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HABITUATION AND SENSITIZATION OF THE MONOSYNAPTIC REFLEX IN IN VITRO SPINAL CORD OF RAT

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INDEX

ABBREVIATIONS

aCSF = artificial cerebro-spinal fluid

aCSFm = modified artificial cerebro-spinal fluid

CNS = central nervous system

CS = conditioning stimulus

EPSP =excitatory post-synaptic potential

FRA = flexor reflex afferents

GWR = gill-withdrawal reflex

ISI = interstimulus interval

MVR = monosynaptic ventral response

TS = test stimulus

ABSTRACT

The capacity to ignore only those stimuli that are irrelevant and to channel behaviour into organized and directed actions in response to meaningful stimuli is necessary to conserve energy and focus behaviour. This behavioural definition of habituation and sensitization allows to understand the important role played by the two simplest forms of non-associative learning in ordinary life. Likely habituation and sensitization are terms denoting different neural processes which work and compete each other, determining the final behaviour. The complexity of the mammalian models used to study these two opposite processes did not clarify the underlain mechanisms. Moreover they have been studied also in invertebrate models at a cellular level but the obtained results did not exhaustive explain these processes in mammalians. This study was aimed to test whether the monosynaptic ventral response (MVR) in *in vitro* spinal cord preparation of rat presents the behavioural features of habituation and sensitization. Furthermore we investigated the variables which affect habituation and sensitization and their interactions to shape the final behavioural outcome. Lastly the study aims to validate the *in vitro* spinal cord preparation as a new model to study learning processes in mammalians.

The spinal cord preparation has been isolated from rats between 5 and 15 days old. The dorsal root has been stimulated and the homologue MVR has been recorded. The MVR amplitude has been used as a parameter to evaluate the learning process. Repetitive application of a test stimulus (low intensity/low frequency, TS) resulted in an exponential decrement of the response, followed by its spontaneous recovery when the stimulation was withheld. The depression degree was directly associated to the stimulus frequency and opposite related to the stimulus intensity. Furthermore habituation of the response occurred faster an at higher degree when more than one training trail has been used (potentiation of habituation). Its spontaneous recovery was longer when the final plateau-like level was reached and further TS were delivered (below zero effect). On the contrary a facilitation of the MVR has been seen when a strong stimulus (high intensity/high frequency, CS) was

applied to a flexor root and the MVR was elicited from a different flexor root (dishabituation). The facilitatory effect of the CS decreased as it was repetitively applied (habituation of dishabituation). Interestingly an additional depression appeared on the habituated response, when the CS was delivered from a flexor root and the MVR was evoke from an extensor root. The stimulus features played a rule in elicit depression or facilitation. In specific conditions, it was possible to elicit a pure depression and a pure facilitation processes, described by a simple mathematical model. A repeated stimulation could result in a depression of the response. However if the stimulus was sufficiently strong depression could be preceded by a transient facilitation or even replaced by enduring sensitization.

A decrement to repeated stimulation is usually termed habituation if it exhibits properties consistent with the behavioural definition of habituation. The central processes underlain habituation and sensitization must to be inferred (Kandel, 1973). The MVR showed all the features of behavioural habituation, indicates by Rankin and colleagues (Rankin et all., 2009) and presented in all the species studied. The parameters of the TS and the CS could be modified to study the complex interactions between these two processes. Lastly this study represents an additional proof for the dual process theory of learning in which the two opposite processes interact to shape the final behaviour. In conclusion, the possibility to stimulate the dorsal roots with a well-known and defined input (the stimulation), to record the activity of the final output of the motoneurons (the behaviour) and, eventually, to gain access to the cells underlain this circuit in a very well controlled conditions (known solutions, temperature, possibility to use drugs, etc.), allow to use the *in vitro* spinal cord preparation of rat as a good model to study learning and memory in mammalian nervous system.

INTRODUCTION

Human learning and cultural evolution are supported by a paradoxical biological adaptation: we are born immature. Young infants cannot speak, walk, use tools, or take the perspective of others. Immaturity comes at a tremendous cost, both to the new-born, whose brain consumes 60% of its entire energy budget[1]. During the first year of life, the brain of an infant is teeming with structural activity as neurons grow in size and complexity and trillions of new connections are formed between them. The brain continues to grow during childhood and reaches the adult size around puberty. Yet immaturity has value. Delaying the maturation and growth of brain circuits allows initial learning to influence the developing neural architecture in ways that support later, more complex learning[2].

LEARNING AND MEMORY

Learning and memory are psychological concepts and refer to the mental processes of acquiring knowledge. Both of them are inferred from enduring changes in behaviour as a result of experience [3]. Thus learning and memory are disposition terms which refer to processes of the central nervous system (CNS), which cannot be observed directly. Most psychologists would agree on the existence of several forms or categories of learning, but at this point, it is useful to keep the basic definition of learning broad. Thus, bacteria have a kind of memory and their behaviour can change as a result of experience (for example, after exposure to certain molecules), and this change can persist after the experience. This example does not fit neatly into any of the common categories of learning but it may serve as a model [4].

Currently, the most productive research strategy for investigating the neural basis of learning is the model systems approach [5,6]: selection of an organism that exhibits a given form of learning and memory and that has a nervous system amenable to analysis. Certain invertebrate preparations are valuable as model systems because some of their behavioural functions are controlled by ganglia that contain relatively small numbers of large, identifiable cells-cells that can be

consistently identified in different individuals of the species [7-9]. With vertebrate model systems, these goals are considerably more difficult to attain. Specialized forms of learning in birds-imprinting and song learning-have proved to be most useful models [10-12]. But we are mammals and the ultimate goal is to understand how the human brain learns. The problem of localizing the neuronal substrates of learning and memory, first explored in depth by Lashley [13] and later by Hebb [14], has been the greatest barrier to progress and remains fundamental to all work on the biological basis of learning and memory. To analyse the biophysical mechanisms that form memory traces it is necessary to localize the memory traces, and this in turn requires identification of the essential memory trace circuits.

To relate cellular plasticity to behavioural change, one must consider what is happening at the neural network level. Because the output of a nervous system is a function of the input into the circuit and the anatomical and functional connectivity, changes in the output can be caused by alterations to any one of these three properties. First, altering the input of a circuit can radically change the functional connectivity. Second, the functional connectivity can be changed by altering the individual strengths of the existing synapses. Third, synapses can be added or eliminated to change the anatomical connectivity. Different network elements may mediate different components of the behavioural response, meaning that plasticity in different network elements will confer learning in different components of the behaviour. Therefore relating learning kinetics at the cellular level to learning kinetics at the behavioural level may be complicated by interactions between learning processes at the network level.

Traditionally, theorists have divided learning into two categories: non-associative and associative. Non-associative learning occurs when an individual is exposed to a single type of stimulus and behaviour is changed as a result of that exposure. Examples of non-associative learning include habituation and sensitization (see below). Associative learning occurs when animals learn to link a stimulus or behaviour with a second temporally associated stimulus. Associative learning

includes classical conditioning and operant conditioning. The most prominent example of classical conditioning is Pavlov's experiments with dogs in which the animal learns to associate the ringing of a bell with food (1927). In operant conditioning, an animal learns to associate one of its own behaviours with a stimulus. For example, in B.F. Skinner's classic operant conditioning experiments, a rat learns to press a lever for a reward of food. Skinner used the term operant to refer to any "active behaviour that operates upon the environment to generate consequences" (1953).

HABITUATION

Habituation is defined as “a behavioural response decrement that results from repeated stimulation. Traditionally Humphrey (1933) is commonly credited with the discovery of habituation, providing the first comprehensive review of the topic and establishing its fundamental importance. He noted that habituation is a ubiquitous process that can be observed through a wide variety of organisms, from the single celled amoeba to humans. Examples of responses include the withdrawal response in the earthworm [15], the body withdrawal in the snail [16], the escape response in the crab [17], the tail-flip response in the goldfish [18], the mobbing reflex of the chaffing [19], the rotation nystagmus in the dog [20], and the startle reflex in the human infant [21].

Lorenz (1965) suggested that habituation is likely to be the phylogenetically oldest process for modifying an organism’s behaviour. The universality of habituation implies that this process, however it may be explained, is fundamental to the adjustment of all organisms in their respective environments and that it has not altered radically through evolution. As Humphrey (1933) emphasized, the pervasiveness of habituation, as an “elementary conservative phenomenon, common to living systems of many different grades of complexity, justifies an inquiry into the nature of the phenomenon without giving a precise description of all the processes concerned. Clearly, the capacity to ignore only those stimuli that are irrelevant and to

channel behaviour into organized and directed actions in response to meaningful stimuli is necessary to conserve energy and focus behaviour.

Several characteristics of habituation have been found in a variety of species, vertebrate and invertebrate alike. Some of the basic properties of habituation were described in the classic works noted above [22-25]. In 1966, Thompson and Spencer surveyed the by then very extensive behavioural literature on habituation and identified some nine basic parametric properties or characteristics exhibited by behavioural habituation. Highlighting such properties is needed to direct and guide the search for underlying mechanisms. Recently it has been revisited to include all the species in which habituation and sensitization have been studied [26]. Scientists indicated the nine following parameters which have been found in all organisms and one (the age-related habituation) which is not included because it is relatively new.

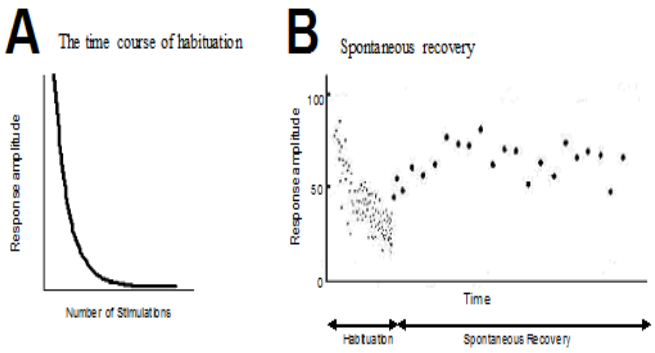


Fig. 1

Repeated stimulation elicits a depression of the response which spontaneously recovers when the stimulation is withheld. **A)** the exponential decay of the response to repeated stimulation. **B)** The response recovers spontaneously when the stimulation is stopped. Adapted from "A Model Phenomenon for the study of Neuronal Substrates of Behavior," by R. F. Thompson and W. A. Spencer, 1966, Psychological Review, 12, p. 24-26

1. *The time course of habituation*. Given repeated applications of a stimulus that initially elicits a particular response, further presentations of the stimulus result in a response decrement depicted by a negative exponential function of the number of stimulus presentation (Fig. 1A). This is the primary operational definition of habituation. In this

sense, it has been widely documented in research with various organisms (for a review see [27,28]).

Distinguishing habituation from adaptation and fatigue forces one to admit that behavioural changes due to an inability to detect a stimulus at the sensory level or to respond to it at the motoric level cannot describe all instances of response decrement. Behavioural and physiological evidence has confirmed that habituation is a learning process that cannot be explained by these simple potential causes of cessation in response. Adaptation is unlikely to be a factor of waning if a same stimulus elicits a different response after having initially faded. Another source of evidence that decreased response cannot be due to adaptation comes from studies whereby a stimulus to which the organism no longer responds again elicits a response when presented in a different context.

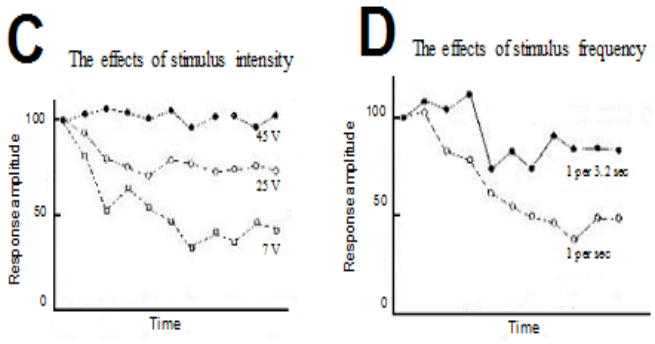


Fig. 2

Effect of stimulus frequency and intensity on the response. **C)** Increasing the stimulus intensity, the response shows less habituation. **D)** increasing stimulus frequency , the response shows a higher degree of depression. Adapted from "A Model Phenomenon for the study of Neuronal Substrates of Behavior," by R. F. Thompson and W. A. Spencer, 1966, Psychological Review, 12, p. 24-26

2. *Spontaneous Recovery*. If the stimulus is withdrawn, the response reappears over time (Fig. 1B). Hinde's (1960) study on the response of wild-caught chaffinches to live owls and owl models demonstrated that the time of response recovery after habituation is relatively rapid. In a comparison of the first trial response of the initial

habituation series with the first trial response of the recovery series, Hinde reported that the rate of recovery reached nearly 50% after 30 min, and grew subsequently for 24 hours. Consequentially the recovery is composed by a first fast phase in which the response recovery increases quickly and a second slower phase in which it reaches the control value.

3. *The Effects of Stimulus Frequency*. The greater the presentation of the stimulus, the faster or the more pronounced becomes habituation (Fig. 2D). In a study of habituation in the nematode Caernorhabditis elegans, Rankin and Broster (1992) found that its tap withdrawal responses were sensitive to the frequency of stimulation. If taps were presented at a short interstimulus interval (ISI) of 10 seconds or less, the tap response waned at a faster rate and more completely than if the taps were given at a longer ISI of 60 seconds. Additionally, they demonstrated that spontaneous recovery occurred faster for worms trained at short ISIs, while those trained at longer ISIs showed a relatively protracted recovery.

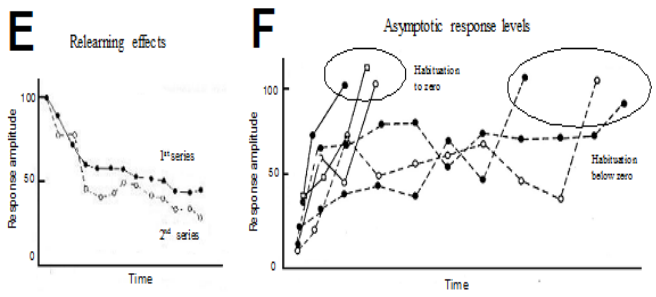


Fig. 3

Habituation can occur faster and can persist longer when the stimulation is protracted. E) If repeated series of habituation and spontaneous recovery are offered, habituation occurs more rapidly each time. **F)** The effects of habituation training may persist beyond the plateau-like level. Even after a response has disappeared or remained at a stable level, the organism will recover more slowly. Adapted from "A Model Phenomenon for the study of Neuronal Substrates of Behavior," by R. F. Thompson and W. A. Spencer, 1966, Psychological Review, 12, p. 24-26

4. *The Effects of Stimulus Intensity*. The weaker the stimulation, the faster or the more pronounced is habituation (Fig. 2C). With a very strong stimulus there may be no

response decrement but rather an initial response increment. In a study of contraction to mechanical shock in the flatworm Stenostomum, Applewhite and Morowitz (1966) reported that more intense stimuli took longer to habituate to and were slower to recover from. However, Sokolov (1963) posited that the relationship between stimulus intensity and the orienting response (also called the orienting reflex, or OR) is not linear. Interestingly, he found that near-threshold stimuli elicit relatively large responses.

5. *Relearning effects*. If repeated series of habituation and spontaneous recovery are offered, habituation occurs more rapidly each time (fig. 3E). This was exemplified in the decline in responsiveness to foot shock found over days in thigh electromyogram of spinal frogs [29]. When sessions of presentation were repeated daily, response amplitudes decreased progressively more rapidly from one day to the next.

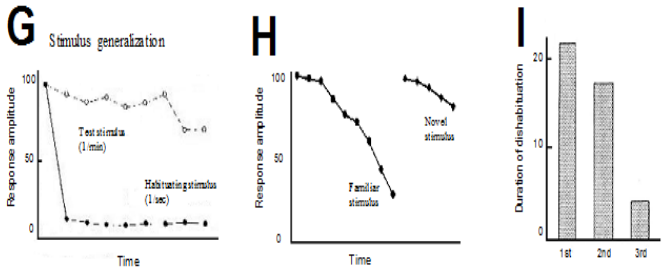


Fig. 4

Habituation is not related with neurotransmitter depletion. **G)** Habituation of a response to a particular stimulus is accompanied by stimulus generalization to other stimuli. **H)** Presentation of a novel stimulus results in recovery of the initial response. **I)** Repetition of the dishabituatory stimulus results in a waning of sensitization rate. Adapted from "A Model Phenomenon for the study of Neuronal Substrates of Behavior," by R. F. Thompson and W. A. Spencer, 1966, Psychological Review, 12, p. 24-26

6. *Asymptotic Response Levels*. The effects of habituation training may persist beyond the zero or asymptotic response level. Even after a response has disappeared or remained at a stable level, the organism will recover more slowly if it is exposed to additional habituation series beyond that point (Fig. 3F). Consequently, there should be less spontaneous recovery with increased number of habituation trials. In an

attempt to dissect the dynamics of the neural systems involved in habituation, Farel and colleagues (1973) studied the depression of the monosynaptic response of the lateral column/motoneurons pathway. Using in vitro preparation of the spinal cord of frog, they showed that the response amplitude after a trial of 50 stimuli recovered slower than the one in a trial of 10 stimuli.

7. *Stimulus generalization*. Habituation of a response to a particular stimulus is accompanied by stimulus generalization to other stimuli (Figure 4G). Hence, habituation is often used to determine which stimuli organisms find similar. For instance, Johnson and Aslin (1995) found that after habituating two-month-old infants to a black rod moving behind a white stationary box, the infants generalized habituation to a solid rod but not to a discontinuous rod. This finding suggests that these young infants treated the familiar stimulus as a continuous rod moving behind the box although the middle section of the rod remained invisible. Interestingly, this feature was not found in monosynaptic system as Aplysia[30,31] or the lateral column/motoneurons in *in vitro* spinal cord of frog[32].

8. *Dishabituation*. Presentation of a novel stimulus results in recovery of the initial response (fig. 4H). In an experiment conducted by Groves and Thompson (1970), rats were exposed to a series of 14 tones to which the rats' startle response habituated. Then a flashing light was presented to the experimental group before the 15th tone whereas the control group received no light. The first group exhibited a sudden return of startle response, although short lived, above the initial response level while the latter group continued on a habituating trajectory. Initially, this type of finding was used to infer that it is a process of inhibition that causes the response decrement which is then disinhibited by the novel stimulus. Yet, it became clear that dishabituation is not a disruption of habituation but rather a superimposed process of response sensitization which overrides the processes underlying the waning[33,34]. This notion formed the basis for the dual-process theory which will be discussed later.

9. *Habituation of Dishabituation*. Repetition of the dishabituatory stimulus results in a waning of dishabituation that is habituation of dishabituation (fig. 4I). For example, Lehner (1941) reported that various responses, including the tail reflex in spinal rats to repeated tapping, the respiratory startle in response to auditory stimulation in intact rats, and the umbilical abdominal reflex in humans, were revived with presentation of an extraneous stimulus once habituated through repetition. These dishabituatory effects themselves waned progressively showing less and less recovery with each habituation series.

Many people will be surprised to learn that, although habituation is termed ”the simplest form of learning” and is well studied behaviourally, remarkably little is known about the neural mechanisms underlying habituation. Researchers, who work on this form of learning, believe that because habituation allows animals to filter out irrelevant stimuli and focus selectively on important stimuli, it is a prerequisite for other forms of learning. Therefore, to fully understand the mechanisms of more complex forms of learning and cognition it is important to understand the basic building blocks of habituation.

SENSITIZATION

Dishabituation and sensitization have commonly been considered to reflect a unitary process: Sensitization refers to a general facilitation produced by strong or noxious stimuli that enhances subsequent responding [35] while dishabituation has been thought to represent a special instance of sensitization in which the facilitation is simply superimposed on a habituated response level [36].

A novel or strong stimulus initiates a general arousal-like process that is widespread in the nervous system, facilitating habituated and non-habituated responses [33,37,38]. Although both reasonable and logically consistent, this explanation does not rule out the possibility that dishabituation and sensitization could reflect separate facilitatory processes that are activated in parallel by a strong stimulus. This hypothesis was proposed by Wagkolov,(1976) who suggested that dishabituation and

sensitization might be separable on the basis of the stimuli used to elicit these processes. Specifically, Wagner proposed that dishabituation might be able to be produced by relatively innocuous stimuli that would not be sufficient to produce sensitization. This suggestion was supported by the results of Whitlow (1975; see also Whitlow and Wagner, 1984), who showed that a vasomotor response in the rabbit could be facilitated by a relatively innocuous "distracting" stimulus only if that response were previously decremented; that is, the response could be dishabituated but not sensitized. Wagner (1976) pointed out that most studies, examining dishabituation and sensitization, would not distinguish between these two processes since strong or noxious stimuli are typically used to produce response facilitation.

Sensitization could be evoked in different ways. When the same innocuous stimulus (primary stimulus) which evokes habituation is used, it has been called primary sensitization or intrinsic sensitization [39-41], warm-up [39,42] or iterative enhancement [43]. When sensitization is elicited with a different , usually strong or noxious stimulus (secondary stimulus) has been called extrinsic sensitization or secondary sensitization. [34,44].

MODELS OF HABITUATION AND SENSITIZATION

The Kupfermann-Kandel model

Habituation has been studied in a huge kind of species but the best known system which has been able to give information about habituation both at behavioural and neuronal level is the so-called gill-withdrawal reflex (GWR). This is the response of the gill to weak tactile stimulation of the siphon in the marine mollusc Aplysia Californica. The major attraction of this system was the hypothesis that the operationally defined behaviour corresponded to a unitary motor pattern, in this case a simple stereotyped withdrawal, and that the neural circuitry involved was simple enough to make it possible to establish a direct link between neuronal changes and the behavioural changes defined as learning [45-47]. The Kupfermann-Kandel model represented a major advance in behavioural neurobiology because it

made clear, falsifiable predictions about the role of individual neurons in observed behaviour and the causation of behavioural plasticity at the cellular level.

