrathecally injected and responses measured. Each doses was separated by 30min.

### *In vivo electromyographic recordings*

Recordings were made in the ipsilateral biceps femoris muscle with two teflon-coated stainless steel electrodes (Phymep, France; diameter 200  $\mu$ m). Electrical stimuli (500  $\mu$ s, single chocks, master 8 stimulator, AMPI, Israel) were delivered with electrodes placed under the paw skin in the region of the sural nerve. A maximum response was

obtained for a stimulus intensity of 60 to 80 V, as determined with single shocks delivered at 30 s intervals to avoid central sensitization. At this stimulus intensity, a slight muscle contraction was elicited without movement of the limb. Data were acquired by a CED 1401 interface, and analyzed with Spike 2 software (CED, UK).

#### *In vivo extracellular recordings*

After urethane anesthesia, rats were placed in a stereotaxic frame to ensure stability during electrophysiological recordings. A laminectomy was performed on lumbar vertebrae L1-L3, for exposition of the L4-L5 segments of the spinal cord. Extracellular recordings of wide dynamic range dorsal horn neurons (DHNs) were made with borosilicate glass capillaries (2 M $\Omega$ , filled with 4% NaCl) (Harvard apparatus, USA). Electrodes were positioned with a microdrive (unimecanique, France). The depth of the neurons from the dorsal surface of the spinal cord was monitored (recordings were performed between 500 to 1000 $\mu$ m from the surface). The response to various natural stimuli (brush, pressure, pinch) in the most responsive part of the receptive field of the neurons was characterized to ensure the wide dynamic range properties of the recorded neurons. We recorded the neurons' responses following transcutaneous electrical stimulation (see above) of the centre of the receptive field. The criterion for the selection of a neuron was the presence of an A $\beta$ -fiber evoked response followed by a C fiber-evoked response. For analysis, we performed post stimulus histogram (PSTH) and separate in 4 zones: an A $\beta$  zone between 0 and 20ms an A $\delta$  zone between 20 and 90 ms, a C zone between 90 and 300 ms and a post discharge zone (PD) between 300 ms and 1s.

#### *Windup protocol*

Sequences of 15 stimuli were delivered at 1 Hz, except in experiment of Fig.1, in which the stimulation frequency was varied from 0.1 to 1 Hz as indicated on the

graph. For Flexion reflex, each stimulus was a single shock (500  $\mu$ s) at ~80% of the intensity eliciting a maximal response (see above). For extracellular recordings, single shock 3 fold the threshold for C-fibres.

#### *Data Analysis and statistics :*

The electromyographic signals were integrated by measuring the area under the curve after rectification of the signal. Windup plots (e.g. Fig 1A) were normalized to the first response of the series. For extracellular recordings, spikes in the C+PD part of the response were counted and Data were plotted with the Prism software (v 5.0, GraphPad Inc.). All values are mean  $\pm$  SEM, and N indicates the number of tested animals. A windup coefficient was calculated by dividing the sum of the 15 responses by 15 times the first recorded response. Comparison of windup plots were performed using a two-way ANOVA. Windup coefficient comparison between two populations was performed using non parametric Mann Whitney test. Windup coefficient comparison between before and after drug applications were compared using paired t test or a one way ANOVA when 2 drugs were applied. A value of  $p < 0.05$  was considered significant.

## **Results**

### **Windup of WDR neurons and of the flexion reflex share similar characteristics.**

In juvenile rats, electrical stimulation of high threshold fibers in the paw triggers a muscular contraction of the flexor ipsilateral muscle. This response called RIII flexion reflex or nociceptive flexion reflex, appears in the electromyogram with a latency of 80-300 ms with high stimulations (Figure 1A). The same type of reflex is also observed in adult rats (figure 1B). Repetitive stimulation at low frequency (here 1Hz) generates a gradual increase in the reflex magnitude during the sequence of

stimulation, with a progressive development of an after-discharge (Fig. 1A and B). This increased response is so-called windup. The amplitude of the windup is similar in juvenile and adult rats since the last response of the series represents  $467 \pm 97\%$  in the adult and  $453 \pm 56\%$  ( $N=22$  and  $26$  respectively,  $p>0.05$ , Mann-Whitney) in juvenile rats. Windup coefficient defined as the sum of the 15 responses subtracted by 15 times the first response is also very similar (Figure 1C, windup coefficient is  $30.38 \pm 5.77$  in adult et de  $32.04 \pm 6.99$  in juvenile;  $p>0.05$ , Mann-Whitney). Windup of the flexion reflex is frequency dependent in adult rats (Figure 1D). Together these results show that windup phenomenon is not modified during development and exhibits the same characteristics at the level of the muscle or the dorsal horn of the spinal cord.

### **Windup of the nociceptive flexion reflex in adult rats is controlled by synaptic and intrinsic components.**

We next study the molecular mechanisms controlling the windup of the flexion reflex in adult rats. First we focused on synaptic components and we observed that application of  $100\mu\text{g}$  AP5 strongly decreased the flexion reflex windup (Figure 2A, B and C). This effect is dose dependent with an  $\text{EC}_{50}$  of  $10,26\ \mu\text{g}$  (not shown). The baseline response was also modified with a significant decrease at  $57\%$  of the control. This result is comparable to previous study in juvenile rats but the sensitivity to NMDA blockers is strongly decreased in adult rats since the dose necessary to obtain a complete suppression of the windup is 10 fold higher than in juvenile rats.

In juvenile rats, windup of the nociceptive flexion reflex is always present if we suppress both excitatory and inhibitory synaptic component (Fossat et al, 2007). We tested for this possibility in adult rats. We first applied an inhibitory dose of  $100\mu\text{g}$  AP5 to suppress the windup (Figure 2D, E and G), we further applied both AP5 and



strychnine and the windup was recovered (Figure 2D, F and G). Thus, we demonstrated in adult rats that windup of the nociceptive flexion reflex depends on a synaptic balance between excitatory and inhibitory component.

### **Windup of the flexion reflex is sensitive to IL blockers in adult rats:**

We assessed the role of ILs in windup of the flexion reflex using two different families of blockers (Lapirot et al, 2018). First, we applied 100 $\mu$ g verapamil a blocker of the phenylalkylamine family. Verapamil blocked the windup of the flexion reflex (Figure 3A-C). The effect was dose dependant with an EC50 of 26 $\mu$ g. Because of lack of specificity of phenylalkylamine, we used dihydropyridine to confirm the involvement of LTCs. Intrathecal application of 50 $\mu$ g nicardipine also suppressed the windup of the flexion reflex (Figure 3D-F). Again, the effect was dose dependant with an EC50 of 16.4 $\mu$ g. Finally, we assessed for the involvement of CAN in windup of the flexion reflex. Intrathecal Application of 200 $\mu$ g flufenamic acid (FFA) completely suppress windup of the flexion reflex (Figure 4 A and B). Effect of FFA was dose dependant with an EC50 of 13.6  $\mu$ g. These results demonstrate in adult rats that LTCs and CAN are two important elements in the expression of windup of the nociceptive flexion reflex.

### **Synaptic component of windup in DHNs of adult rats.**

The nociceptive flexion reflex is the output of a reflex network that comprises two neuronal levels i.e. dorsal and ventral horn. We then studied the properties of the windup of the discharge of DHNs and its sensitivity to a synaptic balance between excitation and inhibition. We first evaluated the characteristics of the windup in DHNs neurons in adult rats. We observed a progressive increase in neuronal discharge in response to repetitive stimulation of the hindpaw at 3-fold the threshold for C fibers in

53% (68/128) of the recorded neurons (Figure 5A). When present, windup was also frequency dependent with no windup at 0.1 Hz and an increased amplitude until 1Hz stimulations (Figure 5B).

Next, we wanted to determine if a balance between excitation and inhibition modulate windup of DHNs. We first confirmed that windup is influenced by NMDA receptor blockers (Figure 6). We showed that intrathecal application of 100 $\mu$ g AP-5 significantly decreases DHNs excitability (Figure 6C) and Windup amplitude (Figure 6 A,B and D). As already shown previously for the flexion reflex, inhibition control the amplitude of windup. To study this phenomenon in DHNs, we used strychnine to block glycinergic receptors (Figure 7). Intrathecal application of 170 $\mu$ g strychnine increased the excitability of DHN (Figure 7C). Windup was also increased (Figure 7A, B and D). Therefore, windup of DHNs of adult rats is potentiated by synaptic excitations and decreased by synaptic inhibitions. In the next step, we wondered if we could restore a windup after blockade of NMDAr by suppressing inhibitory influence (Figure 8). To that issue, we suppressed windup of DHNs by applying 100  $\mu$ g AP-5 and then we applied both 100 $\mu$ g AP-5 and 170 $\mu$ g Strychnine. 100 $\mu$ g AP-5 almost completely suppressed the windup (Figure 8B D and E) and subsequent application of AP-5 and Strychnine restore a windup of DHNs (Figure 8C, D and E). Together, these results demonstrate that expression of windup by DHNs in adult rats depends on a synaptic balance between excitations and inhibitions.