The synapses between sensory neurons and motoneurons of this model exhibit homosynaptic depression and presynaptic facilitation [48,49]. Thus, short-term habituation appears to be presynaptic and seems to result from homosynaptic depression [31-33], which in turn may result from decreased transmitter release [50-52]. The decrease has different causes. These include decreased calcium influx [53] and depleted vesicle pools [54], both resulting in reduced transmitter release from the presynaptic terminal.

Heterosynaptic facilitation is ‘‘branch specific,’’ meaning that output from different sites on the same nervous system can be facilitated to different degrees. In the Aplysia gill withdrawal circuit, a facilitator interneuron becomes activated and acts on sensory neuron terminals to increase the level of intracellular adenosine 3 ',5 '-monophosphate, which, through a cascade of intracellular reactions not fully understood, causes a particular class of potassium channels in the sensory neuron to close, thereby reducing the overall efflux of K^+ at the time of depolarization by the action potential. Because the repolarization of the neuron is due to an efflux of K^+, a decreased outward movement of K^+ ions results in a longer period of depolarization produced by each action potential, which in turn results in an increased influx of extracellular Ca^{++} and transmitter release [55].

Evidence further suggests that conditioning results from activity-dependent amplification of presynaptic facilitation [56] and Hawkins and Kandel (1984) have suggested how the simplest learning processes may combine to produce characteristics of more complex forms of learning.

Mammalian models

Thompson and Spencer described the habituation of the flexion reflex in the acute spinal cat with all nine parametric characteristics (1966). The reflex also

exhibits extrinsic sensitization [33,57] and intrinsic sensitization [41]. Interestingly, both forms of sensitization habituate if the sensitizing stimulus is repeatedly applied [33,37,57]. The experiments performed in acute cat spinal cord did not allow them to show the circuit underlain habituation and to find the cellular mechanisms of habituation and sensitization. Later the monosynaptic response of motorneurones to lateral column stimulation in the isolated frog spinal cord have been used by Farel and Thompson to study phenomena analogous to behavioural habituation in the intact animal[29,32,58]. The decrement of the monosynaptic ventral root response and intracellular recorded responses of identified motorneurones to repeated stimulation of the lateral column exhibit the parametric features characteristic of habituation in the intact animal. The decrement to repeated stimulation appears to be the result of some process of homosynaptic depression limited to the synapses of lateral column fibres on motorneurones. This model of habituation does not involve actions of interneurons and is not due to either pre-or postsynaptic inhibition. The data from the cat experiments and from the frog allowed them to understand better the interaction between facilitation and depression processes and to build the dual process theory of learning (see below).

A study of the rat startle reflex done by Davis (1974) has offered momentous support for the dual-process theory. He presented evidence that repeated presentation of a tone leads to either a decrease or an increase in response depending on the level of background noise: startle responses to a 110-db tone habituate when background noise level is at 60-db, whereas responses rise with a background noise level of 80-db. These results lend themselves to be interpreted in light of the competing processes that are habituation and sensitization. Low sensitization levels arise from the exposure to the low background noise resulting in the net gain of habituation, whereas sensitization prevails over habituation and is maximized by the low background noise.

THE DUAL PROCESS THEORY OF LEARNING

A theoretical approach to habitation and sensitization was proposed by Thompson and Spencer (1966), predominantly on the basis of neurophysiological research on the hindlimb flexion reflex of acute spinal cats. They presented evidence that behavioural responses elicited by repeated stimulation reflect the net outcome of two independent, superimposed processes: habituation and sensitization.

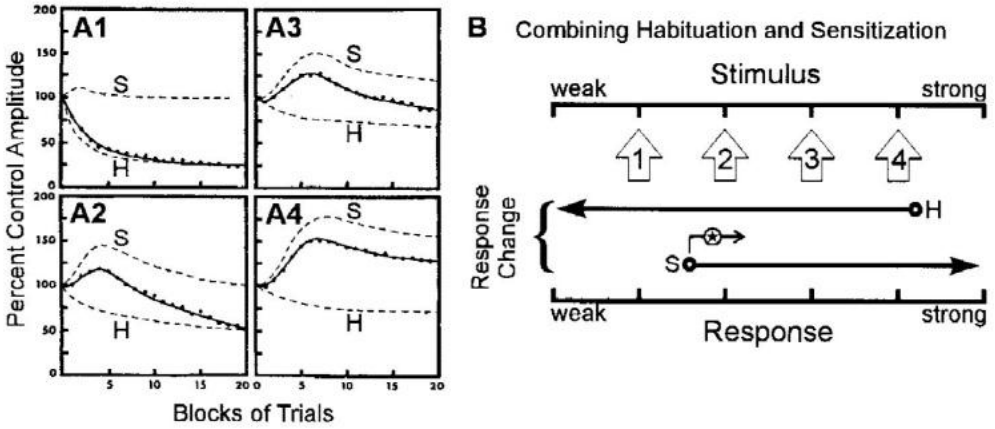


Figure 5

The combination of habituation and sensitization to produce dual-process learning. (A1–A4) Decomposition of dual-process learning curve (solid line) into component habituation learning curve (broken line labeled H) and habituating sensitization learning curve (broken line labeled S) at four different stimulus intensities increasing between A1 and A4. Empirical data are shown as dots. (B) The contribution of opposing processes at different stimulus intensities. Stimulus intensities used in A are illustrated as open arrows pointing along the stimulus axis. Below each of these arrows are shown the learning processes recruited at that stimulus intensity. The arrow marked H indicates habituation (response decrement by depression); the arrow marked S indicates sensitization (response increment by facilitation). Stimulus 1 elicits strong habituation; stimulus 4 elicits only weak habituation but also elicits strong sensitization; stimuli 2 and 3 are intermediate. An important addition to this interaction is that the lower limit for eliciting sensitization increases (indicated by arrow marked with star) consequent to depression early in the neural circuit (see text), meaning that the balance between habituation and sensitization shifts in favor of habituation over time, explaining the bumpy shape of the curves seen in A. Adapted by Groves, Philip M.; Thompson, Richard F. *Habituation: A dual-process theory*. Psychological Review, Vol 77(5), Sep 1970, 419-450.

Sensitization refers to an increase in response magnitude as a result of a stimulus that increases arousal. Habituation and sensitization are often considered opposing processes and, therefore, mutually exclusive. Sensitization increases the salience of strong and/or novel stimuli (high informational value), whereas habituation

decreases the salience of repeated stimuli (low informational value) that, as Brown (1998) points out, serves to further enhance the salience of less frequent, high information stimuli. The combination of sensitization and habituation essentially works toward making behaviour effective but not wasteful. These two processes are assumed to occur in two different parts of the nervous system with the habituation process occurring in the sensory side and the sensitization process on the motor side [59-61]. The first evokes a response by activation of a stimulus response reflex arc from the organism's perceptual encoding of the stimulus to the muscle systems. The second is the arousing system that determines the organism's general level of responsiveness, or readiness to respond. The change in behaviour is determined by whichever process is stronger. As will be discussed later, a common finding in habituation research is that sometimes (it depends by the initial conditions) an initial response increase before declining, implying an initial greater effect of sensitization over habituation, with further presentations producing more habituation than sensitization. This theory can account for many of the behavioural phenomena of habituation and sensitization.

THE RAT SPINAL CORD

As part of the central nervous system (CNS), the spinal cord is the gateway for information transfer between body and brain, as well as a centre for neuronal circuits that integrate and coordinate complex sensory, motor, and autonomic functions. The several hundred thousand neurons per segment are housed within cytoarchitectonically defined anatomical layers called laminae (I–X). These can broadly be divided into: the sensory dorsal horn (laminae I–VI); the intermediate gray (lamina VII); and the ventral horn (VII–IX). Motor neurons are located in lamina IX. The axons of motoneurons and preganglionic neurons exit via ventral (anterior) roots to innervate skeletal muscle and postganglionic neurons, respectively.

The rat sensory systems are immature at birth, except olfaction and temperature sensation [62]. The vestibular and the proprioceptive systems develop gradually; they

reach full maturity more than 3 weeks later. However, some sensory feedback exists very early; the monosynaptic stretch reflex pathway studied by Kudo and Yamada (1985, 1987a) is present at E18.5.

The afferent system

The sensory experience begins in the periphery, where the peripheral terminals of primary afferent fibres respond to a myriad of stimuli and translate this information into the dorsal horn of the spinal cord, where the central ends of these fibres terminate (Fig. 1). There are three main types of sensory fibres: Ia fibres (or Aβ-fibres), Aδ and C fibres.

Muscles spindles in rats' hindlimb contained two types of sensory endings, the primary ending and the secondary ending. The primary or annulospiral endings give rise to the Ia fibres which are large in diameter and highly myelinated, they show low activation threshold and are the only which are connected monosynaptically to motorneurones [63]. The Ia afferent fibres distribute the information from the periphery not only to motorneurones individually but simultaneously to a large population of motoneurons that are thus grouped into a functional entity. Monosynaptic connections from Ia fibres to motoneurons may spread over several segments. It can therefore not be ruled out that the collateral system is much longer than a single motor nucleus and may cover a distance of up to 30 mm. Shortly after entering the spinal cord, the axon of an Ia fibers bifurcates into an ascending and a descending branch, localized in the dorsal funiculus. The areas of Ia terminal arborizations are limited to three regions: I) lamina VI (the intermediate region), 2) lamina VII (the region of the Ia inhibitory interneurons), and 3) lamina IX (the motor nuclei). The number of terminals is largest in lamina IX and smallest in lamina VII, and despite proximity of some collaterals, the areas of terminal distributions of adjacent collaterals show no overlap [64].

The efferent system

The ventral horn comprises lamina VII–IX. Motoneurons are largely cholinergic, with at least some containing glutamate as co-transmitter (yesper and Kiehn,2008). They are among the largest neurons in the CNS with extensive dendritic arbors that receive 20,000–50,000 synapses. Alpha (α) motoneurons supply skeletal (extrafusal) muscle fibers and are responsible for movement; gamma (γ) motoneurons are smaller than α-motoneurons and innervate intrafusal muscle fibers to control muscle tone by regulating the sensitivity of muscle spindles to stretch.

Motoneuronal pools of the lumbar region have been studied anatomically in rat [65]. MNs of a muscle form a discrete longitudinal column in the lateral ventral horn. There is a double gradient in the organisation of motoneuronal pools, a rostro-caudal gradient with the flexor MNs located more rostrally than extensor ones, and a proximo-distal gradient with the MNs innervating hip muscles that are ahead of those innervating knee and ankle muscles.

The pain system

Thin Aδ and C fibres afferents, terminating in the superficial dorsal horn of the spinal cord, are involved in nociceptive transmission. Aδ-fibres are small in diameter and thinly myelinated, making them slower-conducting than Aβ-fibres. They possess higher activation thresholds and respond to both thermal and mechanical stimuli. C-fibres are the smallest type of primary afferents and are unmyelinated, thus making them the slowest conducting. They have the highest thresholds for activation and therefore detect selectively nociceptive or 'painful' stimuli. Collectively, both Aδ- and C-fibres can be termed as nociceptors or 'pain fibres', responding to noxious stimuli which may be mechanical, thermal, or chemical [66-69].

Within the spinal cord there are cells which fire action potentials when a painful stimulus is detected at the periphery. Nociceptive-specific (NS) cells are mostly found superficially and synapse with Aδ and C fibres only. Cells which receive input exclusively from Aβ-fibres are proprioceptive and only respond to touch. A third type of neurone, termed wide dynamic range (WDRs), receive input from all three types of

sensory fibres, and therefore respond to the full range of stimulation, from light touch to noxious pinch, heat, and chemicals. WDRs fire action potentials in a graded fashion depending on stimulus intensity, and also exhibit 'wind-up', a short-lasting form of synaptic plasticity [70].

Several forms of activity-dependent plasticity have been described in the spinal cord. Thompson and colleague revealed the mechanism of the windup [71]. It is a progressive increase in the number of action potentials elicited per stimulus that occurs in dorsal horn neurons and in the large motor neurons of the ventral horn [72] when the stimulus frequency of C-fibers exceeds 0.5 Hz. Increasing the stimulation frequency, the postsynaptic depolarizing responses to individual dorsal root stimuli in

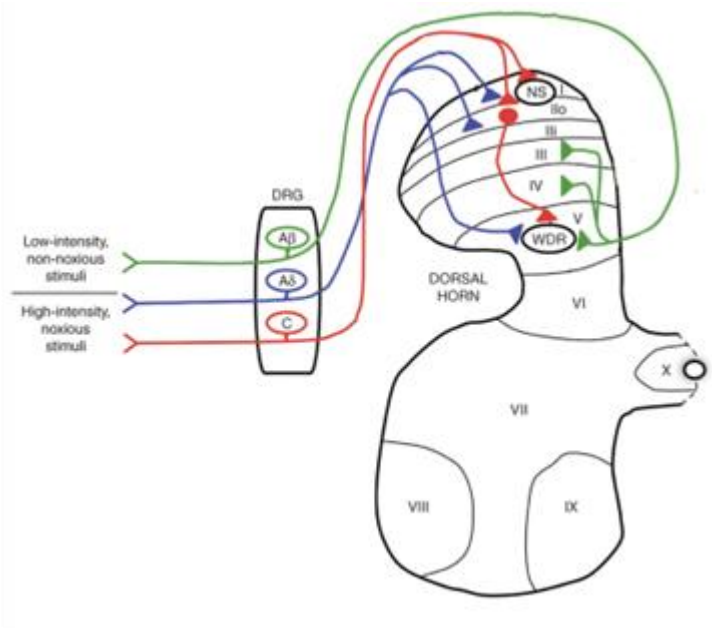


Fig 6

Pain pathways from periphery to brain. Primary afferent fibres (Aβ, Aδ, and C-fibres) transmit impulses from the periphery, through the dorsal root ganglion (DRG) and into the dorsal horn of the spinal cord. Nociceptive specific (NS) cells are mainly found in the superficial dorsal horn (laminae I–II), whereas most wide dynamic ranges (WDRs) are located deeper (lamina V). Figure adapted from D'Mello et all., 2008.

the ventral horn cells summated to produce a cumulative depolarization, which resulted in a burst of action potential discharge instead of a single action potential in the ventral horn cell in response to each dorsal root stimulus. After the high-frequency stimulation was stopped, action potentials continued to fire for as long as the depolarization of the ventral horn cell lasted (approximately 60 s) and then

ceased. Long term potentiation (LTP) was first described in the hippocampus as a long-lasting increase in the efficacy of synaptic transmission caused by brief high-frequency (100 Hz) stimulation of synaptic pathways. Long term depression (LTD) is, as its name implies, a long-lasting decrease in the efficacy of synaptic transmission, which can also be caused by high-frequency conditioning stimulation. More recently, LTP and LTD of synaptic transmission have also been described in several regions of the spinal cord, from the primary afferent [73,74], to the GABAergic transmission [75].

THE "H"REFLEX IN RAT SPINAL CORD

As the neural circuitry of the H-reflex is largely the same as the monosynaptic stretch reflex (tendon-tap reflex), the H-reflex has often been described as the electrical analogue of the stretch reflex. Primary afferents of a particular muscle monosynaptically excite two groups of motoneurons: those that innervate the same muscle (homonymous) and those that contact muscles with related function (synergists). These primary afferents also project onto interneurons that inhibit motoneurons innervating muscles with opposite function [76-79]. Therefore, a specific primary afferent monosynaptically excites homonymous and synergistic motoneurons and disynaptically inhibits antagonistic motoneurons (reciprocal inhibition). Most of the old studies to describe this monosynaptic pathways have been done on cats. The monosynaptic reflex of the rat spinal cord is very similar but there are same differences, due to the species specificity, which are important to know before to use this model for a study on plasticity.

Dorsal root afferents reach the ventral horn by day E17 in embryonic rats, produce functional excitation of motoneurons shortly thereafter, and are well established by postnatal day two [80-82]. Sensorimotor innervation in the spinal cords of neonatal rats imply that, at 4-10 days after birth, appropriate contacts are formed between primary afferents of flexor and extensor muscles and their specific motoneurons [83].

Generally speaking, the efferent conduction is faster than the afferent one in rat peripheral nerve :the former is on an average 65-80m/sec and the latter, 50-70m/sec when measured at the body temperature. These values are definitely smaller than those of cat. The fact that the efferent conduction is faster than the afferent one is quite in line with the result of the fibres analysis, which shows that the efferent path contains larger fibers more profusely than the afferent one and in periphery the largest efferents are larger than the largest afferents [84].

The fast response was inferred to be monosynaptic from its short latency by Nagai and colleagues[85]. In rats the latency itself is not so short as to leave no doubt for its monosynapticity. The duration of the fast synchronous wave is approximately 5 ms which is rather long as well. When the stimulus intensity was raised from liminal to maximal one, the response become more synchronized and shorter [86]. It was due to the increased synaptic drive by augmented action of the concerned afferent fibers, as well known from the experiments on cat spinal reflex [87].

In young rats, the monosynaptic convergence is weak and the synaptic drive is not sufficient to fire motoneurons monosynaptically when the stimulus strength is low. Indeed when the stimulus intensity is weak, the large part of motoneurons pool is insensitive to the monosynaptic drive and barely reaches firing level after polysynaptic convergence, and with the increase of stimulus intensity the monosynaptic drive becomes more and more effective, which leads to the gradual augmentation and forward progress of monosynaptic component and at the same time to the decrease and the dispersion of later polysynaptic components by refractoriness and recurrent inhibition [86]. Indeed the neural circuitry responsible for the H-reflex is predominantly characterized by the monosynaptic projection of the group Ia afferents onto the homonymous motoneurons. The connections are not purely a monosynaptic input from only group Ia afferents, but is confounded by oligosynaptic contributions from the Ia and other large-diameter afferents [88-90]. Thus, the earliest component of the H reflex (the positive peak) is still predominantly

comprised of the monosynaptic Ia effects, but the later portions of the reflex probably include contributions from oligosynaptic Ia pathways.

The rat monosynaptic reflex present long latency, long duration but also other properties which present this animal as a good candidate for our purposes. Indeed it showed a drastic depression to low frequency repetitive stimulation [91-93]. Furthermore in spinalized rats the post-tetanic potentiation and post-tetanic depression have been begun to be investigated [86]. After tetanic stimuli, the monosynaptic reflex did not show potentiation (as in cat[33]) but showed the pot-tetanic depression. Further investigations on motoneurons clarified that the motoneurons had a strong tendency to become silent and firing probability fell severely. Furthermore the depression was attributed both to the cessation and to the temporal dispersion of motoneurons firing [86].

THE *IN VITRO* SPINAL CORD PREPARATION OF RAT

The neonatal rat preparation was presented for the first time by Otsuka and Konishi (1974). They demonstrated the presence of a polysynaptic reflex in an isolated hemisected spinal cord superfused with a Krebs solution. Suzue (1984), who was the first to use the complete dissected preparation from the brainstem to the sacrococcygeal spinal cord, investigated the respiratory rhythm. For locomotion, the rhythmic behaviour obtained with different neuroactive substances, was described by Smith and Feldman (1987) and Kudo and Yamada (1987b). This *in vitro* preparation corresponds to a very immature central nervous system (Clarac et al. 2001) but in the last years, preparations from 5 months old rats have been started to use [94](see materials and methods for further informations).

AIMS OF THE STUDY

Habituation and sensitization are likely the phylogenetically oldest processes for modifying an organism's behaviour. Clearly, the capacity to ignore only those stimuli that are irrelevant and to channel behaviour into organized and directed actions in response to meaningful stimuli is necessary to conserve energy and focus behaviour. These non-associative forms of learning have been studied in invertebrate models at a cellular level but the obtained results did not exhaustive explain these processes in mammalians. It is most probable that habituation and sensitization are terms denoting different processes which work and compete each other, determining the final behaviour. In mammalians, the complexity of the used models by Thompson [33]and Daves [40,95]did not give the possibility to understand the underlain circuit and mechanisms of these processes. Furthermore depression has begun to be studied also in in vitro preparation of embryonic chick spinal cord (Lee and O'Donovan, 1991), rat spinal cord (Ziskind-Conhaim, 1990), neonatal rat spinal cord (Kudo and Yamada, 1985; Fulton and Walton, 1986), and kitten (Eccles and Willis, 1965) but no one of these studies showed the mechanisms responsible of these processes in mammalian CNS.

Consequently this study is aimed to:

1. test wheter the MVR *in vitro* spinal cord of rat presents the features of the behavioural habituation and sensitization, found in all species studied and indicated Rankin and collegues (2009);

2. investigate the variables which affect the reflex amplitude during the learning process, trying to obtain the experimental conditions to activate only the depression process (pure habituation) or the facilitatory process (pure sensitization). Furthermore a simple mathematical model will be used to described the final reflex learning kinetics, as a result of the complex interactions between the short and intermediate effects of the two processes;

3. To discuss and to compare data with literature, both from vertebrate and invertebrate models;

4. To update and to use the dual process theory of learning, proposed by Thompson and Spencer in 1966, to explain all our results. The implication of habituation and sensitization, as basic processes underlain other more complex types of learning, will be discussed as well.

5. To verify whether the *in vitro* spinal cord preparation of rat is a good model to study the cellular and molecular mechanisms underlain learning and memory in mammalians.