## **Discussion :**

This study shows that windup of a nociceptive flexion reflex depends on both synaptic and intrinsic components in adult rats *in vivo*. We confirmed the role of LTCs

and CAN in this form of short term sensitization to pain. We also show similar characteristics between the windup of nociceptive flexion reflex and the windup of DHNs and we observed a global decrease in sensitivity to blockers of the molecular components of windup in adult rats.

### **Adult and juvenile windup**

These results demonstrates that the windup of the flexion reflex is not different between juvenile and adult rats. Indeed, as in juvenile rats, windup in adult also depends on stimulation frequency and is sensitive to synaptic modulators and plateau potential blockers. Windup in adult rats is also sensitive to synaptic NMDA blockers and its onset depends on a balance between synaptic inhibitory and excitatory inputs. However, we show here a general decrease in drug sensitivity in adult rats as compared to juvenile. These modifications in drug sensitivity have already been found for NMDA receptors blockers [16]. One may consider that differences between juveniles and adults could reflect a modification of neuronal phenotypes with altered expression in channels or receptors subunits. However, such a general effect for all tested substances is more probably due to an increased difficulty for any drugs to reach their targets as the areas concerned are deep and adult tissues have a complex extracellular matrix and are enriched in myelin.

### **Importance of LTCs in windup.**

Windup is an activity dependent short term plasticity involved in pain processing. It represents a form of input/output amplification mechanism, which strengthened contrast between background activity and relevant stimuli. Windup is known to depend on synaptic plasticity exerted through NMDAr and neurokinin[10, 17, 18]. *In vitro* recordings in juvenile rats also revealed the role of intrinsic properties of deep dorsal horn neurons in windup [12]. In brief, two ionic channels involved in the

expression of plateau potentials are also necessary to trigger windup of the discharge. These two channels are LTCs and CAN. LTCs is necessary in the early phase of plateau whereas CAN promote afterdischarge [19]. However, the role of LTCs in short term central sensitization to pain in adult rats is not fully demonstrated. For instance, in the formalin model of short term sensitization, LTCs blockers have no effect [20] while LTCs blockers suppress DHNs windup [11]. Here, we show that, in adult rats, windup of a flexion reflex depends on LTCs since windup is blocked by blockers of LTCs belonging to two different families in a dose dependent manner. Moreover, a recent study suggest that one specific type of LTCs is involved in windup in juvenile rats. Indeed, Two LTCs forming channels are expressed in dorsal horn of the spinal cord, Cav1.2 and Cav1.3. Blocking Cav1.3 expression with an antisense strategy suppress the windup of the flexion reflex [13, 14]. The participation of LTCs in pain depends on the type of pain. For instance, they are not involved in acute nociception but they are clearly important for windup, central sensitization resulting from joint inflammation [21] and in long term changes that accompany nerve injury [22] but not in sensitization induced by formalin injection [20].

We also show here that CAN are also involved in the windup of the flexion reflex in adult rats. CAN promote another important conductance of plateau potentials. Indeed, CAN is triggered following LTCs activation and elicit a larger depolarisation responsible for prolonged after discharge [12, 19]. The role of CAN in windup has been demonstrated in DHNs neurons in slices from juvenile rats [19] or in anesthetized juvenile rats [11] and we show here their involvement in the windup of flexion reflex in adult rats.