MATERIALS AND METHODS

PREPARATION

The *in vitro* rat spinal cord is a preparation in which the CNS is isolated from thoracic level to the end of the cord and it is placed in a recording chamber to perform the experiment. The surgery to take out the spinal cord from the rat body has to minimize the mechanical damages to the cord and the neuronal death due to the great mechanical stress of the surgery itself. Furthermore it has to be as fast as it is possible in order to minimize the neuronal death due to the oxygen rate decrease. Indeed the artificial solution in which the cord will be placed for the experiment, is highly oxygenated but the diameter of the cord is an important factor which rule the oxygen rate which reaches the cells: the greater the diameter, the lower the oxygen which will reach the deeper cells. For these reasons we set up different surgeries to take out the spinal cord from rats of different age.

The literature (for a review see [96]) and our data have been used to distinguish three classes of rats on the basis of the age. It has been done in order to have a preparation in which the parameters (inhibitory system, consume of oxygen, length of the cord and ecc.) do not change during the rat developing, affecting the results of the experiments. The parameters we took into account to divide the rats in different ages class, are briefly summarized:

- Motoneurons are produced on embryonic days (E) 13–14 in the lumbo-sacral cord (birth occurring at around E22). Roughly half of the motoneurons produced degenerate and die [97](67% in mouse [98]);

- The stretch reflex appears at E19–20 [80]. The initial monosynaptic connections are not all specific: at E19–21, 29% of the motoneurons receive monosynaptic innervation from primary afferents of antagonistic muscle [99]. The proportion of such inappropriate contacts peaks at P0–2 and then decreases; the majority of monosynaptic connections are appropriate within 1 week after birth. [100];

- A transient widespread electrotonic coupling is found between motoneurons in the perinatal spinal cord [101]. However, electrical and dye coupling decrease with age and are no longer present after the first postnatal week [102].

- Receptive field areas are large at birth and nociceptive withdrawal reflexes are often misdirected, bringing the stimulated area of the skin towards the stimulus instead of away [103]. The size of receptive fields decreases with age, and from the 2nd postnatal week, adequate withdrawal reflexes can be elicited, suggesting that some inappropriate connections become depressed or eliminated.

- In embryonic and neonatal rat lumbar motoneurons, application of GABA and glycine induces membrane depolarization. Activation of $GABA_A$ and glycine receptors causes the opening of Cl^- channels with consequent outward movement of Cl^- because the intracellular Cl^- concentration is maintained at a relatively high level by an inwardly directed Cl^- active transport mechanism coupled to Na^+ and K^+ [104]. This efflux transiently drives the transmembrane potential toward the equilibrium potential for chloride (ECl) which is above the resting membrane potential in immature motoneurons. The subsequent reduction in the amplitude of glycine and GABA-induced depolarization reported during development is likely due to a shift of ECl toward more negative potentials. The relative importance of glycine as compared to GABA increases during spinal cord maturation. The density of the GABA current is larger than that of the glycine current before birth. The density of the glycine current increases significantly after birth, whereas there is little change, if any, in the density of the GABA current [105];

The rat spinal cord undergoes a significant continuous transformation during perinatal development. All these modifications have to be taken into account using this preparation and pooling the data obtained over a time window of only a few days. Therefore we divided the rats in three classes: very young, considered from postnatal day 0 (P0, it indicates the first 24 hours after birth) to P7, young (between P8 and P13) and older (after P14). All the showed experiments have been performed

in young rats (between 8 and 13 days old), unless a comparison of the habituation at different ages should be done.

Advantages of this preparation

The neonatal spinal cord *in vitro* preparation is the first mammalian nervous system to be totally isolated in vitro from the brainstem to the caudal end of the spinal cord. Because of the size of a neonatal rat, the preparation is very small and it is possible to use whole spinal cord preparation or part of it (see below). However the whole preparation shares some similarities and advantages with other preparations (e.g. the sliced preparation) but not its disadvantages.

The surgery for the preparation can be done quickly (about 10 minutes) in conditions that are easier than for other mammalians and the length of the spinal cord is short, although the size increases rapidly (from 3 cm at P0 to 5 cm at P6). So an adequate circulating system is needed to keep the nervous tissue alive. Anyway the CNS during the first postnatal week is still not myelinated. The spinal cord has a small diameter (4 mm/8 mm) and can be easily perfused, allowing the direct contact between neurones and solution. Furthermore in the *in vitro* preparation there is no hemato-meningeal barrier as in the acute animal. In fact some neuroactive substances do not cross the bloodbrain barrier either cannot be studied or can be studied only if precursors are used, like L-Dopa for the dopamine [96,97] and 5-HTP for 5-HT 98. In the in vitro preparation all the substances can circulate through the different spinal internal structures. This enables us to characterize their actions and to measure the effects, the latency and the duration of their effects.

The preparation can be used routinely for several hours when it is maintained in an appropriate physiological medium. Indeed all the chemical parameters of the medium are totally controlled. The solution is continuously bubbled with a 95% O2/5% CO2 mixture and the pH is around 7.4. To prevent a metabolism that is too high, which increases the acidity of the medium, most experiments are performed at around 27° C. At birth the neonatal rat does not regulate its body temperature

properly; the CNS therefore supports a large variation of temperature. The use of an artificial celebro-spinal fluid (aCSF) gives also the possibility of ion manipulations. By the application of some specific solutions, the number of synapses (polysynaptic or monosynaptic connections) and the different ion channels involved can be characterised.

The overall development of the rat lasts for at least 6 weeks with about 3 weeks in utero. In the *in vitro* spinal cord all the central structures, the different nuclei, their inputs and outputs remain in place; it is possible to have intracellular recordings from different cells of the spinal cord: motoneurons and interneurons (Endo et all., 2010) and dorsal root ganglia cells. It is also possible to record extracellularly from the dorsal and ventral root. The dissection becomes easier and is routinely used after birth from P0 to P6. Later, the development of myelin and the increased size of the spinal cord prevent a sufficient oxygenation of the tissue. Some authors, however, have performed in vitro experiments on spinal cords isolated from animals older than 6 month, recording reflexes from sacral and caudal roots and motorneurones [95].

The preparation can be modified to be used for different purposes. For instance to correlate the nervous central activity with the muscular output, the dissected spinal cord could be done with one or two hind limbs attached (Hochman et all., 2006). For the aims of this study, totally isolated hemisected preparation with a particular focus on the lumbar region, which has the longest ventral roots, has been used. Anyway, for further investigation at cellular level, the preparation could be isolated and the dorsal or the ventral horns could be removed. It is possible also cut only one ventral or dorsal horn and left the other hemisection intact in order to record from motoneurons or interneurons (Dougherty et all 2010) . In the last ten years this preparation has been used also from rats older than 5 months [94]

In parallel to the studies performed on the *in vitro* preparation, an extensive amount of data has been obtained behaviourally in the young rat [84,86,91,94,95,106-111]. A systematic comparison between *in vitro* and *in vivo* data greatly increases the value

of the neonatal rat model. Furthermore the comparison between cat and rat is updated continuously. Finally human and rat development appear to be quite different. This is largely due to huge differences in the duration of prenatal and postnatal development: The human baby is born after 9 months (270 days) in utero and adulthood is reached at around 20 years, whereas rat development lasts about 6 weeks, 3 weeks before [embryonic day (E) 0 to 21] and 3 weeks after [post-natal day (P) 0 to 21] birth. However, striking similarities can be observed between human and rat motor development when taking a time scale of one day in the rat corresponding to one month in the baby (Fig. 2)[106].

Before the beginning of a study with this preparation it is important to consider that the spinal cord changes each day. The development of the cord happens and the different systems are subjected to variations. For instance the inhibitory system is immature at birth [112]. Furthermore the GABAergic system is predominant during the first postnatal week while the glicinergic system become predominant after the second one [113]. The cortico-spinal tract reaches the lumbar spinal cord between the first and the second postnatal week [109]

SURGERY

All experimental procedures were conducted in accordance with guidelines for the ethical treatment of animals issued by the Italian Council on Animal Care and approved by the University of Milan Ethic Committee.

Dorsal and ventral approaches to young rats spinal cord

The animals were decapitated and spinal column was isolated and placed in a dissection chamber with ice slurry bath of oxygenated, high-Mg2+, choline-containing artificial cerebral spinal fluid (aCSF), composed by (in mM): choline chloride 125; KCl 1.3; CaCl2 0.5; KH2PO4 1.2; MgCl2 7; HEPES 10; D-glucose 25. Low temperature were used to reduce temperature of the spinal cord and high concentration of choline and Mg2+ for reducing the surgical damage to the preparation. Vertebral bodies were removed with special care using a ventral

approach which is simpler than the dorsal approach for different reasons. First of all the position of the spinal cord in the column and the position of the dorsal root ganglia (DRG, which are closer to the ventral side than the dorsal side of the cord) allow less mechanical damages to the preparation when the operator has not experience. Indeed the scissor, cutting the vertebral body, are farer from the tissue than using the dorsal approach. Furthermore this approach allow to have longer dorsal and ventral roots, giving the possibility to use more than only electrode on the root. The dorsal approach is suggesting only when the spinal cord has to be taken out faster (for instance in older rats) and it has to be hemisected to prevent neuronal death.

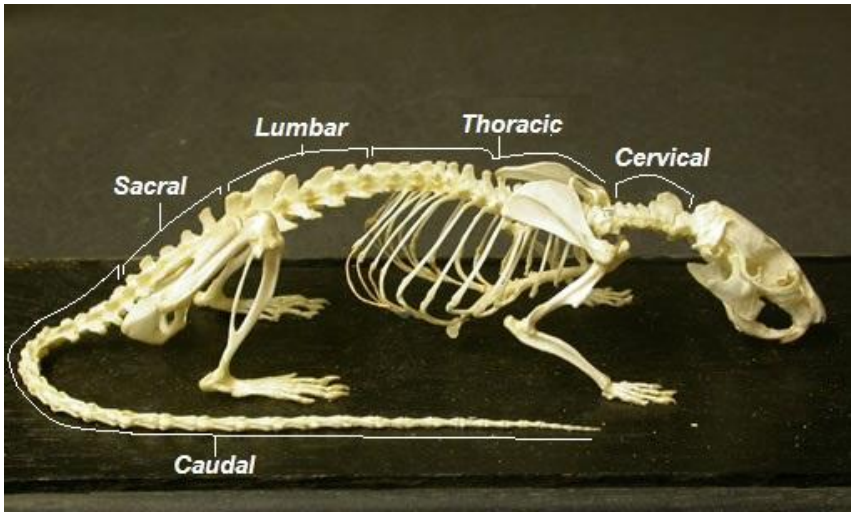


Fig.7

The rat vertebral column. The rat vertebral column presents 6 vertebrae at cervical level, 13 at the thoracic, 6 at the lumbar, 5 at sacral and the final caudal end.

By the way, the spinal cord was exposed by removing the vertebral lamina from the ventral surface (or from the dorsal one), using a pair of fine eyes scissor working from rostral to caudal. After the laminectomy was performed, the process is the same in the two approaches. The Dura mater was opened and the spinal cord was exposed directly to the aCSF. After cutting the spinal nerves, peripheral to the dorsal

root ganglia, the cord was floated free from the vertebrae. Immediately it was taken out from the vertebral bodies and pinned down, ventral side up, in a Sylgard-coated experimental chamber, superfused at 6 ml/min with oxygenated (95% 02/5% C02) aCSF solution at room temperature (28°C) containing (in mM): NaCl 130; KCl 3; MgSO4 1.3; CaCl2 2; NaH2PO4 0.58; NaHCO3 25 and D-glucose 10. A sagittal hemisection of the cord was performed using insect pins and each hemisection was transferred to a separate dish.

Dorsal approach to old rats

Rats were deeply anesthetized with Equitensine (sodium pentobarbital, 58.5 mg/kg) and under a surgical microscope the skin was cut and the vertebral column was isolated from the muscles, taking care not to cut important arteries or veins (for example iliac artery, which is very close to the spinal column and its big diameter could provoke the death of the animal, if it was cut). When the vertebral column was cleaned from muscles and connective tissue on dorsal side and it was possible to see clearly the processes of the vertebrae, a laminectomy was performed, starting from the thoracic 4 (C4) vertebra to lumbar 6 (L6) one (corresponding to spinal segment T6 to the end of the cord). When the cord was clearly visible, it was continuously wetted with modified artificial cerebrospinal fluid (aCSFm) and the Dura mater was opened. The last step has to

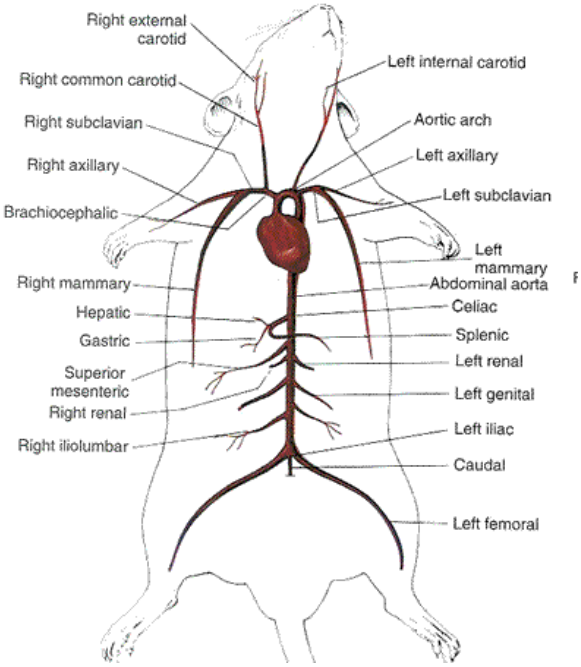


Fig.8

The rat arteries system. The most important arteries of the rat circulatory system. During the surgery it is important pay attention not to damage the abdominal aorta and the two iliac arteries. When the vertebral column is isolated, the carotid artery is cut to decrease the bleeding when the spinal cord will be take out.

be done paying attention not to cut arteries or veins, which are very close to the cord and delicate. At this point pure oxygen had been given to the rat with a mask until the dorsal vein turned bright red. Afterwards the carotid was cut and the cord was quickly removed and immersed in a dissection chamber with aCSFm. At this point the Dura mater was completely removed from the cord and the roots. Slowly the Pia mater was opened to allow a higher rate of oxygenation to the tissue and a sagittal hemisection of the cord was performed using insect pins. After that, each hemisection was transferred to a separate dish to performed the experiments.

The most important factor which affect the in vitro spinal cord preparation is the diameter of the cord that enabled it to survive whole (unsliced) in vitro when it was acutely isolated from new-borns, young or adult rats. When the spinal cord is taken out from the spinal column, the oxygen and nutrients only diffuse 300 mm into tissue [114] and it is the limit to maintain the spinal cord in an artificial conditions.

STIMULATION AND RECORDING

Bipolar glass suction electrodes were attached usually to L3 dorsal roots (SD_{L3} in fig 9) to allow for electrical stimulation as well as to L3 ventral roots (RV_{L3} in fig 9) to record evoked reflexes. In all experiments, an additional suction electrode was attached to the L3 dorsal root (RD_{L3} in fig 9) to record the afferent volley. In same experiments different roots (from T10 to C1) were tested to verify whether different monosynaptic pathway segments present the same properties. Voltage current stimulators (Grass S8 stimulator) delivered single-shock stimuli of defined stimulation parameters to the dorsal roots. Raw data were collected with Clampex software (pClamp.v. 9.0, Axon Instruments, Union City, CA) and stored in a PC for off-line analysis.

SPINAL REFLEXES

The viability of the preparation was assessed at the beginning of each experiment. The MVR on L3 ventral root (VR_{L3} in fig. 9) was elicited by mean of electrical stimulation of the homologue dorsal roots (SD_{L3} in fig 9). In addition, an

electrode was placed on L3 dorsal root to record the incoming dorsal root volley (RD_{L3}in fig. 9). Stimuli of low intensity (50 μs, about 500 mV) were delivered to activate only the Ia afferent fibers to evoke the early response (4-5 ms latency at room temperature, MVR in fig 9) which is known to be monosynaptic [86]. Typically the reflex had two components, a fast rising positive-negative response generated by the monosynaptic reflex pathway, followed by a second slower, small positivity which could represent the monosynaptic activity of the motoneurons at a subliminal fringe

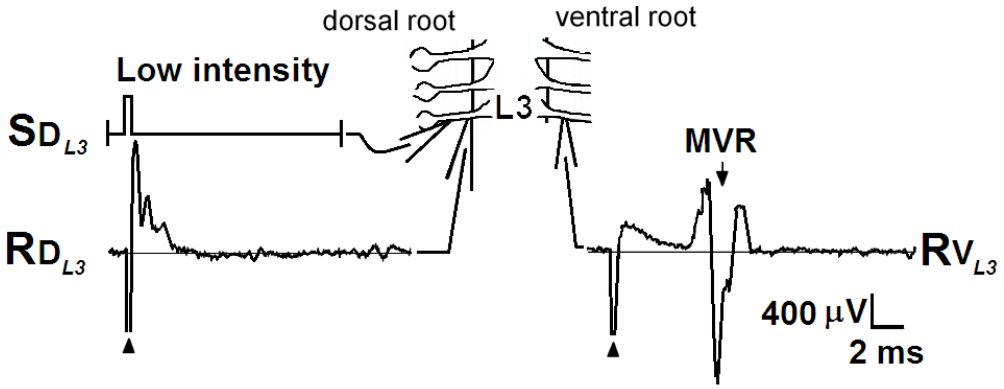


Fig. 9

Habituation experiments. Schematic view of the preparation with the lateral side of the spinal cord up with the dorsal root in the left side and the ventral roots on the right one. Two suction electrodes were attached to dorsal root: one to stimulate (SD_{L3})and one to record the fibres activation (RD_{L3}). The homologue ventral root response (RV_{L3}) is showed in the right side of the panel. MVR is only about 3 ms after the stimulus artefact. The area under the triphasic response of the MVR has been used to measure MVR amplitude during the experiments.

or oligosynaptic activity (MVR in Fig. 9). Indeed an electrical stimulus, usually at low intensity, causes a few motoneurons to fire but it induces sub-threshold excitation to many other motoneurons that constitute the subliminal fringe and their discharge could not be evoked at all or could be delayed [115]. While for oligosynaptic inputs, they have ample time to contribute to the delayed H-reflex wave, given that the composite EPSPs of the motoneurons have a sufficiently long rising phase to permit oligosynaptic inputs to reach the motoneurons at the subliminal fringe [116]. So the amplitude of the MVR was evaluated measuring the underlying area of the first positive-negative wave. Furthermore we use this wave to have the possibility to

relate possible changes in the slope of the positive-negative wave to changes in the number of the α-motoneurons that are considered to be at a subliminal fringe [117], as a result of the learning process. Indeed the depression or the facilitation process underlain habituation and sensitization could decrease or increase the number of the subliminal fringe motoneurons which are able to discharge. It could be seen, for instance, in fig. 10B, where a low intensity stimulus and a higher intensity stimulus have been used. The slope of the higher stimulus elicited response is stepper than the lower stimulus-induced response.

These data were used to calculate the percentage of the reduction of the reflex at any tested stimulus interval, using the general equation:

$$Percentage\ of\ the\ reduction = Ri/Rmax$$

where Ri is the response at a given stimulation interval and $Rmax$ is the average responses elicited at a 30 or 60 s stimulation interval (control recording) in each experiment. After an initial stabilization period (about 30 minutes), no time-dependent changes in reflex amplitude were observed at this frequency.

At the beginning of each experiment, the dorsal root was stimulated at increasing intensities (50 μs, 60 s) and the intensities with the relative responses were plotted in a graph to obtain the stimulus/response relationship (fig. 10A). The MVR amplitude is expressed in the Y axis as % of the maximum monosynaptic ventral response at which, enhancing the stimulus intensity, the MVR did not increase anymore. The stimulus intensity is indicated in the X axis as a multiple of the voltage used to evoke the smallest MVR amplitude. The activation curves of the MVR is very steep between 1 T and 2 T. Between these values, the slope reaches the highest value of the tested intensities. The slope of the stimulus/response curve warrants attention because it provides information about the reflex gain, the so called input–output relationship [118,119]. Further, this slope is best fitted with a sigmoid function that takes into

consideration the amplitude of the maximal H-reflex (H_{max}) and the stimulus required to evoke a response equivalent to half the H_{max} [120].

The response reached the 95% of the maximum response when the stimulus intensity was 3 T and intensities greater than 4 T did not increase the monosynaptic response size. Furthermore in rats older than 9 days, when the stimulus was higher than 3 T, different types of fibers (like tactile or Aδ fibers) begun to be activated. In

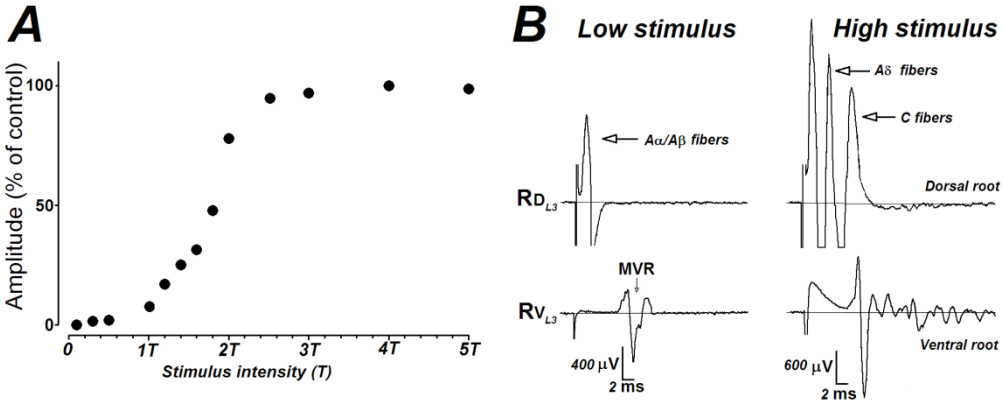


Fig.10

A) Stimuli used for the experiments. The low intensity stimulus (50 µs, 1.4 T) elicited the activation of only Ia fibers and the homologues monosynaptic response (respectively RD_{L3} and RV_{L3} on the left). The high intensity stimulus (500 µs, 10-30 T) evoked the activation of all fibres in the root with the mono and polysynaptic response (right side). Note has two components: the monosynaptic reflex followed by the polysynaptic response, which lasts many ms after the stimulus. **B) Input/output relationship.** The L3 monosynaptic ventral root responses evoked by stimuli (0.016 Hz, 50 µs) of increasing intensity. The intensity of the stimulus is indicated in the X axis as multiple of the voltage needed to evoke the smallest response (threshold). The amplitude of the reflex in the Y axis is expressed as a percent of the maximum monosynaptic ventral response (MVR_{max}), at which, increasing stimulus intensity, MVR did not grow up anymore.

rat between 6 and 8 days old, the threshold of tactile fibers was lower and their activation started using intensities higher than 2 T. When rats were younger than 5 days, the threshold of other fibres overlapped the monosynaptic ones very early. Furthermore in this young rats there are some unspecific monosynaptic connection between tactile fibers and motoneurons [100]. For this reasons all experiment have been performed in rats older than 7 days old.