## Synaptic component of windup

The other major component of windup *in vivo* is synaptic. NMDAR and neurokinin receptors are activated during trains of stimuli and this lead to the onset of windup. Here, we show that both windup of flexion reflex and windup of DHNs are completely suppressed by NMDAR blockers *in vivo* in adult rats, thus confirming classical results of the literature [10]. This blockade is also accompanied by a decrease in the baseline response and DHNs excitability indicated an effect of NMDAR not only in sensitization but also in acute nociception [11]. We also show here that windup depends on inhibitory synaptic influences. Blocking glycine receptors increase the amplitude of windup of DHNs. This effect was accompanied by an increase in DHNs excitability. This effect is comparable to that previously observed in nociceptive flexion reflex [7, 11]. This result confirm that inhibitory interneurons control the onset of central sensitization by exerting a tonic inhibitory tone. Finally, we show that windup depends in adult rat on a dynamic balance between excitation and inhibition. Indeed, when blocking NMDAR, we suppressed windup that can be restored by the subsequent blockade of glycine receptor. This suggest that intrinsic properties of spinal neurons are a the key element that elicits windup as suggested in juvenile rats [11].

## Neural substrate of windup

Two neuronal levels can elicit windup of their discharge within the flexion reflex circuit: motoneurone and DHNs neurons [8, 9, 12, 15, 23]. We previously showed that in control conditions, the windup of the flexion reflex was strictly correlated with DHNs windup [11]. In this condition, motor neurons seems to follow amplification properties expressed by DHNs neurons. Here, we show that the windup of the nociceptive flexion reflex and the windup of DHNs share exactly the same properties

suggesting that the dorsal level is crucial to elicit reflex windup. Indeed, we show that both windup of flexion reflex and windup of DHNs are modulated by NMDAr, Glycine and controlled by LTCs and CAN. We cannot exclude a role of windup of motor neurons in other motor tasks but our results strongly suggest that the flexion reflex is first controlled at the dorsal horn level.

### **Windup and central sensitization to pain.**

Windup has long been studied in various models of pain. It has been defined as a model of short term sensitization to pain and it is considered as a good model for the study of mechanisms leading to central sensitization to pain. Moreover, windup is still used to assess level of pain in many models. For instance, several evidences show the usefulness of windup as a cue of central sensitization in patients suffering of idiopathic pain [5, 6]. However, windup is a short term sensitization with a time scale of few seconds and cannot be responsible for long term central modifications leading to neuropathic pain [24]. On the one hand, time scale of windup is too short to trigger neuronal modifications appearing in long term changes leading to an increase in spontaneous activity and an increased response to both innocuous and noxious stimuli. On the other hand, it seems that windup is not affected by nerve lesions-induced neuropathy or slightly decreased [13, 25].

Nevertheless, recent studies have shown that in SNL rats, the number of neurons expressing plateau potentials increases dramatically as compared to naïve animals [26]. In conclusion, even if windup is not responsible for the deep changes appearing in central sensitization to pain, an increased capacity to generate windup in such pathology cannot be excluded on chronic pain syndromes and controlling windup onset in the dorsal horn of the spinal cord is a potential way to limit exaggerate pain in chronic pain syndrome.

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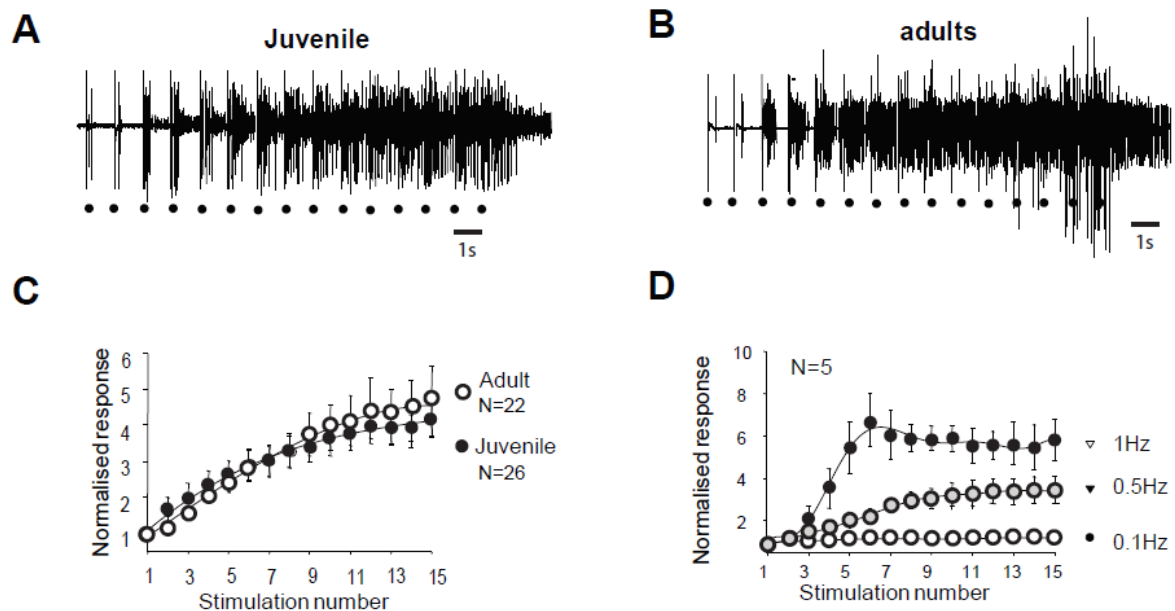
## Figure legends :

### Figure 1 :

Flexion reflex in adult and juvenile rats (A et B) Electromyographic recordings (EMG) of windup triggered by a series of electrical shocks in the sural nerve peripheral receptive field in juvenile (A) and in adult rats (B). Each dot indicates an electric stimulation (500  $\mu$ s, 80% of the maximal response). (C) Normalised windup curves in juvenile (open dots) and adult rats (filled dots). Note that the two curves are strictly

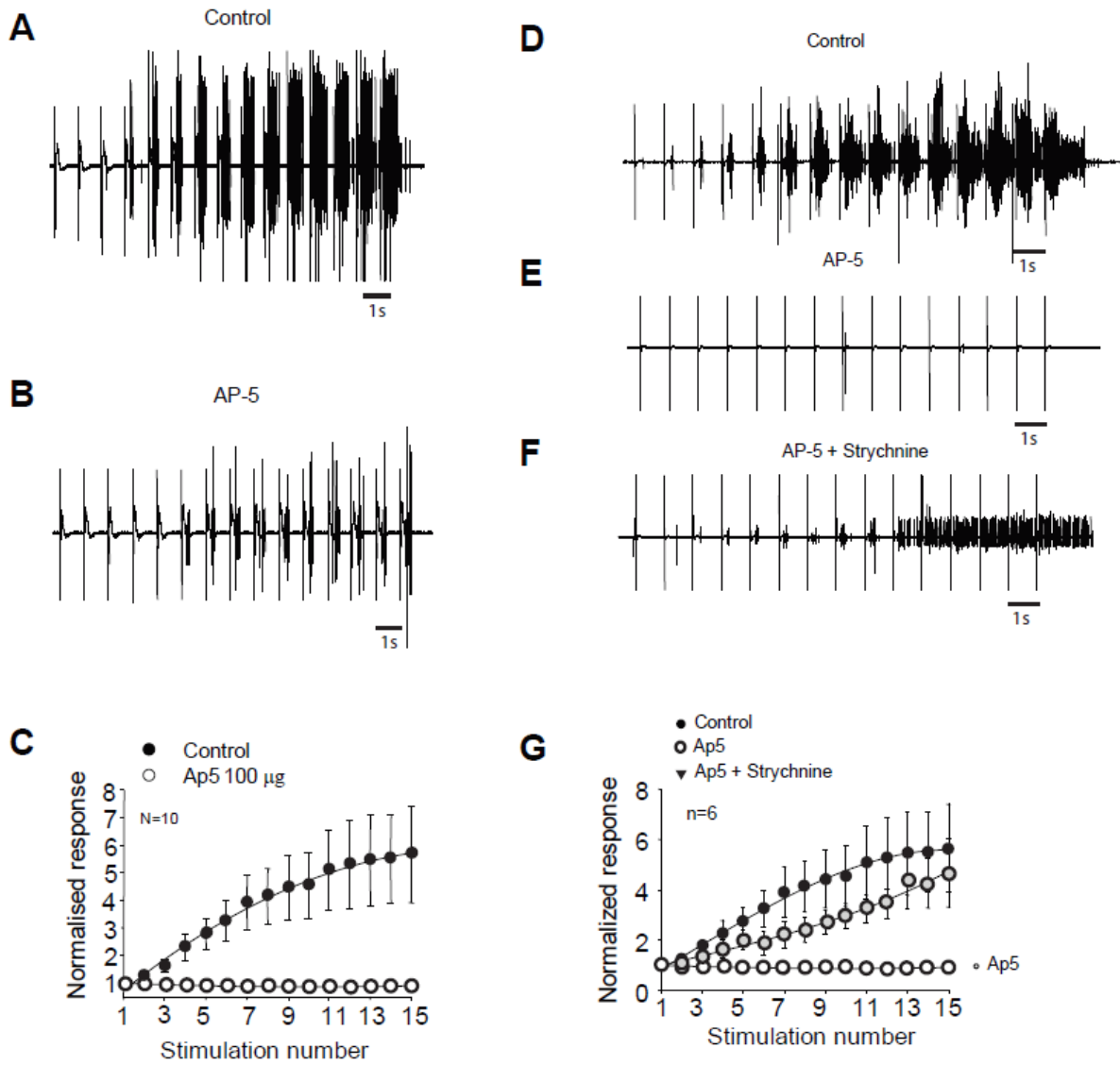


superimposed showing a comparable sensitization. (D) In adult rats, windup is frequency dependant with no windup for frequency <0.1Hz and a progressive increase until at least 1Hz.



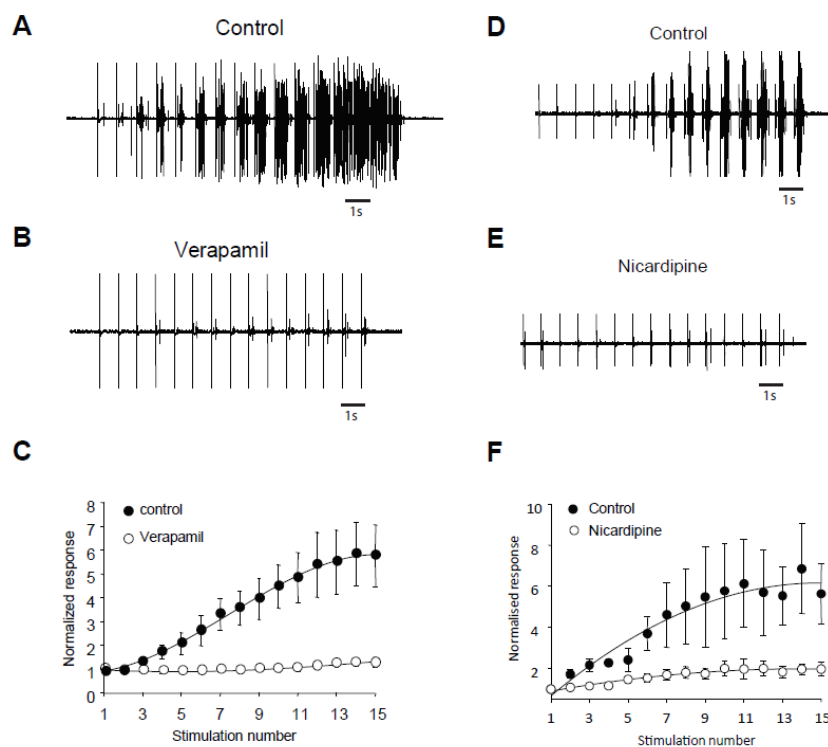
**Figure 2 :**

Windup of the nociceptive flexion reflex in adult rats is controlled by NMDAr and glycine receptors. A) EMG in control or B) after application of 100µg AP5. C) Windup is significantly decreased by AP5 (windup coefficient :  $20.4 \pm 4$  in control vs  $1.2 \pm 0.5$  in AP5,  $n=10$ ,  $p < 0.01$ , Paired t test). D) EMG in control, E) after 100µg AP5 and F) after 100µg AP5 + 170 µg strychnine. G) Application of AP5 suppressed the windup that was restored after subsequent application of AP5 and Strychnine (windup coefficient;  $41 \pm 13.8$  in control vs  $-1.39 \pm 1$  in AP5 and  $24.2 \pm 7.2$  in AP5+Strychnine,  $n=6$ ,  $p_{\text{control vs AP5}} < 0.01$ ;  $p_{\text{control vs AP5+strychnine}} > 0.05$ , Dunn's post hoc test).