The stimulus, which activates only Aα and Aβ fibres and elicits just the MVR, was called low intensity stimulation or test stimulus (TS, left in fig. 10B). High voltage stimulus (70 Hz, between 10 e 40 T, T indicates the voltage used to evoke the smallest MVR) were delivered to activate Aδ and C fibers and it was able to elicit not only the monosynaptic response but also a long lasting response which lasts more than 100 ms (right in fig 10B). It was called high stimulus or conditioning stimulus (CS).

VIABILITY OF THE PREPARATION

The state of excitability of the afferent fibres and the α-motoneurons pool play a significant role in determining the H-reflex magnitude [121]. In order to maintain a stable afferent fibres/motoneurons excitability and to minimize the mechanical stress effects-induced by the dissection surgery, the

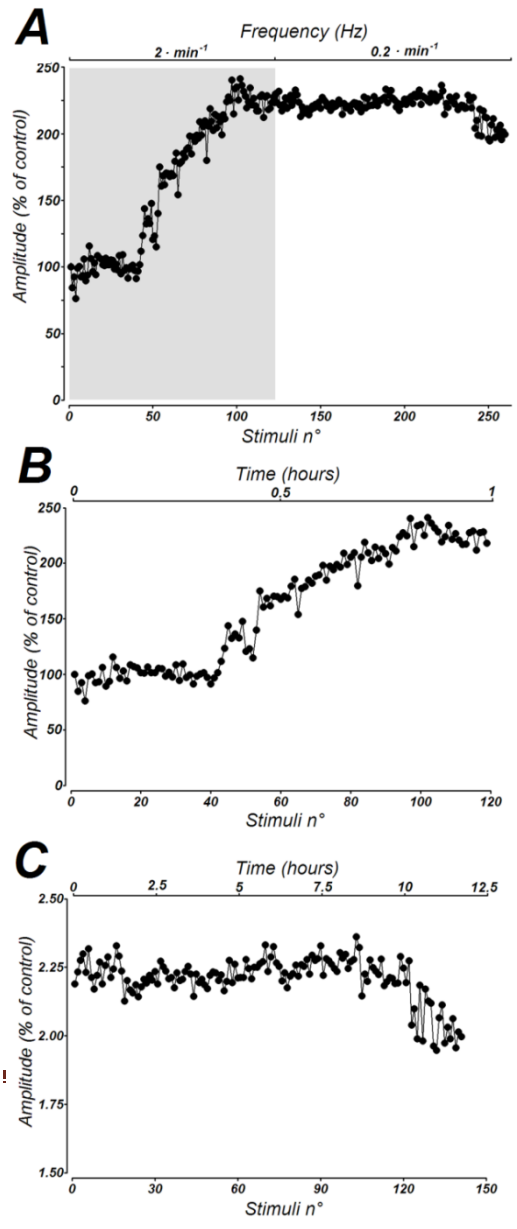


Fig. 11

Viability of the preparation. The stability of the monosynaptic reflex has been evaluated all over 12 hours, using a TS (50 μs, 1.2 T). **A)** the L3 dorsal root has been stimulated and the homologue MVR has been recorded. The underlain area has been measured and its value has been normalized to the amplitude average of the first 10 minutes of control recording. During the first hour after the surgery, the MVR showed a continuous increase in amplitude (grey zone). Then the reflex was stable for about ten hours and the amplitude begun to decrease (white zone). **B)** The increase of the reflex amplitude during the first hour of recording after the surgery (grey zone in A). The responses were stable during the first 20 minute of recording and then the response started to enhance, reaching the final value of 250% of the control. **C)** MVR amplitude during 12 hours (white zone in A). It was very stable but after 10 hours, its amplitude started decreasing. In order to performe experiments of plasticity it is important to pay attention to the physiological oscillation of the reflex amplitude during a day.

preparation was allowed to rest at least one hour before any manipulation. Indeed this model presents a very good stability of the elicit responses. Usually it has been used for 12 hours without any problem. When longer survival times are required, the lower temperatures could be used and the cords could be kept alive for as long as 50 hr at 18-20°C [122].

Furthermore the monosynaptic reflex is well known to have physiological amplitude oscillations during a day. These physiological oscillations could affect our purpose to evaluate learning by mean of the reflex amplitude. Indeed the oscillation in reflex amplitude could be seen as effect of learning (decrease for depression and increase for facilitation). We have to measure the physiological oscillations of the reflex amplitude in order to be able to exclude this phenomenon from learning.

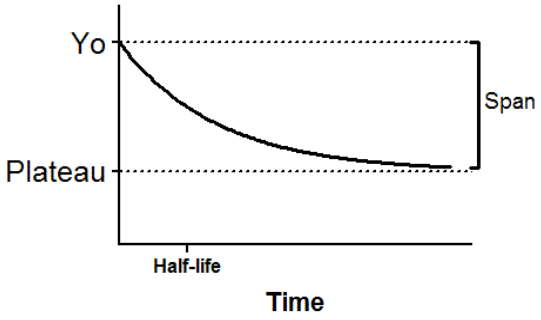
Same experiments have been performed to verify the stability of the H reflex amplitude in this preparation since its isolation for the next 12 hours (fig. 11). The MVR was elicited from the homologue L3 dorsal root, using a TS (50 μs, 1.2 T). During the first hour, the MVR increased its amplitude until to reach a stable state in which the new final value was greater more than twice the initial control one (fig. 3B). it was maintained for the next 10 hours (fig. 11C) and presented physiological oscillations (about 1/5 of the final value). A deterioration of the preparation usually occurred after 10 hours by its isolation (right side in fig. 11C) with a decrease in the amplitude value. Interestingly the physiological oscillations during the deterioration increased to 1/3 of the final value.

STATISTICAL ANALYSIS

Exponential fitting

The depression curves of the reflex are fitted using the equation

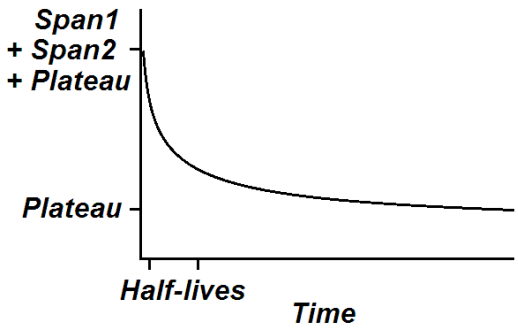
$$Y = (Y0 - Plateau) * \exp(-K * X) + Plateau \; [1]$$



where X is the time, Y0 e Plateau are the MVR value at the start and at the end of each experiment and K is the speed constant expressed as the inverse of the X axis unit (s). Using this equation, τ (indicates the speed of MVR decrement) and the plateau amplitude (the steady state of the decrement at the end of the experiment) have been calculated for all experiments.

The sensitization curves have been fitted using a two phase decay equation:

$$Y = Span1 * e - k1 - x \; + \; Span2 * e - k2 - x \; + \; Plateau \; [2]$$



This equation describes a two phase exponential decay. Y starts out equal to Span1+Span2+PLATEAU and decays to PLATEAU with fast and slow components. The two half-lives are 0.6932/K1 and 0.6932/K2. In the figure, the two rate constants differ tenfold, but the spans were equal. The curve is not obviously biphasic, and it takes a very practiced eye to see that the curve does not follow a single phase model. But when the equation has been used to fit the point of sensitization experiments, the two components are very clearly visible (see in results).

The among group difference of the tested parameters were examined using the one way analysis of variance (ANOVA) or the Student' s T test. All the offline analysis are performed using Clamfit 9 (Molecular Devices, USA) and GraphPad Prism 5 (GraphPad Software, USA).

T test and one way ANOVA

T tests, and related nonparametric tests, has been used to compare two sets of measurements (data expressed using an interval or ratio scale). If three or more groups have been analysed, one-way ANOVA has been used. A confidential interval of 95% has been used, unless it is different indicated. When P value > 0,05, the data are significantly different.

F test for unequal variance

The unpaired t test depends on the assumption that the two samples come from populations that have identical standard deviations (and thus identical variances). We have tested this assumption using an F test. First compute the standard deviations of both groups, and square them both to obtain variances. The F ratio equals the larger variance divided by the smaller variance. So F is always greater than (or possibly equal to) 1.0.

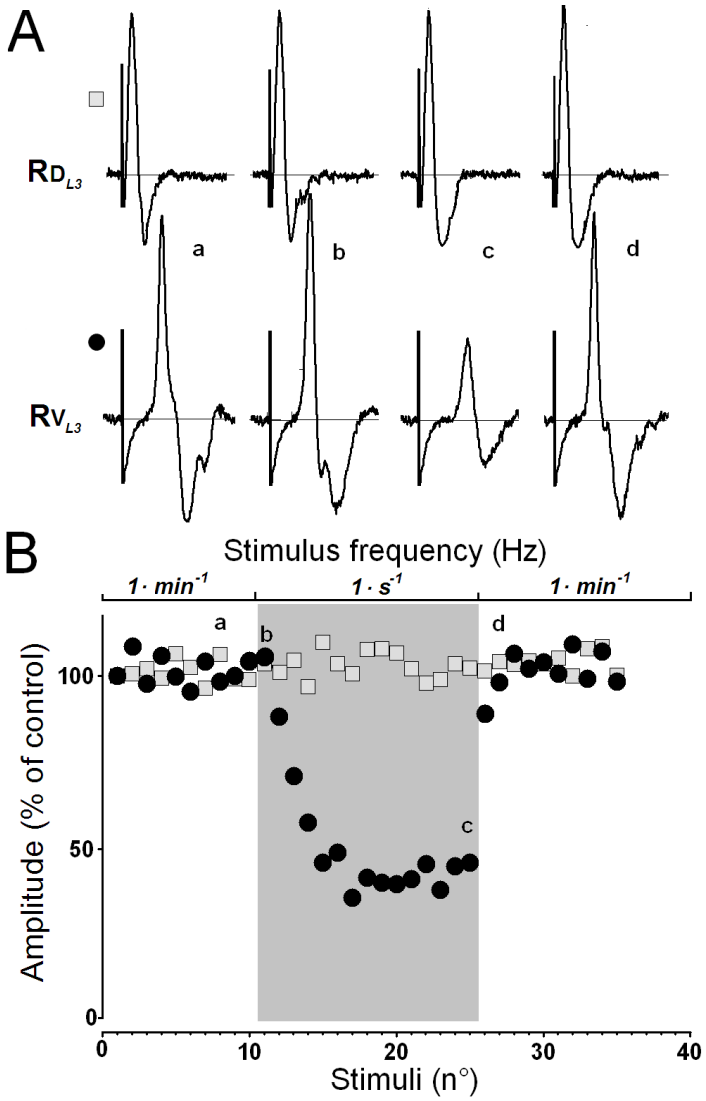
If the P value is large (>0.05), it suggests that there is no evidence that the variances differ. If the P value is small, the variances differ significantly.

RESULTS

HABITUATION

Given that a particular stimulus elicits a response, repeated applications of the stimulus result in decreased response. This is the most general property of response habituation which shows a decline in response, usually exponentially, to a plateau like level during the repetition of a meaningless stimulus. In the *in vitro* spinal cord of rat, the repetition of a low intensity stimulus on the dorsal root depressed the homologous early ventral

Fig 12



Repeated stimuli to the dorsal root decrease the homologue monosynaptic ventral response (MVR) in isolated rat spinal cord. The repetition of a low intensity stimulus (50 µs, 1.4 T, 1Hz) on the L3 dorsal root causes the decrease of the MVR_{L3} in one preparation of 11 days old rat spinal cord. **A)** Recordings of the L3 dorsal root activation (RD_{L3}, grey squares) and the L3 ventral root responses (RV_{L3}, black circles) during the experiment; a) control recording using stimulation frequency 1*min^{-1} Hz; b) the first stimulus and c) the last stimulus of 15 stimuli at 1 Hz; d) control recording at 1*min^{-1}. **B)** The graph shows the dorsal root activation and the MVRL3 during the entire experiment. The lower X axis indicates the number of stimuli, the upper X axis indicates the stimulation frequency (Hz) and the Y axis the reflex amplitude, expressed as percent of the maximum reflex amplitude recorded during the control stimulation frequency of 60 s stimulus interval. The letters are the same of the ones in the upper panel. Note the stability of the dorsal root activation while the MVR decreases when frequency stimulation changes to 1 Hz. MVR recovers spontaneously when stimulation frequency returns to the control value

root response (monosynaptic ventral response, MVR). A typical decrement of the MVR is shown in fig. 12 where low voltage stimuli were delivered to the L3 dorsal root. After ten minutes of control recording (a in fig. 12), 15 stimuli were delivered at 1 Hz (b and c in fig. 12) and then the stimulus frequency returned to the control value (d in fig 12). Dorsal fibers activation (grey squares) and the homologue ventral root responses (black circles) are plotted as functions of the stimulus number and the stimulus frequency. The MVR amplitude did not change during the control recording, as the dorsal root activation, but it decreased when the frequency was changed to 1 Hz. MVR amplitude decreased quickly during the first seven stimuli and then it reached a new value which was maintained for the rest of the stimulation. This level of the response will be indicated as “the plateau-like level”. It is important to note that the dorsal root activation was stable throughout the experiment. When the stimulation frequency returned to the control value, the MVR stopped decreasing and it came back to the initial value after the first two stimuli. It should be noted that the more the stimulation progressed, the more was the latency delayed and the more the duration of the monosynaptic reflex wave was prolonged. Thompson and Spencer (1966) stated that spontaneous recovery of a response to the level seen at the start of an experiment, had become the most common method of demonstrating that a given response decrement is an example of habituation opposed to nonspecific declines in response. However, they were careful to note that defining habituation based on spontaneous recovery alone would not be useful as a number of variables can greatly affect whether and when spontaneous recovery is observed.

Features of the test stimulus on habituation

As habituation is the reduction of a response to a repeated stimulus, it is expected that manipulating variables having to do with the repeated stimulus might modify habituation, either in magnitude or in speed of development, or both. These variables include the number of the stimuli, their intensity or frequency, the number of habituation trials, and the frequency of the trials. The spontaneous recovery of the response in the previous experiment is due to a break in the stimulation frequency

but it could be affected by the time frame of the stimulation as well. If the frequency does not change, the number of stimuli affects the spontaneous recovery: the greater the number of stimuli, the longer it takes for the spontaneous recovery. The MVR decline and its spontaneous recovery are shown in fig. 13, after 10, 30 or 50 stimuli (50 μs, 1.4 T, 1Hz). The stimulation, using the same frequency, changed in duration (10, 30 and 50 s). MVR after 10 test stimuli (black triangles) decreased, reached the plateau-like level only with the last three responses and recovered to the control value soon after the first control recording stimulus (τ = 0.38 s). Using 30

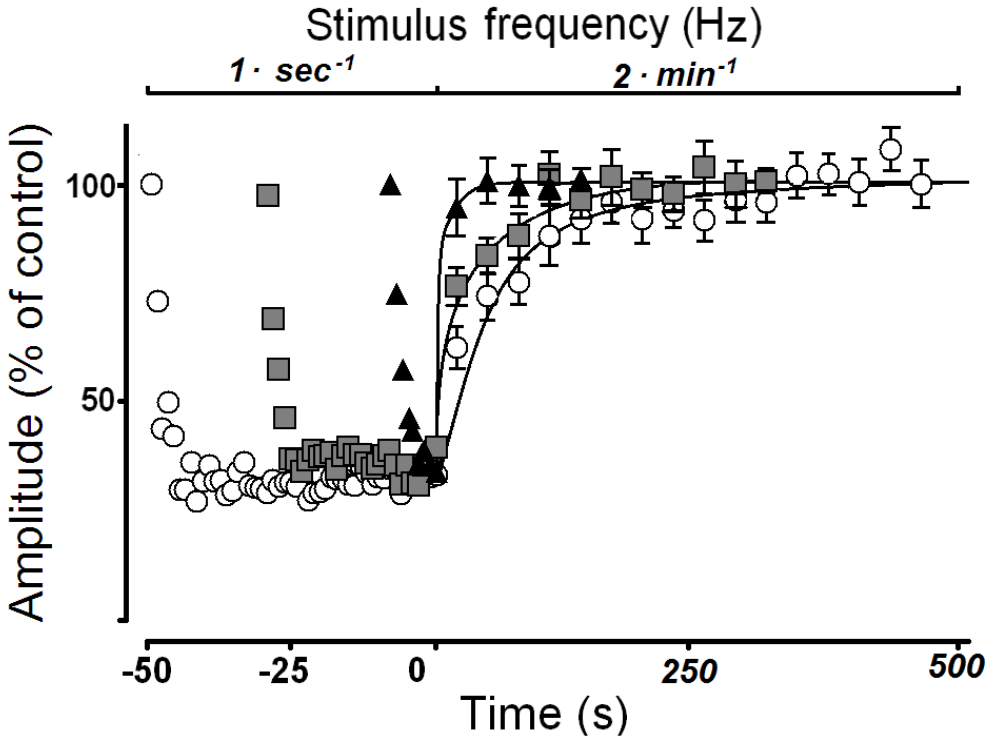


Fig. 13

MVR decrease and spontaneous recovery using 10, 30 and 50 stimuli. Data are average of ten preparations of 10 days old rats. On the left of the graph, MVR declined exponentially to a plateau-like level, using a stimulation (50 μs, 1.4 T, 1Hz) for 10s (10 stimuli, black triangles), 30 s (30 stimuli, grey squares) and 50 s (stimuli, white circles). On the right, the spontaneous recovery when the stimulus was withhelds. Each preparation received the trials in the same conditions and in a random order. At least 15 minutes were allowed to elapse between the complete recovery from one stimulation trial and the presentation of the next one. All the points are fitted by the equation in [1] (see materials and methods). Note the inverse relation between the number of stimuli and the time frame of spontaneous recovery: the tau is 0.38 s for 10 stimuli, 122 s for 30 stimuli and 208 s for 50 stimuli while the amplitude at the plateau-like level is the same for all of the trials (about 42% of the control amplitude).

stimuli (grey squares), MVR reached the plateau level and recovered in about 3 minutes (τ = 122 s). Lastly after 50 stimuli, MVR reached the plateau level and it was held constant until the end of the stimulation. The recovery after 50 s of stimulation was slower than before and it peaked the control value in about 6 minutes (τ = 208 s). Note the inverse relation between the number of stimuli and the recovery rate. The number of stimuli (and so the time of the stimulation) affects only the recovery of the response but not the run down or the plateau-like level.

Habituation was, in general, more rapid or more pronounced to weaker stimuli than to stronger stimuli. Thompson and Spencer noted that this pattern was characteristic of most habituating responses. In fig. 14A the graph shows different degree of MVR depression when different stimulus intensities are used. The experiments were performed on 10 rats between 9 and 10 days old. Trials with a constant number of stimuli at the same frequency (50 μs, 1Hz) but with increasing intensities (1.2 T, white triangles; 1.4 T, grey squares; 1.8 T, black circles) are delivered to the preparations in a random order. The intensities evoked, respectively, about the 20%, 40% and the 60% of the maximum response of the MRV. During the stimulation, MVR_{L3} decreased and it reached different constant levels (ANOVA test, $p < 0.0001$). The degree of the MVR depression was related to the stimulus intensity, the higher the stimulus intensity, the smaller the final habituation degree. Using the equation in [1], the τ and the plateau have been calculated for 8 different intensities and placed (respectively in fig. 14B and C) as a function of the stimulation intensity, expressed as multiple of the voltage (T) used to evoke the smallest response. The value of the tau (expressed in s) has been indicated in logarithmic scale while the plateau value (as % of the control value) is shown in a linear scale. Using further voltage increases over 3 T, usually did not depress the reflex. The stimulus intensity did not affect the spontaneous recovery of the reflex (fig. 14D, unpaired t test, $p = 0.09466$). The experiment in fig. 14D shows the recovery of the response, using 1.1 T and 1.8 T stimulus intensities. Although responses amplitudes at the end of the trial were very different, the recovery took the same time to reach the control value.