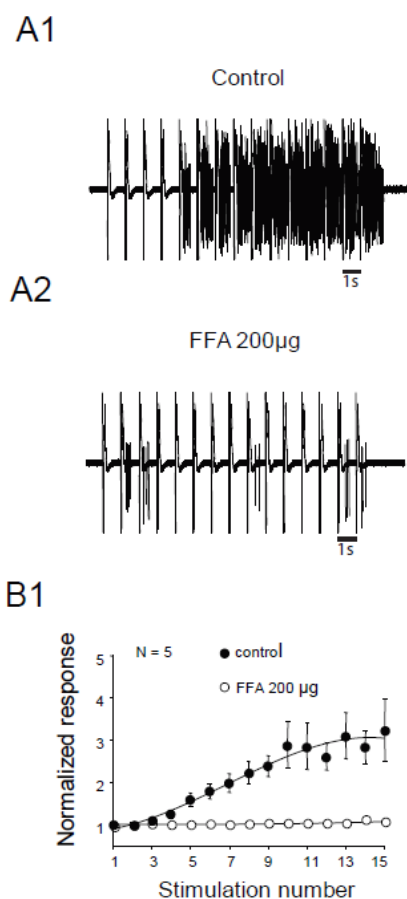


**Figure 3 :**

Flexion reflex windup depends on LTCs in adult rats. A and B) EMG in control and after application of 100 $\mu$ g Verapamil. C) Normalised response showing a significant decrease of flexion reflex windup (windup coefficient;  $31\pm 9.8$  in control vs  $5.4\pm 4$  in verapamil,  $n=5$ ,  $p<0.05$ , paired t test). D and E) EMG in control and after application of 100 $\mu$ g Nicardipine. F) Normalised response showing a significant decrease of flexion reflex windup (windup coefficient;  $62.5\pm 12$  in control vs  $11\pm 3.3$  in nicardipine,  $n=5$ ,  $p<0.05$ , paired t test).

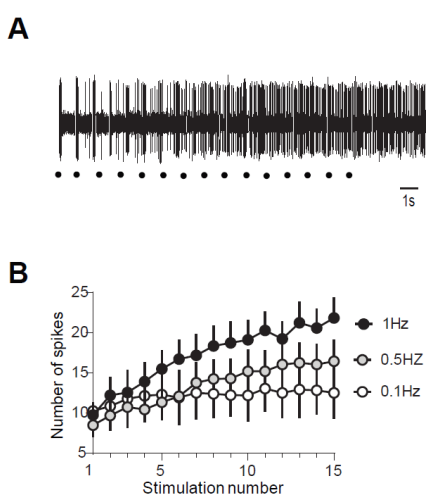
**Figure 4**

Flexion reflex in adult rats is sensitive to CAN currents. A and B) EMG in control or after application of 200 $\mu$ g of FFA C). B1 Normalised response showing a significant decrease of flexion reflex windup with 200 $\mu$ g FFA (Windup Coefficient ;  $16.9\pm 4.7$  in control vs  $0.16\pm 0.1$  in FFA,  $N=5$ ,  $p<0.05$ , Paired t test).



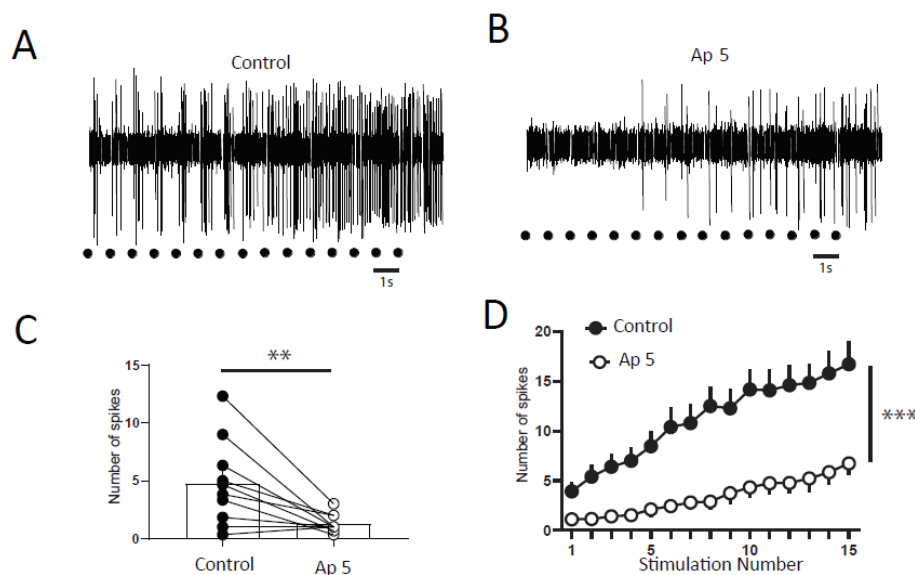
**Figure 5**

(A) Repetitive stimulations of the paw at three times the threshold for C-fibre induce a progressive increase in DHNs discharge showing up a windup. (B) windup of DHNs is frequency dependant. Windup Coefficient was  $130 \pm 15.7$  at 1Hz,  $86.7 \pm 24$  at 0.5 Hz and  $29 \pm 7$  at 0.1Hz,  $n=7$ ,  $p_{1\text{Hz vs } 0.1\text{Hz}} < 0.05$ , Dunns multiple comparison test).

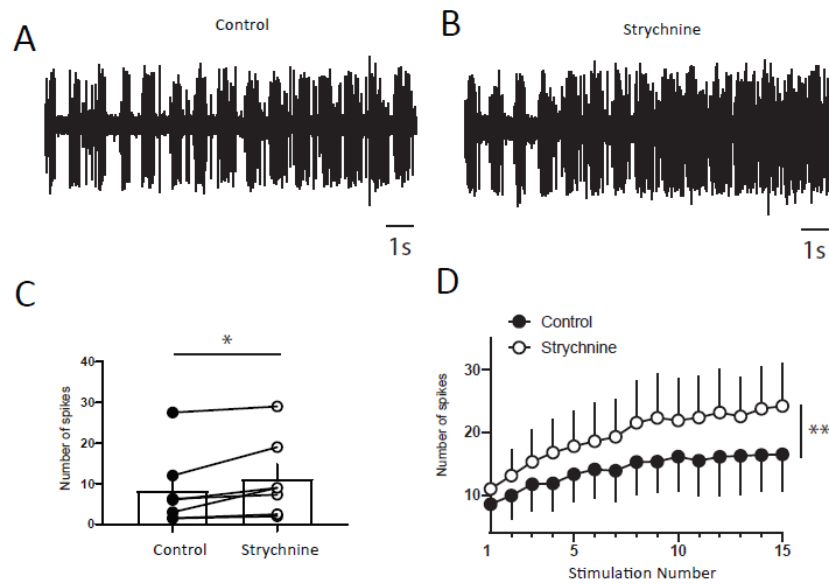


**Figure 6 :**

DHNs windup depends on NMDAR. A and B) Extracellular recordings of DHN response to a series of electric shocks before and after 100 $\mu$ g AP5. C) AP5 decreases DHNs excitability, since the response to the first nociceptive stimulation is significantly decrease (Response to the first stimulation  $4.7\pm 1.2$  spikes in control vs  $1.3\pm 0.25$  spikes in AP5, N=10,  $p<0.01$ , Paired t test). D) Amplitude of windup is significantly decrease (windup coefficient;  $103\pm 15.7$  in control vs  $31\pm 9.2$  in AP5, N=10,  $p<0.001$ , paired t test).

**Figure 7 :**

DHNs windup depends on glycinergic receptors. A and B) Extracellular recordings of DHN response to a series of electric shocks before and after 170 $\mu$ g strychnine. C) strychnine increases DHNs excitability, since the response to the first nociceptive stimulation is significantly increase ( $8.5\pm 3.5$  spikes in control vs  $11\pm 3.6$  spikes in Strychnine, N=7,  $p<0.01$ , Paired t test). D) Amplitude of windup is significantly increased (windup coefficient;  $83\pm 28.6$  in control vs  $130\pm 43.3$  in Strychnine, N=7,  $p<0.05$ , Paired t test).



**Figure 8 :**

Dynamic synaptic balance control DHNs windup. A) Extracellular recordings of DHN in control showing a windup. B) After 100µg application of AP5, windup is decreased. C) Subsequent co-application of AP5 and strychnine restored a windup. D) Windup curve showing difference between control and AP5 and recovery with AP5 and Strychnine. E) Windup coefficient is significantly decreased after AP5 and partially restored after AP5 and strychnine (windup coefficient  $139,0 \pm 47,07$  in control;  $0,2500 \pm 5,155$  in AP5;  $84.33 \pm 42.77$  in AP5+Strychnine,  $N=8$ ,  $p_{\text{control vs AP5}} < 0.001$ ,  $p_{\text{control vs AP5+Strychnine}} > 0.05$ , Dunn's post hoc test.)