Frequency can be defined both in terms of real time and in terms of number of stimuli delivered. In other words, habituation should be affected both by stimulating an absolute number of times more rapidly (15 stimuli at 1 Hz versus 10 Hz) or by stimulating more frequently within a set period of time (1 minute of stimuli at 1 Hz

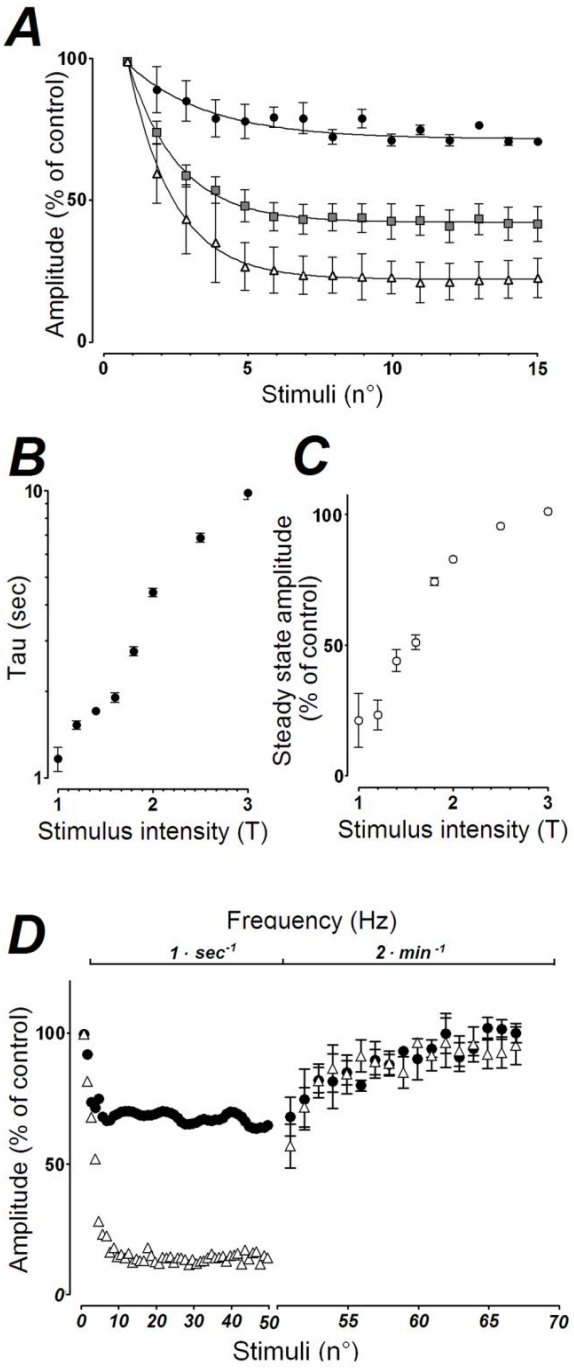


Fig 14

Inverse relation between the stimulus intensity and the degree of MVR depression. The depression of MVR is affected by the stimulus intensity. The experiments were performed on 10 rats between 9 and 10 days old. **A)** Trials with a constant number of stimuli at the same frequency (50 µs, 1Hz) but with increasing intensity of 1.2 T (white triangles), 1.4 T (grey squares) and 1.8 T (black circles) are randomly delivered to the preparations (ANOVA test, $p < 0.0001$): **B)** The τ has been calculated in *s* for eight different intensities (1, 1.2, 1.4, 1.6, 1.8,2 and 3 T), using the equation [1] (see materials and methods). It is plotted in logarithmic scale as a function of the stimulation intensity, expressed as a multiple of the voltage used to evoke the smallest response. **C)** The plateau level amplitude of the MVR has been calculated as previously for the τ and plotted as a function of the stimulus intensity. The S.e.m is greater in the first three points than in the rest of the intensities used. **D)** After 50 stimuli (50 µs, 1Hz) at 1.1 T (white triangles) and 1.8 T (black circles), the frequency of the stimulus changes to $2*min^{-1}$ in order to evaluate the spontaneous recovery. The recovery time is not different (unpaired t test, $p = 0.09466$, F test to evaluate variance, $p = 0.8261$).

versus 1 minute at 10 Hz). Increasing frequency in either way modifies MVR habituation to repeated stimuli. Data in fig. 15 shows MVR depression using different stimulation frequencies with the same number of stimuli. 18 hemisected spinal cords of 10 days old rats were used and the preparations randomly received the stimulation trials with at least 15 minutes between each other. Fig.

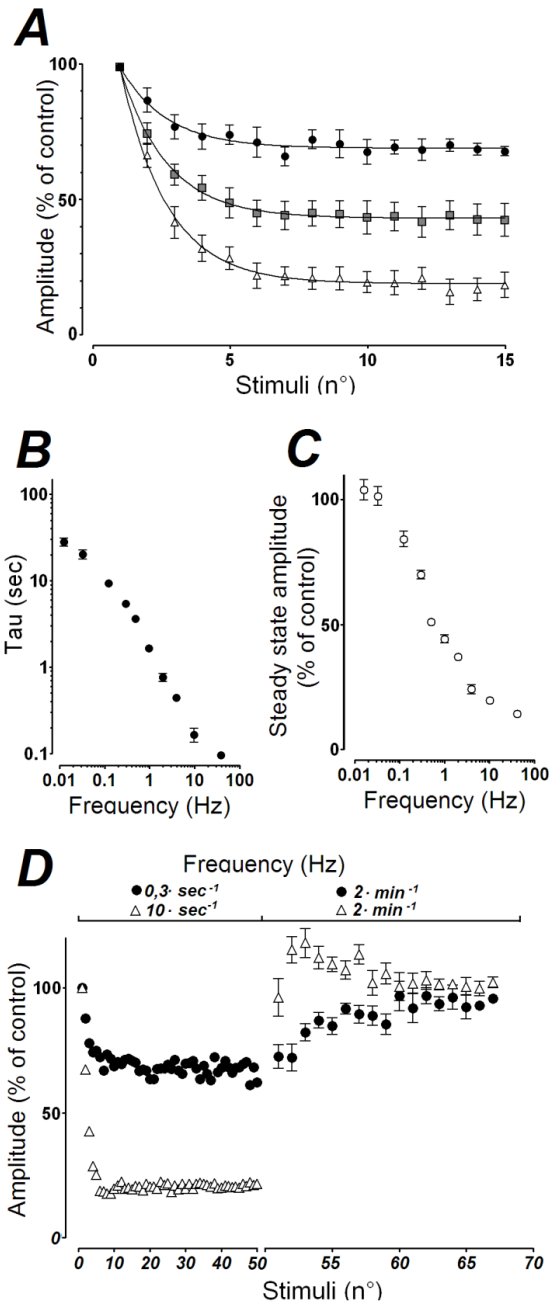


Fig. 15

Direct relation between degree of habituation and stimulus frequency. The depression of the MVR is affected by the stimulus frequency. The experiments were performed on 18 rats at 10 days old. **A)** Trials with 15 stimuli (50 µs, 1.4 T) at different frequencies (10 Hz, white triangles; 1 Hz, grey squares; 0.3 Hz, black circles) are randomly delivered to the preparations. Note that the x axis indicates the number of the stimuli but the duration of the stimulation changes, depending on the frequency. **B)** The τ has been calculated in s for ten different frequencies (0.016, 0.033, 0.125, 0.3, 0.5, 1, 2, 4, 10, 40 Hz), using the equation [1] (see materials and methods). It is plotted as a function of the stimulation frequency, expressed in a logarithmic scale**. C)** The plateau level amplitude of the MVR has been calculated as previously described for the τ and plotted as a function of the stimulus frequency. **D)** After 50 stimuli (50 µs, 1.4 T) at 10 Hz (white triangles) and 0.3 Hz (black circles), the frequency of the stimulus changes to 2*min-1 in order to evaluate the spontaneous recovery. The recovery time is affected by the frequency of the stimulation (unpaired t test, $p < 0.0001$, F test to evaluate variance, $p = 0.4490$).

15A shows the different degrees of MVR depression, using different frequencies. The lower frequency (0.3 Hz, black circles) caused only a 20% of reduction in MVR depression, 1 Hz (grey squares) frequency caused a 55% reduction and, the higher frequency (10 Hz, white triangles) reached an 80% reduction. The graphs 15B and C show respectively the tau and the plateau for ten frequencies (0.016, 0.033, 0.1, 0.3, 0.5, 1, 2, 10, 40 Hz), calculated with the equation in [1]. The different stimulation frequencies elicited responses which are significantly different between them (one way ANOVA, $p<0.0001$). Stimulus intervals above 30 s did not depress the response, at least in the experiment time. The recovery time of MVR with two different frequencies are shown in fig 15D. The lower frequency (0.3 Hz, black circles) depressed the reflex response less than the higher one (10 Hz, white triangles) but the recovery time took more time to return to the control value. It is important to note that after the stimulation at 10 Hz, the amplitude of the reflex is higher than the control amplitude and it needed about 5 minutes to decrease to the control value. Comparing data with different stimulation intensities and frequencies, it has to be noted that the stimulation intensity affected the reflex more in the range of the smaller responses (see the S.e.m. in fig. 14B and 14C) than in the greater ones. In contrast, the frequency of the stimulation seems to affect the reflex in the opposite way. By decreasing the frequency, the elicited responses become more different between them and the s.e.m. increases in value as the frequency decreases.

Potentiation of habituation and effects after the asymptotic level

The third criterion as defined by Thompson and Spencer, potentiation of habituation, is the enhancement of habituation to a test stimulus after repeated trials of habituation training followed by spontaneous recovery. This phenomenon requires repeated habituation training interspersed by periods of no disturbance to allow for spontaneous recovery. Criterion 6 also implies that if stimulation is continued beyond the point of stable habituation (the plateau-like level), slower recovery from the habituated response would ensue when the stimulation eventually ended. Some preparations have been used to see the effect of ten consecutive trials

of 15 stimuli each (50 µs, 1.2 T, 10 Hz) on MVR depression and recovery. Fig. 16 shows a single trial (black circles) and the last of ten trials (grey squares) with the associated recoveries. In the ten trials experiment, a trial followed the previous one after 1 minute. The first response of the single trial (a in fig. 16) is about 100% of the control amplitude while the first response of the last of ten trials (c in fig. 16) has lost more than 40% of the initial value. Moreover the run down phase in the last of ten trails (grey squares) was more rapid than the single trial (black circles). Indeed the response just reached the plateau level at the second stimulus while the response in the single trail needed 5/6 stimuli before it was stabilized to the plateau-like level. Lastly the plateau level of the 10th trial (d in fig. 16) is lower than the single trail one (c in fig. 16). The use of ten trials affects not only the decrement of the response but also the recovery. The spontaneous recovery in the single trial session took 2 minutes to reach the control amplitude while the recovery after ten trials needed more than

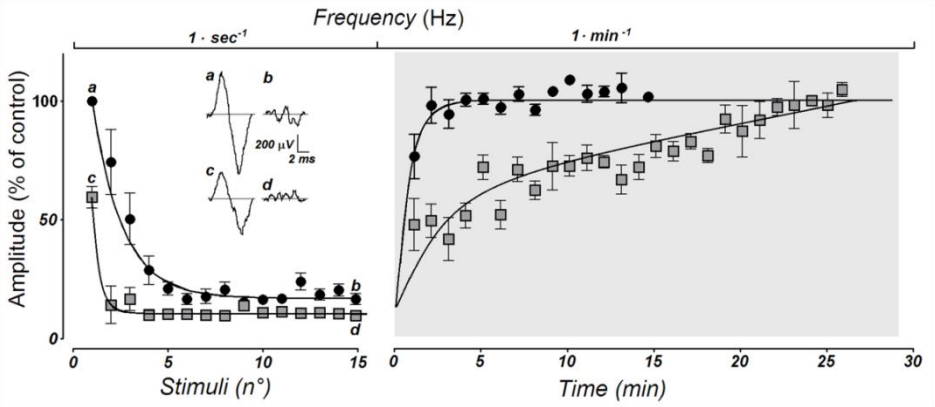


Fig. 16

The response decrement can be greater and faster using more consecutive stimulation trials. The depression of the MVR becomes greater and faster using 10 consecutive trials of 15 stimuli (50 µs, 1.2 T, 10 Hz). The graph shows the average of 3 preparations. The graph represents a single trial session with the recovery (black circles) and the last of ten trials (in which, each trial followed the previous one after one minute) with the spontaneous recovery (grey square). Inset: a and b are the first and last response of the single trial, c and d the first and the last ones of the last of ten trials. The responses decrements are significantly different using one or ten trials (unpaired t test, p = 0.0399, F test, P 0 0.0137) as are the spontaneous recoveries (unpaired t test, p = 0.0024; F test, P = 0.4955)

25 minutes to return to the control value.

Habituation to one stimulus may generate habituation to other stimuli

In order to fulfil the criterion of stimulus generalization, the habituated levels of MVR_{L3} amplitude have to be reached also when it is exposed to a stimulus that is similar, though not identical, to the original habituating stimulus. An experiment to

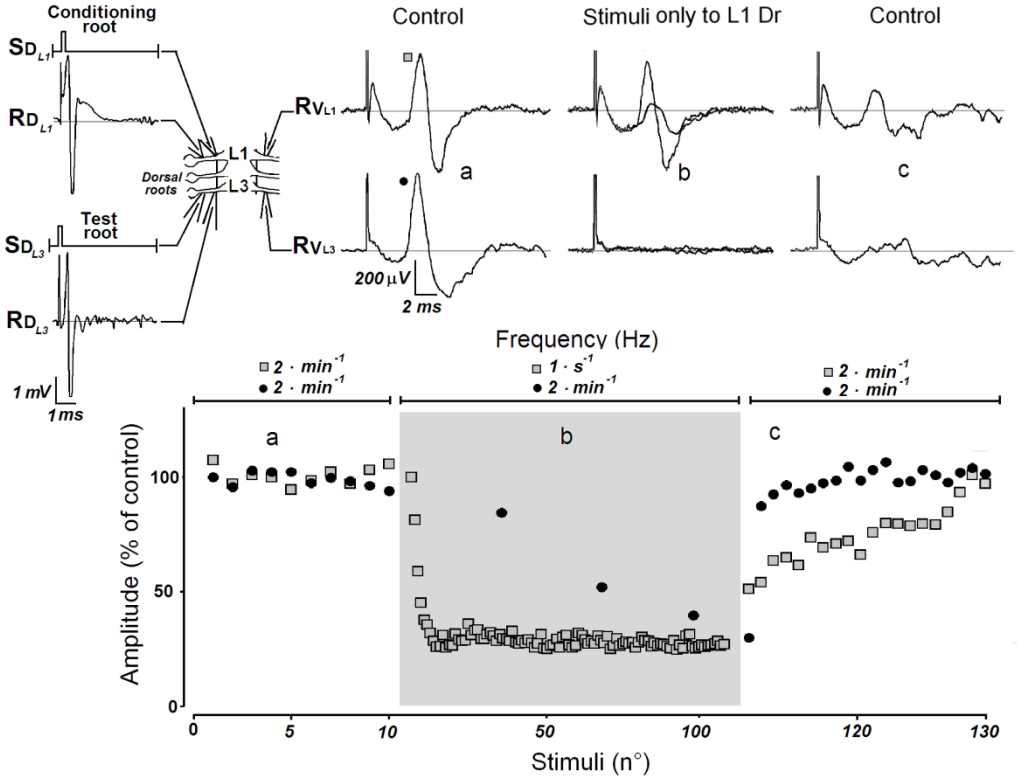


Fig 17

Habituation to one stimulus may generate habituation to other stimuli. The MVR_{L1} (grey squares) and MVR_{L3} black circles) have been recorded during the stimulation of the homologues dorsal root. The experiment on one preparation is shown. On the left of the upper panel, the schematic view of the preparation is presented, with the dorsal roots on the left and the ventral roots on the right. Two electrodes were used for stimulating L1 and L3 dorsal root (SD_{L1} and SD_{L3}) and two others to record the activation of Iα fibers (RD_{L1} and RD_{L3}). The homologues ventral roots have been recorded with the last two electrodes (RV_{L1} and RV_{L3}). After 5 minutes of control recordings from L1 and L3 with test stimuli (50 µs, 1.4 T) every 30 seconds, the stimuli test frequency on $L1_{DR}$ changed to 1 Hz while the frequency of $L3_{DR}$ remained the same of the control recording. After 100 stimuli on $L1_{DR}$, the frequency was returned to the control value in order to measure the spontaneous recovery. The recordings in the upper panel are the control (a), the first and the last responses during the 1 Hz frequency stimulation on L1 (b) and the final control (c).

test this criterion should have to identify two stimuli that would not differ significantly in modality while still being somehow different. Stimulus generalization of habituation across independent inputs is evaluated for the in vitro preparation of the rat spinal cord, testing whether the repeated Ia fibers activation from a different dorsal root (L1) could affect and depress MVR_{L3} evoked by the L3 dorsal root. 5 preparations of 10 days old rats were used. The left part of the upper panel in fig. 17 represents a schematic view of the preparation, with the dorsal roots on the left side and the ventral roots on the right one. Two electrodes were used for stimulating L1 and L3 dorsal root (SD_{L1} and SD_{L3}) and two others to record the activation of Ia fibers (RD_{L1} and RD_{L3}). The response of the homologues ventral roots has been recorded with the last two electrodes (RV_{L1} and RV_{L3}). After 5 minutes of control recording from L1 and L3 with test stimuli (50 μs, 1.4 T), the test stimulus frequency on $L1_{DR}$ was changed to 1 Hz while the stimulation frequency for $L3_{DR}$ remained the same of the control recording. After 100 stimuli on $L1_{DR}$, the frequency returned to the control value to monitor the recovery of the response amplitude. Fig. 17 shows the amplitude of MVR_{L1} (grey squares) and MVR_{L3} (black circles) during the entire experiment. The responses are stable during the control phase (a in the upper and in the lower panel of fig.17) but soon after the stimulus frequency was changed on L1 dorsal root, the MVR_{L1} was depressed and the monitoring of MVR_{L3} showed a decrease in response as well (b in fig. 17). The response depression of L3 was slower but it continued until the end of the stimulation on L1 dorsal root. When the frequency was brought back to the control recording, the MVR_{L1} recovered to the control amplitude in ten minutes while MVR_{L3} recovered in about 5 minutes. Note that MVR_{L3} first response of the final control was lower than the one of MVR_{L1} value, while the second one reached almost the control value.

Age on habituation process

The depression of the monosynaptic ventral root response is affected not only by the stimulus features but also by the age of the animals. Some experiments are performed using the same protocol (15 TS of 50 μs, 1.4 T, 1 Hz) with preparations of

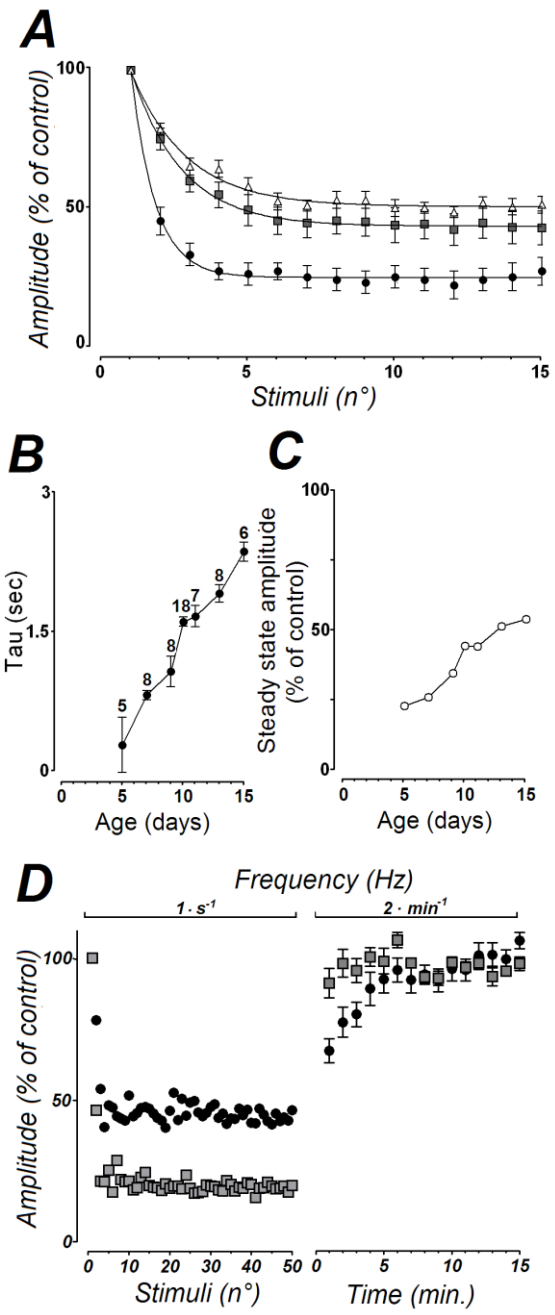


Fig. 18

Animal age affects reflex depression and its recovery. The depression of MVR is affected by the age of the animals. The experiments were performed on 60 rats between 5 and 15 days old. **A)** Trials with 15 stimuli (50 μs, 1.4 T, 1 Hz) have been delivered to L3 dorsal root of 5 days old (black circles n=5), 10 days old (grey squares, n= 18) and 15 days old rats (white triangles, n=6). The data population are significantly different (one way ANOVA, $p<0.0001$) **B)** the τ has been calculated in s for seven different ages (5, 7, 9, 10, 11, 13, 15 days old), using the equation [1] (see materials and methods). It is plotted as a function of age, express in days. **C)** The plateau level amplitude of MVR has been calculated as previously described for the τ and plotted as a function of the age. **D)** After 50 stimuli (50 μs, 1.4 T, 1 Hz) on a 6 day old rat spinal cord (white triangles) and 11 days old rat spinal cord (black circles), the frequency of the stimulus changes to 2*min-1 in order to evaluate the spontaneous recovery. The recovery time seems to be affected by the different age of the preparations (unpaired t test, $p < 0.035$, F test to evaluate variance, $p = 0.4490$).

different animal ages. The reflex depression in *in vitro* spinal cord of 7 days old rats (black circles, 8 preparations), 11 days old rats (grey squares, 18 preparations) and 15 days old rats (white triangles, 8 preparations) are shown in fig. 18A. Increasing the animal age, the depression became slower and it reached a higher plateau-like level. Moreover the run down phase and the plateau level amplitude are more different between 5 and 10 days old preparations than between 10 and 15 days old ones. In fig 18B and 18C, the graphs show the tau and the plateau level of MVR, calculated for seven different ages, using the equation in [1], as a function of the animal age, expressed in days. Increasing the animal age, the run down phase become slower (the response to the second stimulus was smaller in the 7 days old rats than in 10 or 15 days old rats) as well as the plateau level, which reached 75% of the control in the 7 days old rats and 45% of the control in the 15 days old rats.

Dishabituation and habituation of dishabituation

The stimulus test features and the animal age could change the reflex depression and the spontaneous recovery. In contrast to the reversal of habituation due to spontaneous recovery, which is by definition spontaneously generated, the reversal of habituation via the application of a novel, dishabituating stimulus, is called dishabituation. The recovery of the response can be faster if another stimulus, different from the TS is used simultaneously. Some experiments are performed using the electrodes arrangement of fig 19A. After ten minutes of control recording, 30 test stimuli (50 μs, 1.4 T, 1 Hz) were delivered to L3 dorsal root (DR_{L3}). Simultaneous to the 15^{th} test stimulus on L3 dorsal root, a brief train of 0.5 s (conditioning stimulus, CS) of stimuli with the same pulse duration and amplitude but much higher frequency (70 Hz) was delivered to L3 dorsal root (black triangle in fig. 8C). The MVR_{L3} did not change during the control recording and started to depress with the firsts 15 test stimuli (a and b in fig. 19C). Soon after the end of the CS, the MVR immediately recovered and exceeded the control value (c in 19C).Then it started to decrease again (d in fig 19C). When the stimulus frequency returned to the control value, the MVR spontaneously recovered in about one minute. The responses were greatly

potentiated for a brief period after the CS and the amplitude exceeded the control value by about 50%. The CS elicited an instantaneous increase of the response but changed the kinetic of the following reflex depression. Indeed the MVR reached the plateau level with a number of stimuli greater than before the CS. Therefore the effect of the CS is to change the τ of the depression curve of the response (before CS, τ = 2.2 s; after CS, τ = 3.6 s), reducing the depression development. Moreover the CS affects the reflex spontaneous recovery. The amplitude of the reflex reached the control value soon after one minute of the stimulation change in frequency while

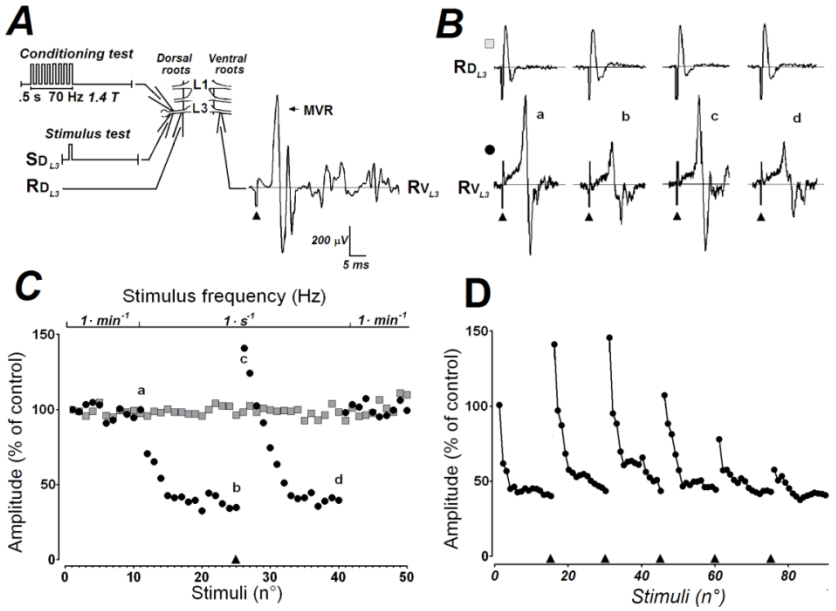


Fig. 19

Dishabituation and habituation of dishabituation in spinal cord in vitro. The figure shows the experiment in one preparation of 11 days old rats spinal cord. **A)** Schematic view of the preparation, with the dorsal roots on the left and the ventral roots on the right. Three electrodes are placed on the L3 dorsal root to stimulate (SD_{L3}) and to record (RD_{L3}) the activation of Aα fibers, using low stimuli (50 µs, 1.4 T). The third electrode has been used to deliver the conditioning stimulus (CS, train of 0.5 s of stimuli of 50 µs, 1.4 T, 70 Hz). The last electrode records the homologue ventral root response (RV_{L3}). **B)** examples of dorsal root fibers activation ((RD_{L3}) and homologue ventral root responses (VR_{L3}) during the experiment: a) first stimulus at 1 Hz, b) last stimulus at 1 Hz before the CS, c) first stimulus after the CS, d) last stimulus at 1 Hz; **C)** the activation of dorsal root fibers (grey squares) and the homologue MVR (black circles) throughout the experiment. The small letters refers to the figure in panel B. CS is indicated by the black triangle, close to the x axis. The first ten stimuli and the last ten are the control recordings at 1 * min^{-1}. The dorsal root activation was stable during the experiment while the MVR was depressed when the frequency was changed to 1 Hz. Soon after the CS delivery, MVR recovered instantaneously and decreased again. **D)** The effect of the CS was attenuated when the CS was repeated. A CS was delivered every 15 test stimuli. The responses are greatly potentiated for a brief period, but after a while the effect of the CS was attenuated or habituated.

with 30 TS without the CS the recovery was longer (grey squares in fig. 13).

The recovery effects of the CS decrease and disappear if it is repeated. Fig. 19D shows the repetition of the CS on the L3 dorsal root every 15 stimuli test. The response was potentiated for a brief period after each CS and then the MVR quickly reached the plateau level. The reflex amplitude recovered soon after the CS presentation and then it started to become weak again. The potentiation effect could be seen throughout the experiment, although the MVR_{L3} recovery became smaller for each CS repetition after the third.

SENSITIZATION

Dishabituation and sensitization

Sensitization refers to a general facilitation produced by strong or noxious stimulus that enhances subsequent responding. Dishabituation has been thought to present a special instance of sensitization in which the facilitation is simply superimposed on a habituated response level. In order to see whether the in vitro spinal cord shows not only dishabituation but also sensitization, a high voltage (30 T), high frequency (70 Hz) train of 0,5 s (conditioning stimulus, CS) has been delivered to the L1 dorsal root (SD_{L1} in fig. 20A) to elicit the activation of the Aδ and C fibres while TS were given to L3 dorsal root to elicit the monosynaptic response in L3 ventral root (RV_{L3} in fig. 20A, see fig. 20A and its caption for electrodes arrangements). The general state of arousal, in which the network stays after a specific meaningful stimulus (the activation of the pain fibres), should have to increase the response,

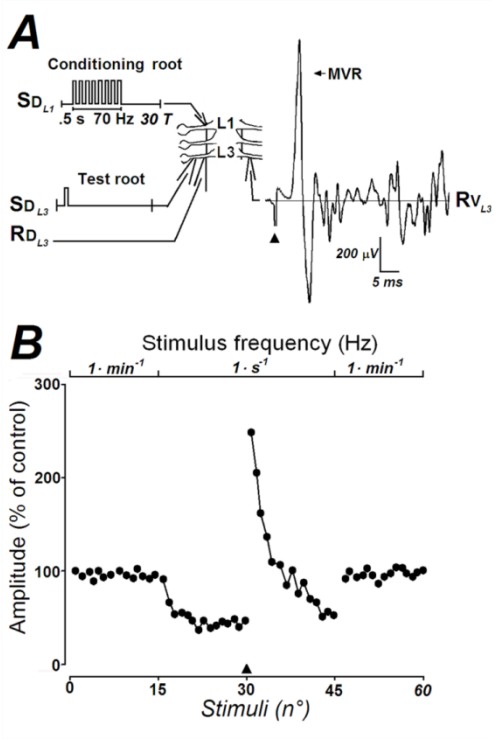


Fig 20

Sensitization of MVR. Using a high voltage, high frequency train to evoke the activation of the L1 dorsal root Aδ and C fibers, the recovery of MVR_{L3} is instantaneous and exceeded more than twice the control value. The figure shows data from 1 preparation of an 11 days old rat spinal cord (same preparation of the experiment in fig. 8) **A)** Electrodes arrangement for sensitization experiments in which one electrode was placed on the L1 dorsal root to activate all the root fibers (SD_{L1}), using a train (0.5 s,) of stimuli (500 µs, 30 T, 70 Hz). Two electrodes were positioned on the L3 dorsal root to stimulate and record the fibers activation. Another electrode was on L3 ventral root (RV_{L3}) to record the ventral response. The trace on RV_{L3} is the first response of the ventral root after the CS. Note the activation of the polysynaptic components. **B)** The graph shows the MVR_{L3} amplitude (black circles) during the experiment. After 15 minutes of control recording ($1*min^{-1}$), 30 test stimuli were delivered at 1 Hz. Then the frequency came back to the control value. At the 15^{th} test stimulus on L3 dorsal root, a CS was delivered to L1 dorsal root (black triangle).

independently from the root used to deliver the conditioning or the test stimulus. In the experiment in fig. 20B, the MVR was elicited with test stimuli and it showed the usual depression process until the CS (black triangle in fig. 20B). After that, the MVR_{L3} recovered instantaneously and exceeded more than twice the control value. Furthermore the depression after the CS was slower than before and it did not reach the plateau level. Lastly the recovery was faster than the experiment in fig. 11 when the stimulus frequency decreased to the control value.

Intensity and frequency of the CS on MVR sensitization

The intensity and the frequency of the test stimulus affect the rate of MVR_{L3} habituation, both the run down phase and the final plateau level. Some experiments have been performed in order to verify if the intensity or the frequency of the CS could affect the MVR sensitization as well. Fig. 21 shows the MVR sensitization using CS with the same pulse duration and frequency but with different intensities. As in fig 20, L1 dorsal root was the conditioning root and the L3 was the test root. 45 TS were delivered to the L3 dorsal root and a CS was given to L1 every 15 TS. Without a CS

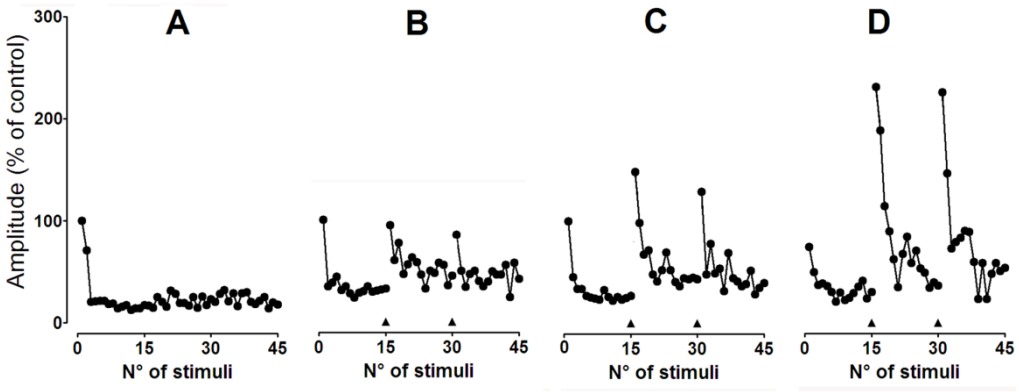


Fig 21

Effects of increasing CS intensity on MVR_{L3} sensitization. The recovery rate of MVR_{L3} is affected by the conditioning stimulus intensity. Using the same electrodes arrangement as fig. 9, MVR_{L3} recovery was evaluated, after a CS of increasing intensity (black triangles). All intensities have been delivered to the same preparation of an 11 days old rat, in a random order. At least 15 minutes were allowed to pass between trails with different CS intensities. **A)** MVR_{L3} depression without the CS; **B)** MVR_{L3} depression and facilitation with 5T CS intensity; **C)** MVR_{L3} depression, using 10 T CS intensity; **D)** MVR_{L3} depression and sensitization with a CS of 30 T.

(fig. 21A) the habituation of the reflex was complete and continued until the end of the stimulation. When the CS was delivered at 5 T (fig. 21B), the response increased to the control value and then decreased again. Using the 20 T intensity (fig. 21C), the CS evoked an augmenting of the reflex but using a CS intensity of 30 T (fig. 21D), the reflex exceeded the control value more than twice. Usually intensities between 10 and 30 T are accepted to be a noxious stimulation (Vinay et all., 2001). The MVR recovery reached higher value as the CS intensity increased. Further voltages of 30 T did not increase more the reflex recovery amplitude. It is important to note that the MVR needs more stimuli to reach the plateau level after the noxious stimulation as the CS intensity increases.

Also the CS frequency affected the MVR recovery. Using conditioning trains of increasing frequency, the response of the ventral root became greater as the frequency enhanced (fig. 22). The CS effects had a peak at 70 Hz and then the amplitude of the reflex did not further enhance, rather the recovery become smaller

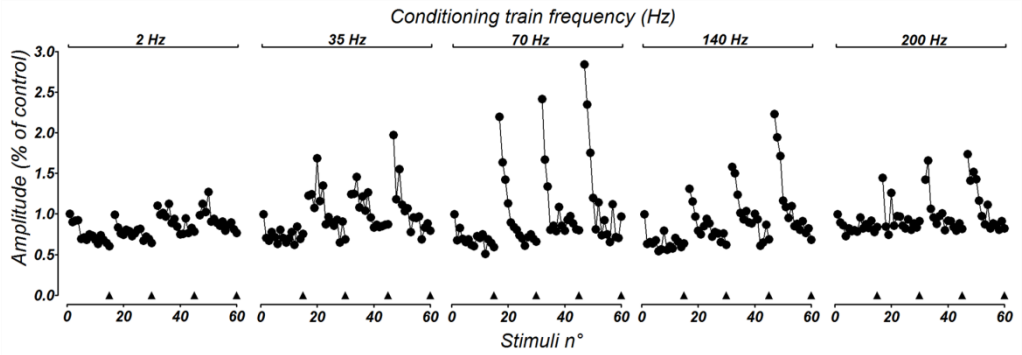


Fig. 22

The rate of recovery of MVR_{L3} is affected by the conditioning stimulus frequency. Using the same electrodes arrangement as fig. 9, MVR_{L3} recovery was evaluated, after a CS (train of 0.5 s of stimuli of 500 μs, 30 T,) of increasing frequency (black triangles). The frequency of the CS is indicated in the upper x axis. All frequencies have been delivered to the same preparation of a 11 days old rat, in a random order. At least 15 minutes were allowed to pass between trails with different CS frequencies.

than before (at least with the number of stimuli used in the experiment). The effect of the conditioning train affected not only the responses after the first three or four stimuli, but the plateau level as well. The CS frequency between 35 Hz and 140 Hz extended the number of the stimuli to reach the plateau level.

Intensity and frequency of the TS on the MVR sensitization

Some experiments have been performed with the different TS intensities or frequencies in order to see whether the recovery of the monosynaptic reflex after

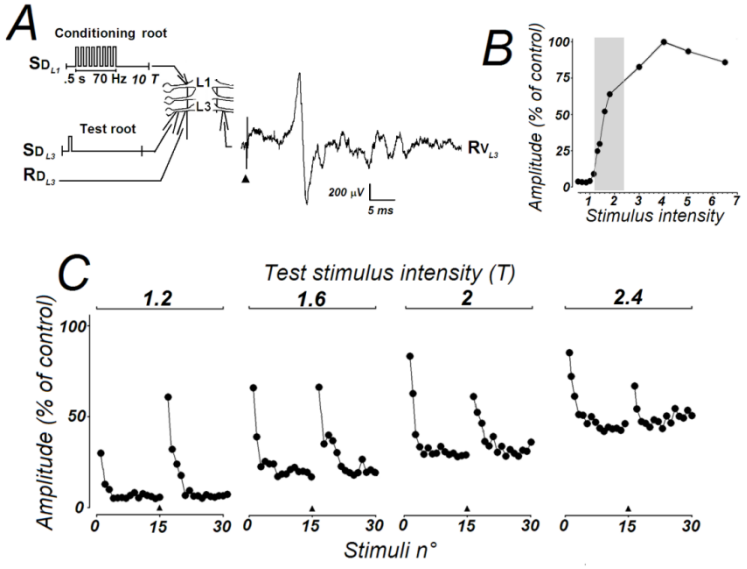


Fig 23

Effects of increasing test stimulus intensity on MVR sensitization. Using the same CS, the test stimulus intensity affects the recovery rate of the response. L3 dorsal root has been stimulated using stimuli with the same pulse duration and frequency (50 µs, 2 Hz) but different intensities. Data are from one preparation of a 12 days old rat. The different intensities have been delivered in a random order and at least 10 minutes passed between one trials and the following. **A)** Electrodes arrangement used in experiments: one electrode was placed on L1 dorsal root to activate all the root fibers (SD_{L1}), using a train (0.5 s,) of stimuli (500 µs, 10 T, 70 Hz); two electrodes were placed on the L3 dorsal root to stimulate and record the fibers activation; the last electrode is on L3 ventral root (RV_{L3}) to record the homologue ventral response. The trace from RV_{L3} is the first response of the ventral root after the CS. Note the activation of the polysynaptic components. **B)** Stimulus/ response graph for the preparation. The points in the grey zone are the intensities used for the experiment. The response activation curve shows the sensitivity of the reflex at intensities between 1 and 2 T. Further increasing of the voltage affects MVR amplitude less than low intensity; **C)** Trials with different TS intensities. All of the responses have been compared to the maximum reflex response, obtained with 4 T intensity stimulation. The CS delivery is indicates by the black triangle in the x axis. The plateau level becomes higher when the TS intensity is increased. MVR recovery, after the conditioning stimulus, became smaller as the intensity increased.

the sensitization could be affected not only by the CS but also by test stimulus features. Using the electrodes arrangement shown in fig 23A, the effect of different intensities of the test stimulus have been tasted. Fig. 23B shows the stimuli/responses curve for the *in vitro* spinal cord used for this experiment. The response activation curve shows that the reflex amplitude increased quickly when the stimulus intensities were between 1 and 2 T and less when the intensity was higher than 3 T. the L3 dorsal root has been stimulated using TS with the same pulse

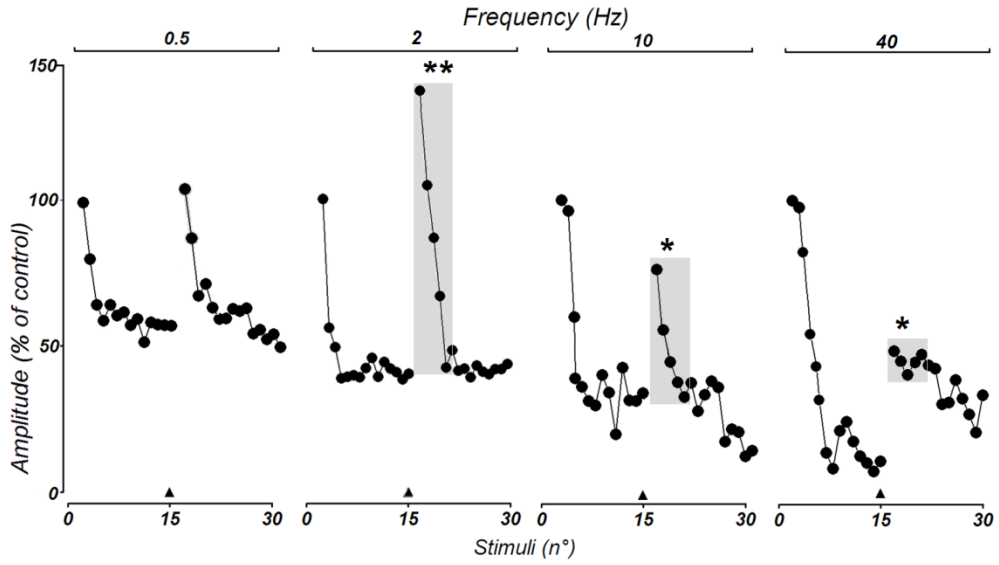


Fig. 24

Effects of frequency test stimulus on MVR sensitization. Using the same CS, the test stimulus frequency affects the recovery rate of the response. Test stimuli with the same pulse duration and intensity (50 µs, 1.4 T) but different frequencies have been delivered to the L3 dorsal root. Data are from one preparation of 12 days old rat. The different frequencies have been delivered in a random order and at least 10 minutes passed between one trials and the following. The CS delivery is indicated by the black triangles in the x axis. Note that the higher the frequency, the higher the plateau level reached by the depression while the greatest recovery has been obtained using 2 Hz test stimulus frequency. The first five responses at 2 Hz, 10 Hz and 40 Hz) after every CS have been used to compare the amplitude of the responses with one way ANOVA test (P = 0.00251).

duration and frequency but different intensities (fig. 23C). All of the responses have been compared to the maximum reflex response, obtained at 4 T (see the graph Stimulus/response in fig. 23B). The amplitude of the recovery after the CS was the

same for all of the intensities tested when they were compared to the maximum amplitude reached at 4 T. If the amplitude of each intensity was compared to the control of that intensity (and not with the maximum response at 4 T), the highest recovery (comparing the responses at one intensity to the first response at that intensity) would have been obtained at the lowest intensity, in which the response after the CS exceeded the control value more than twice (fig. 23C, first graph on the left).

Using the same CS, the test stimulus frequency affected the recovery rate of the response (fig. 24). The L3 dorsal root has been stimulated using TS with the same pulse duration and intensity (50 μs, 1.4 T) but different frequency. The greatest response after the CS (black triangles in fig. 13) has been obtained with a stimulus frequency of 2 Hz. Frequencies higher than 2 Hz elicited a lower recovery as the frequency was increased.

Flexor conditioning root effects on an extensor response

Sometimes the MVR, instead of increasing, showed a higher depression, under the plateau level after the CS. This effect has been seen all the times that an extensor root has been used as test root and the CS has been applied to a flexor root. At

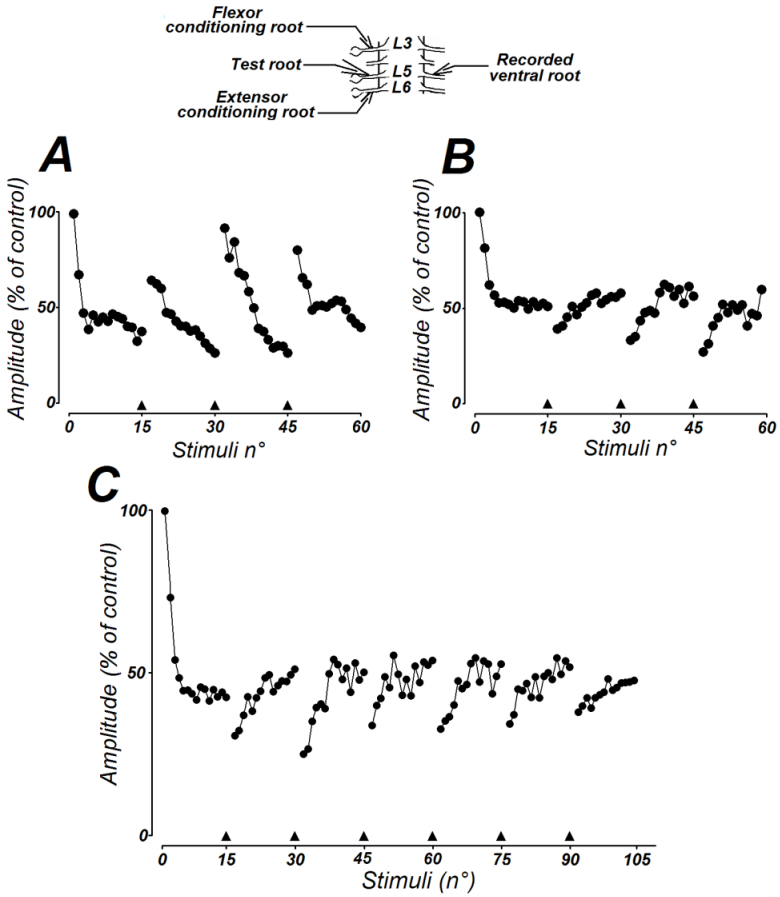


Fig. 25

Flexor and extensor conditioning root on extensor root. When the CS was delivered to an extensor root (L6), MVR_{L5} immediately exceeded the control value and then decreased. When the CS was given to a flexor root, L3, the L5 reflex amplitude did not increase, but rather it decreased under the plateau level and then came back to the plateau level value during the rest of the stimulation. A preparation of 9 days old rat has been used. The L5 segment has been used as test root and a CS (train of 0,5 s composed of stimuli of 500 µs, 15 T, 70 Hz, black triangles in graphs) was delivered or to L6 dorsal root or to L3 dorsal root (upper panel). **A)** Effects of four CS delivered to L6 dorsal root every 15 s on MVR_{L5}. Note the continuous increasing of the response amplitude during the repetition of the CS; **B)** Effects on MVR_{L5} of a CS given on L3 dorsal root. The amplitude of the reflex decreased instantaneously just after the CS and it returned to the plateau value after a while; **C)** Using a CS (0, 5 s, 500 µs, 70 Hz) with a lower intensity (8T), the sensitization effect on MVR_{L5} was attenuated with the repetition of the CS, even though it was present until the end of the stimulation.

lumbar level in the rat, L1, L2, L3 are considered flexor roots because most of the axons in the root, innervate flexor muscles while L5 and L6 are considered extensor roots. The L4 segment is comprised of both extensor and flexor axons in the same proportion. Fig. 25 shows an experiment in which the L5 segment (extensor root) is the test root while the conditioning roots are L6 (extensor root) and L3 (flexor root). A typical sensitization-induced rise of MVR_{L5} amplitude was elicited when the CS was given to L6 (extensor) dorsal root (fig. 25A). When the CS was delivered to L3, the depression of MVR_{L5} did not recover but, rather, it showed an additional depression (fig. 25B). Soon after the additional depression evoked by the CS, the response increased in amplitude up to reach the plateau-like level. The further repetition of the CS elicited an

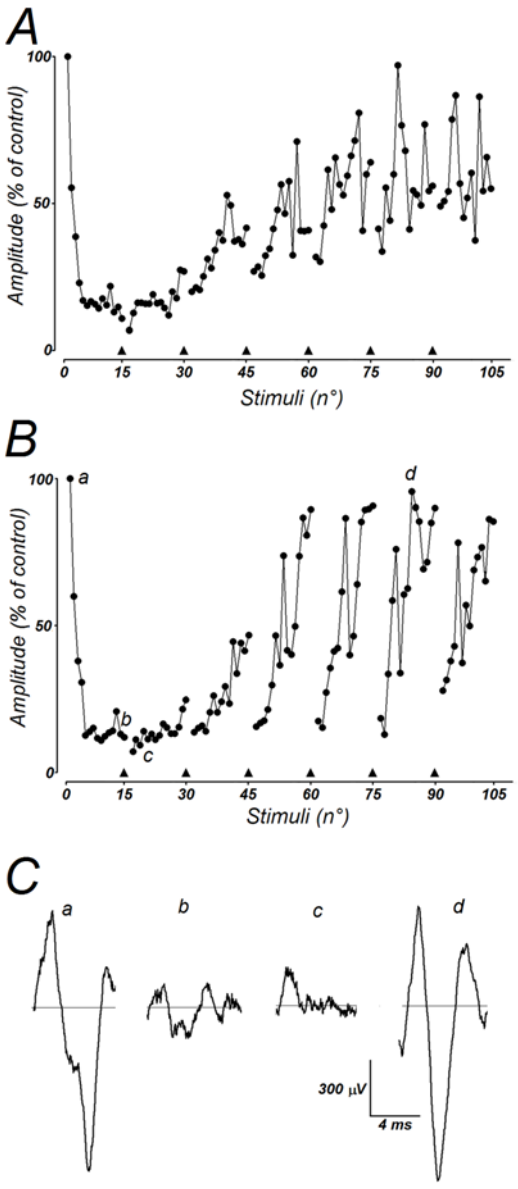


Fig. 26

CS intensity from a flexor root affects the MVR of an extensor root. Using L3 to deliver the CS, MVR_{L5} was further depressed by the CS. A preparation of 8 days old rat has been used for the experiment. **A)** Trial of 105 test stimuli (50 µs, 1.2 T, 10 Hz) to L5, using Cs (0. 5 s, 500 µs, 70 Hz) on L3 with an intensity of 20 T; **B)** trial of 105 stimuli (same of panel A) to L5, using a CS of 30 T on L3. Note the reflex rise after the CS. It became faster and stepper, using the higher CS intensity than the lower. **C)** MVR_{L5} is shown during the sensitization protocol in B: a) first response; b) last response before the CS; c) first response after the CS; d) maximum response of the reflex, reached after 5 repeated CS. The small letters are indicated also in the graph of panel;

additional depression of the reflex, which always recovered to the plateau value .

The response depression, induced by the CS, showed the same characteristics of the usual dishabituation, the habituation of dishabituation (fig. 25C). When a CS (0.5 s, 500 μs, 70 Hz) at 8 T intensity (lower than that one used in experiment in fig. 25B) was used, the sensitization effect on MVR_{L5} decreased with the repetition of the CS. The sensitization effect (which elicited a higher depression) had a peak after the third CS and then the additional depression evoked by the CS became less intense as the stimulation continued, although it was present until the end of the experiment.

As the intensity of the CS affects the recovery rate of the extensor induced sensitization on L5, the intensity of the CS on a flexor root affected the response of the extensor monosynaptic response (fig. 26A and B). Increasing the CS intensity, the responses of the ventral root usually become greater and greater. When a CS of 20 T of intensity (fig 26A) was delivered, the response decreased in amplitude soon after the first CS and then increase to the plateau level. Soon after the second CS, the reflex amplitude was depressed again but then increased and exceeded the plateau level, reaching the control amplitude with the next conditioning stimuli. When the CS intensity was farther increased to 30 T (fig. 26B), the response showed the same behaviour as before, but this time it reached the control amplitude faster than before. When a CS was delivered to a flexor root, the rise in the reflex amplitude was delayed and the sensitization effect lasted until the presentation of the next CS. It can be seen in fig. 26B at the end of the trial, when the last CS was given. The amplitude of the value before the CS was close to 100% of the control but soon after the CS, the first response was the 20% of the control. It is unlikely that the response amplitude could reach the plateaus level with only one stimulus test after the CS. It suggests that the sensitization effect of the CS was present also at the last presentation and its action was as strong as at the beginning of the experiment. Most likely the sensitization effect of one CS did not disappear before the presentation of the next one and the effect of the previous CS was added to the action of the next one.

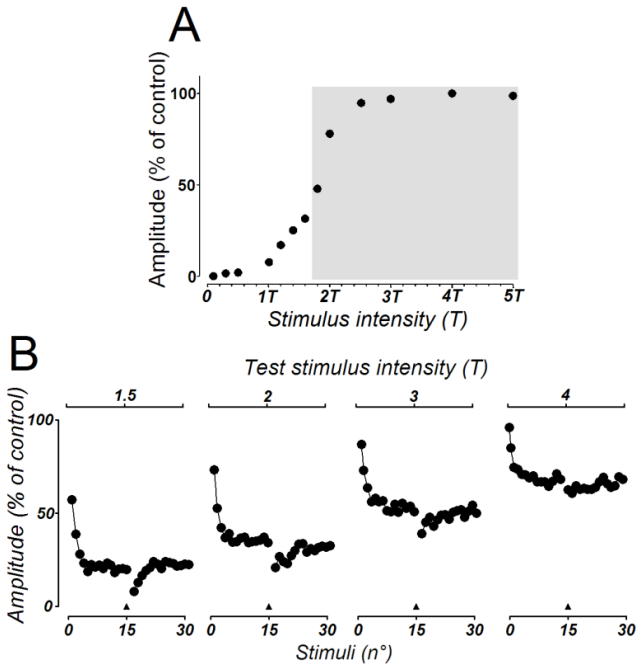


Fig. 27

Test stimulus intensity effect on the sensitization induced by a flexor root. The added depression elicited by the CS became smaller and smaller, with increasing TS intensity. A) Stimulus/response relationship of the preparation used for the experiment. The grey area indicates the test stimulus intensity used for the experiment in panel B; B) trials with TS (50 µs, 1 Hz) of increasing intensity are used. The test stimulus intensities used in each trial are shown in the upper x panel. Increasing the intensity, the reflex was depressed by the first 15 test stimuli and then, after the CS (0, 5 s, 500 µs, 70 Hz, 10 T), a further depression appeared. The effects of sensitization disappeared and the reflex did not show any additional sensitization effect when a stimulus intensity of 4 T was used. Until 3 T intensity, the test stimulus should activate only Ia fibers, but further increasing of the voltage starts to activate different fibers (most probably tactile).

The features of a TS on a flexor root affect the MVR recovery when the CS is given to a flexor root (fig. 23 and 24, stimulus test intensity and frequency, respectively). When a CS is applied to a flexor root, likely the TS intensity can modify the response of the extensor root. Fig. 27A shows the stimulus/response relationship of the preparation used for the experiment in fig. 27B. The stimulation intensities in the experiment are able to activate only the monosynaptic reflex (the lower ones) and tactile fibers and, maybe, fast pain fibres (the highest one). The MVR was depressed by the repeated test stimuli soon after the trials beginning and the depression increased in amplitude when the Cs was applied (fig. 27B, experiment at 1.5 T). Increasing the intensity of the TS the sensitization induced depression became

smaller and smaller (fig. 27B, experiments at 2 and 3 T). When the TS reached a value higher than the one necessary to activate only Ia fibers, the further depression after the CS could not be seen anymore (fig. 27B, experiment at 4 T). At the highest intensity shown, it seems that sensitization did not affect the response. Probably the intensity used (4 T) started to activate different fibres, such as tactile and fast pain fibres. The sensitization action of the CS (which was applied to a flexor root) could be balanced by the afferent information of the test root (which is an extensor root). The outcome of the motoneurons of the root suggests that the its own test root) reached a balance in which the response did not change its behaviour, compared to the amplitude before the CS.

Comparing flexor and extensor induced sensitization

The possibility to affect differently the monosynaptic reflex of one segment, depending by on the relationship between the test and the conditioning roots and by

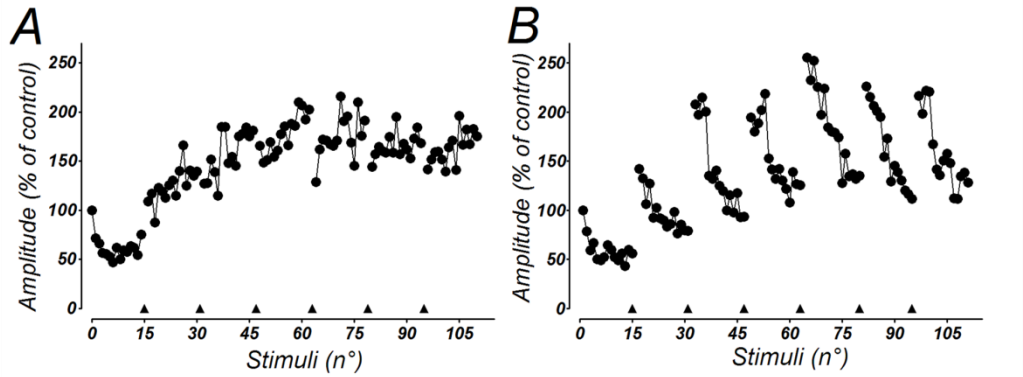


Fig. 28

Maximum rise of MVR_{L5} after a CS on L6 or L3. 13 experiments have been performed in order to compare the greatest increase in amplitude of the reflex, using a CS from a flexor and from an extensor conditioning root. The two different trials have been performed on the same spinal cord with 20 minutes of rest in between. Further increasing of CS intensity did not result in an additional increase or decrease in the reflex amplitude. The order of conditioning root stimulation (first the flexor and then the extensor one, or the opposite) has been inverted in every experiment. **A)** Trial with a CS (0, 5 s, 500 µs, 30 T, 70 Hz) to L3 dorsal root. **B)** Trial with a CS (0, 5 s, 500 µs, 30 T, 70 Hz) to L6 dorsal root. Both of the conditioning roots were able to elicit the same recovery after the CS. Note that the flexor sensitization reached the maximum amplitude close to the next CS while the extensor sensitization reached the maximum amplitude soon after the CS and then decreased. The effects of the CS are present not only in the run down phase but also in the plateau level.

changing the features of the test stimulus and the conditioning stimulus, suggested that the monosynaptic reflex could be evoked only by the activation of the Ia afferent of the homologue dorsal root (test root) but it could be shaped by different fibers coming from another part of the nervous system. Habituation and sensitization protocol have been tested using different roots (from T10 to S2) and have been found in all the tested roots, although they showed different features such as a different number of the test stimuli to reach the plateau level or a different plateau level using stimuli with the same features.

In order to see if a conditioning root could elicit a greater response than the other ones, 13 experiments on rats between 9 and 12 days old have been performed, using L5 as the test root and L3 and L6 as the conditioning root (fig. 28). In fig. 28A the CS has been delivered to L3 dorsal root while in fig 28B the conditioning root was an extensor. The CS (0, 5 s, 500 μs, 40 T, 70 Hz), used during the experiment, elicited the greatest response and further increasing of CS intensity, frequency or duration did not further enhance the MVR_{L5}. The responses elicited during the experiments with both flexor or extensor roots were able to augment the MVR amplitude to the same degree. The amplitude of the reflex became greater until its value reached 250% the control value. The difference between the flexor and extensor conditioning stimulation was given by the kinetic of the response amplitude rise. When the CS was delivered on an extensor (fig. 28B), the response increase quickly and then was depressed. When the CS was given by a flexor root (fig. 28A), the response begun to increase after a while and reached the greatest amplitude only right before the next CS. The only difference given by the relationship of the root is the way in which the sensitization process overrides the habituated response. The same experiments have been performed on L3 as the test, and L5 or L1 dorsal root as the conditioning roots. The obtained results have been the same, the CS could be applied to a flexor or an extensor root and it elicited the same increase in amplitude. The data suggest that the reflex amplitude could be sensitized strongly using both extensor and flexor root.

Generalization of sensitization

In contrast to the comparative input-specificity of habituation, a heterosynaptic mechanism allows the facilitatory effect to generalize between input pathways. Indeed facilitation occurs not only in the stimulus/reflex pathway but affects all central pathways (Hagbarth and Kugelberg 1958, Thorpe 1963). In order to see if monosynaptic response in in vitro rat spinal cord shows generalization of sensitization, same experiments have been performed recording L3 and L5 ventral roots (fig. 18). Two stimulating electrodes have been used: one on L3 dorsal root to

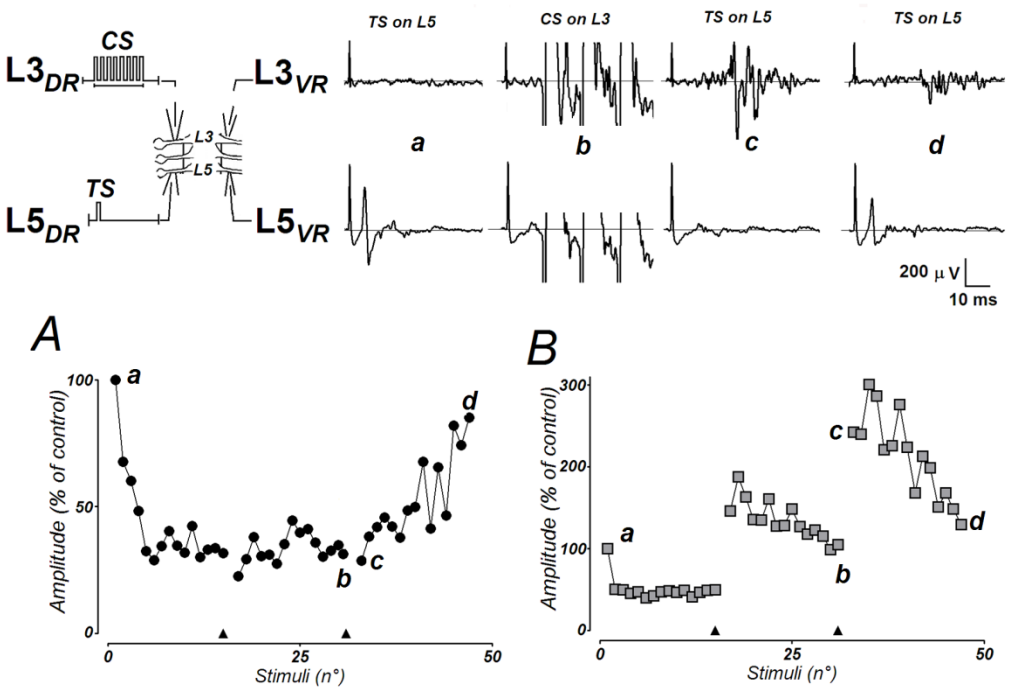


Fig. 29

Generalization of sensitization. Facilitatory effect of sensitization is widespread throughout the NS. The in vitro spinal cord of rat showed generalization of habituation. Upper panel: the electrodes arrangement is showed on the left side. A stimulating electrode ($L3_{DR}$) is on L3 dorsal root to provide the CS (0, 5 s, 500 µs, 30 T, 70 Hz); another stimulating electrode ($L5_{DR}$) is on L5 dorsal root to deliver the TS (1.2 T, 1 Hz). The responses of L3 and L5 ventral roots are recorded with the last two electrodes (respectively $L3_{VR}$ and $L5_{VR}$). Every 15 TS on L5, a CS was delivered on L3 dorsal root. On the right are showed the recordings from L3 and L5 ventral root during the experiment: a)response to the first TS on L5, b) delivery of the CS on L3; c) first response to L5 TS after the CS; d) last response to L5 TS. The letters are the same in the graphs. **A)** MVR on L5 ventral root during the experiment. **B)** Recordings from L3 ventral root all over the experiment. L3 ventral root showed no responses to the first 15 stimuli on L5 dorsal root. When the conditioning train was provided, a long lasting response appeared between 10 and 40 ms after the TS artefact and was depressed by the repetition of the TS. Note that sensitization effect lasted at least 15 s (the time of the stimulation).

deliver the CS and one on L5 dorsal root to deliver the test stimulus. The responses of L3 and L5 ventral roots have been recorded as well (the electrode arrangement is shown on the left side of the upper panel in fig.29). 45 TS at low intensity (1.2 T and 1 Hz) have been delivered to L5 dorsal root. Every 15 TS on L5, a train of 0.5 (40 T, 70 Hz) is delivered to L3 dorsal root in order to activate Aδ and C fibers. In L5 ventral root, the monosynaptic reflex showed depression to the repetition of the TS and facilitation soon after the CS delivery to L3 dorsal root with the usual kinetic for a sensitization induced by mean of a flexor root (fig 29A). L3 ventral root did not show any response during the first 15 TS to L5 dorsal root but soon after the CS to L3 root, a polysynaptic response appeared on L3 ventral root between 10 and 40 ms after the TS artefact, in reply to the TS on L5 dorsal root (fig. 29B). Furthermore the polysynaptic response on L3 ventral root showed habituation and, again, facilitation after the second CS. The stimulation of L5 (it is important to remind that TS activate only the monosynaptic reflex in L5 ventral root and nothing else in L5 and in L3) was able to evoke a polysynaptic response in L3 ventral root after the delivery of the CS, but not before. The facilitatory effect of the CS increases not only the monosynaptic response elicited directly from their Ia fibres. The general state of the system's arousal unmasked the relationship between Ia afferents of a segment and polysynaptic pathways of a different root.

THE DUAL PROCESS THEORY OF LEARNING

Repetitive stimulation often results in habituation of the elicited response. However, if the stimulus is sufficiently strong, habituation may be preceded by transient sensitization or even replaced by enduring sensitization. Thompson and

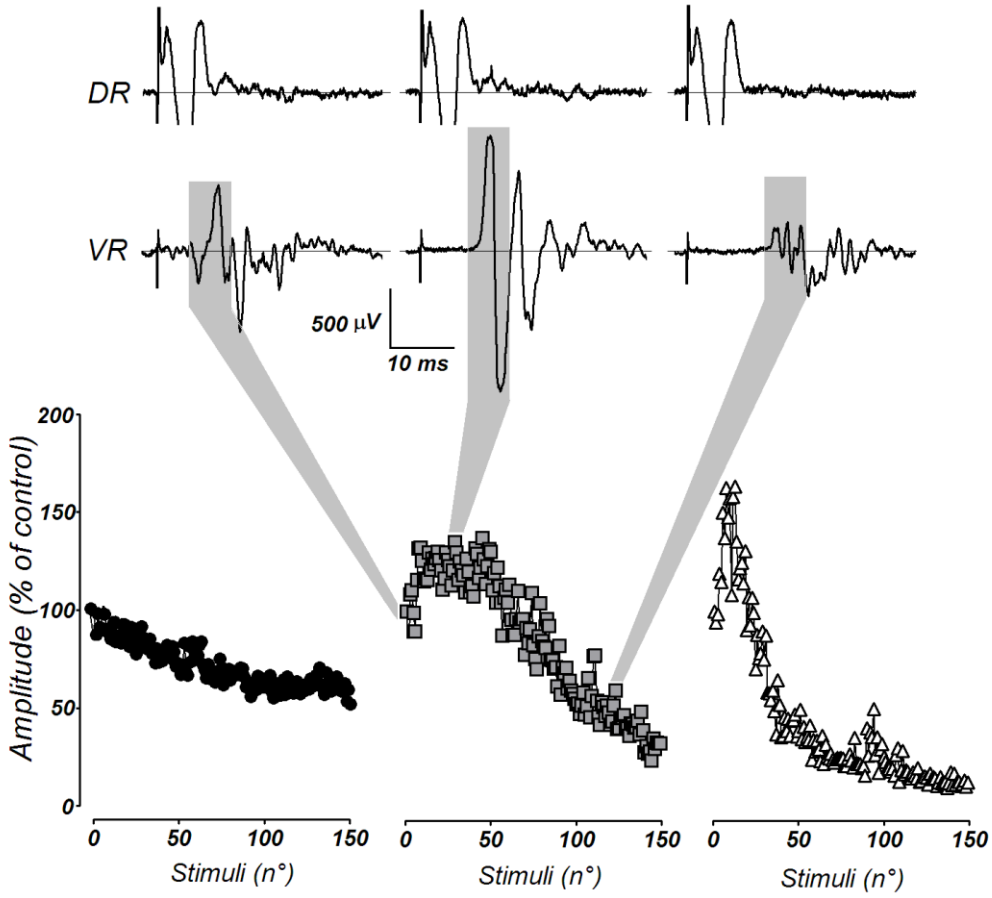


Fig. 30

If the stimulus is sufficiently strong, habituation may be preceded by transient sensitization. When the stimulus test intensity is 5T, the habituation is much slower and the response reaches the plateau like level with a greater number of test stimuli. Therefore a transient sensitization can appear before the usual habituation process when the frequency is increased. When stimulus frequency is 1 Hz (black circles), the response shows a depression process slower than the one in fig 4. When the frequency is 4 Hz (grey squares) the response shows a long lasting sensitization (more than 50 stimuli over the control amplitude) and then the response almost reached the plateau-like level. Lastly, when 10 Hz (white triangles) is used, the sensitization process lasts about only 30 stimuli and then habituation begins and reached the plateau-like level before the end of the trial. In the upper panel recordings of the ventral root (VR) and the dorsal root (DR) of the 4 Hz trial are shown. Note the synchronization of the motorneurones during the sensitization process.

Spencer in 1970 formulated the dual process theory of learning to explain these characteristic behavioural changes on the basis of the competition between decremental plasticity (depression) and incremental plasticity (facilitation) occurring within the neural network[37]. Data from both vertebrate and invertebrate systems indicate that effects of depression and facilitation rather than being mutually exclusive, presumably interact with each other in a complex manner.

In the *in vitro* spinal cord preparation of the rat it is possible to induce both forms of learning processes using different strategies. The simplest way is to use a strong test stimulus which leads to the activation of not only Ia but also the tactile fibres (IIb). Fig. 30 shows an experiment in which a strong test stimulus (5 T) is applied to the L3 dorsal root. When the frequency of the high intensity test stimulus is 1 Hz (150 test stimuli, black circles in the left panel of fig. 30), the response was depressed and reached the same plateau value as the response triggered with only 15 low intensity stimuli, described in fig. 11. The fast run down phase disappeared and the plateau level was reached at the end of the trial. If the stimulation frequency is increased to 4 Hz (grey squares in the middle panel of fig. 30), the response amplitude augmented during the first 50 stimuli and then gradually decreased to reach the same plateau level as described in fig. 15, using low test stimulus at the same frequency. The facilitation process shown in fig. 18 (middle panel), presumably, was also present with the frequency of 1 Hz (left panel of fig. 30) and masked by a stronger habituation. When the frequency was further increased to 10 Hz (white triangles, right panel of fig. 30), the amplitude of the response reached a higher value faster than the one evoked with 4 Hz. At the same time the phenomenon of depression was observed earlier. The top panel of fig. 30 shows the corresponding recordings at 4 Hz. MVR_{L3} (VR in fig. 30A) was faster during the sensitization phase (middle panel) and slower during the depression phase (right panel) compared to the control (left panel). In parallel with this observation the MVR was steeper during the facilitation process, indicating synchronization of motoneuronal discharge, while during the depression phase the discharge was desynchronized. Throughout the experiment the

dorsal root recordings were consistent suggesting that the activation of Ia and IIb fibres did not change.

This experiment showed an additional proof for the theory of Thompson and

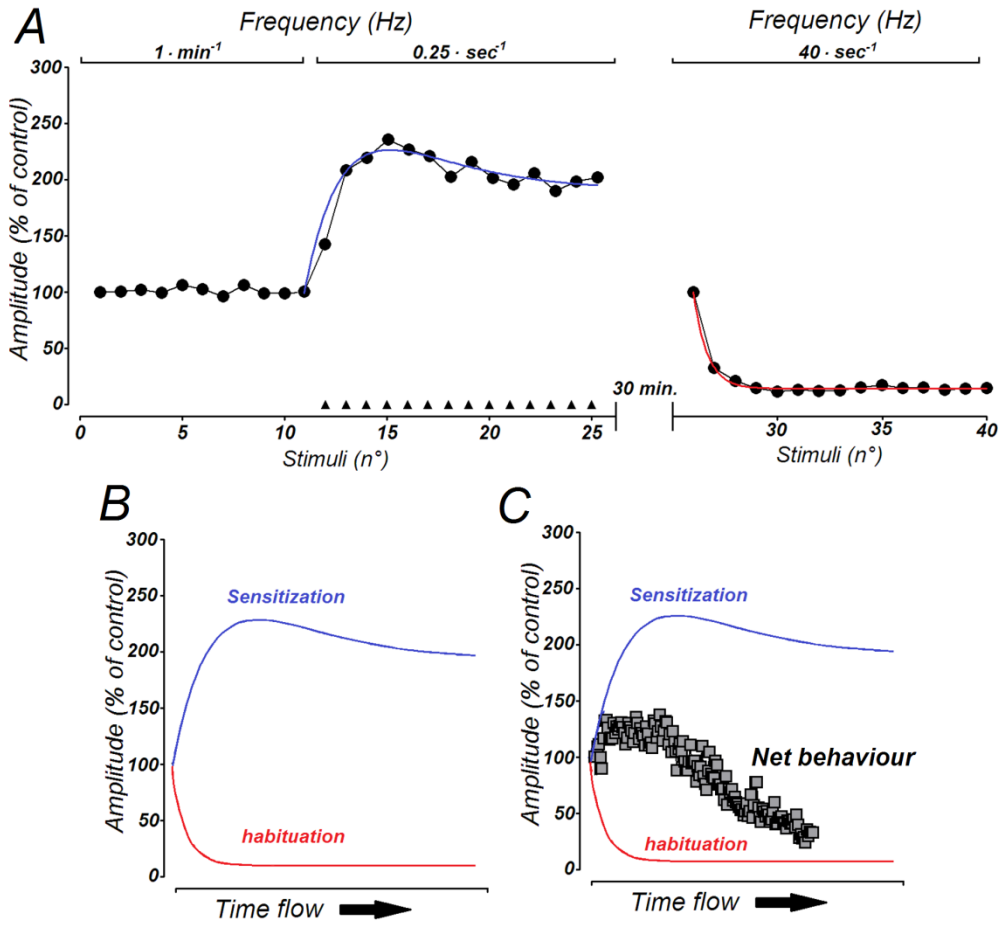


Fig. 31

Pure habituation and pure sensitization processes. In order to see the maximum response amplitude that could be reached with the sensitization process and the lowest level reached with the habituation process, the same spinal cord of fig. 21 experiment has been used. L3 segment is the test root and L1 the conditioning root. A) After ten minutes of control recording (50 µs, 1.2 T), a CS (0, 5 s, 500 µs, 30 T, 70 Hz, black triangles) is delivered to L1 dorsal root every 7.5 s , followed by only one stimulus test on L3 dorsal root. After 30 minutes of rest, in order not to add sensitization effects on habituation process, 15 test stimuli are given to L3 dorsal root at 40 Hz. B) Pure sensitization (blue line) and habituation (red line) process curves. The responses in A) are fitted using equation [1] for habituation process and [2] for sensitization process (see material and methods). C) Modifying test stimulus parameters, the two opposing processes, depression and facilitation, compete to determine the final behavioural outcome.

colleagues (1966) that two antagonist processes, such as depression and facilitation, induced by series of stimuli, compete to determine the final behavioural outcome. This example indicated that the balance between the two processes is set by the intensity of the stimulus. In order to determine the relative contribution played by sensitization in the complex behavioural context, the changes in stimulation frequency were used to uncover the facilitation process and to define the corresponding changes in plasticity both at the level of network and behaviour.

In order to understand if it is possible to separate the two processes, whose rate is modified by the stimulus parameters, some experiments have been performed. Stimulus parameters which increase at the greatest level both habituation (lowest intensity to evoke the smaller response and the frequency in which the response shows the greater habituation degree) and sensitization (high intensity high frequency stimulation on a root and low stimulus and very low frequency for the test stimulus) have been used. This strategy was aimed to have “pure” sensitization process (sensitization without a previous superimposed habituation process, only one test stimulus to see the amplitude of the reflex after every CS) and a “pure” habituation process (maximum habituation degree). The graph in fig. 31 shows the reflex sensitized every 7.5 s with only one stimulus to test the amplitude of the reflex after the CS and the depression process using a low intensity test stimulus at 40 Hz. The data obtained have been fitted with two different equations. The habituation points have been fitted using equation [1] (see materials and methods) which has only one variable because the depression process divide the neural signal with a constant rate which is determinate by the features of the stimulus. The sensitization curves have been fitted using the equation [2] which is an exponential function with two variables. In fact sensitization presents two phases: a facilitation which increases the neural signal with a constant rate and then the habituation of facilitation in which the response tends to wane with the repetition of the conditioning stimulus. The habituation of the facilitation means that the facilitatory effect is still present but not as great as at the beginning of the process, indicating that the neural signal is still

multiplied but by a progressively smaller facilitation. So the two variables of the equation [2] represent the different rate of facilitation during the two phases.

Now keeping in mind pure habituation and pure sensitization curves, it is possible to understand the experiment in fig. 30. The experiment at 4 Hz (middle panel of fig. 30) has been placed inside the maximum habituation and sensitization processes. Because depression and facilitation are, respectively, dividing and multiplying the neural signal by some factor and acting simultaneously on the network, the net change in transmission efficacy (set by intensity and frequency of the stimulation) affect the expression of learning. In this way the signal amplitude (the reflex response) changes and gives rise to different learning kinetics and, ultimately, changes in behaviour. If the dual process theory is applied to all the curves of the experiment in fig. 19, the different kinetics, obtained changing the stimulus parameters, are the expression of two parallel processes which work simultaneously and independently on the neural signal but with a stronger or weaker strength. The difference in the net behaviour is given by the different rate of depression and facilitation set by the stimulation parameters. Indeed specific subcellular events, affecting synaptic plasticity, including vescicles depletion and mobilitation as well as calcium current down and up regulation[123], may interact in a complex way.

Playing with the dual process theory

The experiments until now show that the *in vitro* rat spinal cord is a good model to study habituation and sensitization and the data obtained are in accordance with the literature and the dual process theory of learning (for a review see Prescott et all., 1998). The habituation process is affected by different parameters which are connected with the stimulus test. Facilitation is affected by both the test stimulus parameters and the conditioning stimulus ones. The processes could be evoked simultaneously and independently, as in the experiment before, or the depression could be induced before facilitation. It means that depression starts its action before facilitation and it can result in different behaviours of the reflex. Indeed the factors which affect the net outcome from motorneurones, can interact in a very complex manner. Furthermore the

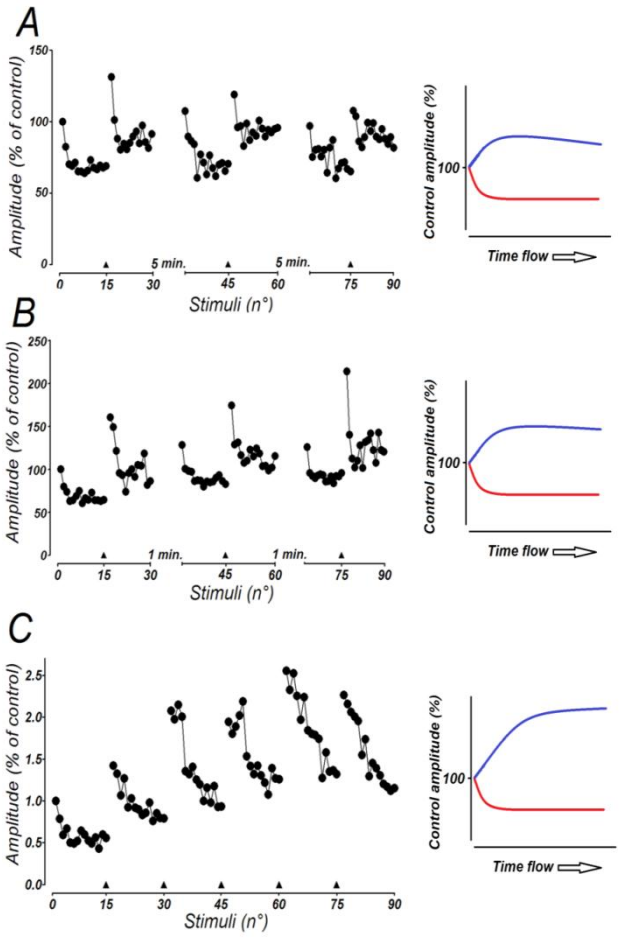


Fig. 32

Facilitation is increased when time between trials is reduced. The effect of a CS could increase MVR amplitude for a short time (15 s) or longer (90 s). It depends on the features of the test stimulus and the CS and on the rest between trials. **A)** Trials of 30 test stimuli (50 µs, 1.6 T, 1 Hz) with 5 minutes of rest in between. The CS (0, 5 s, 500 µs, 30 T, 70 Hz) is applied during the 15th test stimulus. **B)** Trials with one minute of rest; **C)** trials with no rest. The panels on the right of every graph represent the effect of habituation (red line) and facilitation (blue line) during each experiment. Note that the CS effect increases when rest between trials decreases while the depression action is grater when the rest gets longer. The repetition of the CS at shorter intervals between the series has an additive effect on the facilitatory process of the previous CS, and probably, in graph C, the previous CS sensitization effect was not finished when the next one was delivered.

protraction of the stimulation could add new effects which can increase the complexity of the interaction between all the factors, playing a role in shaping the reflex response.

Some experiments have been performed using low test stimulus to stimulate a dorsal root in order to evoke the monosynaptic response, and therefore pure habituation, and a strong conditioning stimulus on a different root to evoke only the sensitization process. Using this arrangement, the two processes interacted in a more complex manner than before, giving rise to different and multiple learning kinetics. Using 105 TS and a CS every 15 of those, the usual increasing response changed when the time between trials was reduced. The response amplitude reached the same plateau like level before and after the CS when the rest between trials was five minutes (in fig. 32A). Furthermore the first response after the CS decreased with the repetition of the trials (habituation of dishabituation). When the rest between trials decreased to 1 minute (in fig. 32B), the CS evoked a greater response, which is higher in amplitude both in the run down phase and in the plateau-like level. It is important to note that the response amplitude came back to the control amplitude after the rest period. The response, after the break in stimulation, seems not to be affected by the depression and facilitation of the previous series. When the CS is delivered without rest between trials (fig. 32C), the response presented a continuous increasing in amplitude after every CS and the CS repetition does not elicit the habituation of dishabituation but rather it evoked a continuous increasing of the response (at least during the stimulation time). The sensitization effect was amplified by the repetition of the conditioning stimulus (which should evoke habituation after a while but it was delivered only five times during the experiment) and by the additive effect of it as the rest between trials became shorter. The depression process was also present (the response amplitude began to decrease soon after the CS) but its effect was weaker than sensitization because of the TS number (the greater the number, the higher the habituation degree fig. 13 and 16) and the TS intensity (the lower the intensity, the greater the depression effect, fig. 15). The net

outcome was a general increasing of the response. The depression and facilitation are, respectively, dividing or multiplying the neural signal of some fixed value and the stimulation time affects this fixed value in a direct way: the longer the rest, the weaker the effect of the previous stimulation on the next one. The balance between sensitization and habituation changes during the course of the experiment. In conclusion, in this experiment the break in the stimulation affects sensitization much more than habituation. It important to remember that habituation is a separate phenomenon from sensitization, in that habituation is not erased by sensitization, but merely temporarily overridden (Groves & Thompson, 1970), indicating that mere exposure to a dishabituating stimulus is not sufficient to eliminate the habituated response. The opposite is also true for sensitization which is a general state of arousal in which the

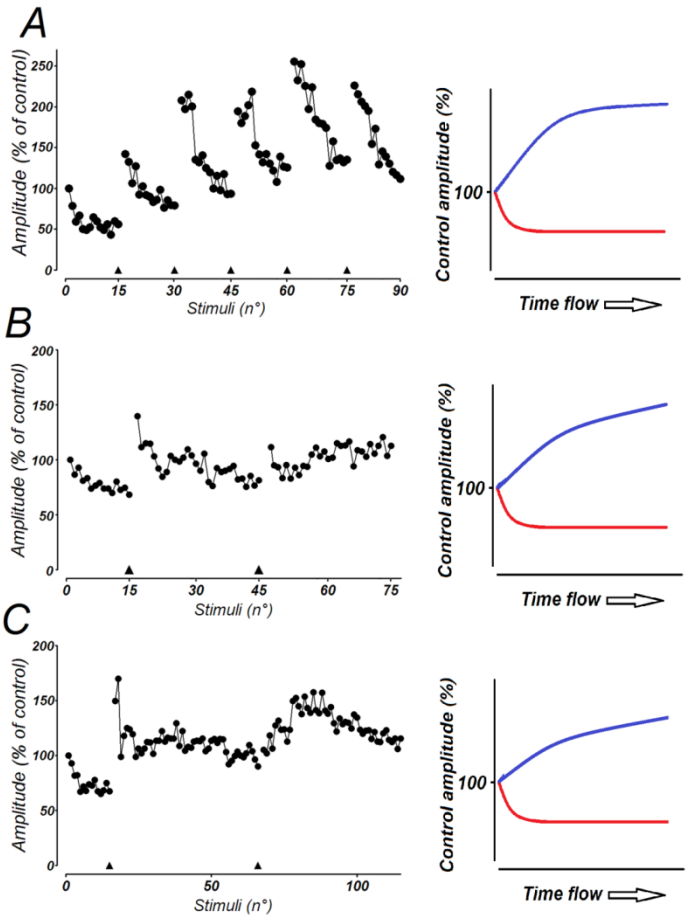


Fig. 33

The number of the test stimuli affects the habituation and sensitization processes. MVR amplitude shows a different kinetic when the stimuli number is changed. Protocol: the first CS (0, 5 s, 500 µs, 30 T, 70 Hz) is always delivered after 15 test stimuli (50 µs, 1.6 T, 1 Hz) to show the start habituation level. Then the number of the test stimuli before the CS increases in each experiment. **A)** CS was delivered every 15 test stimuli. **B)** CS has been delivered every 30 stimuli; **C)** CS delivered every 50 test stimuli. The test stimuli number affects the habituation and sensitization processes and therefore the net behaviour.

network stays after a specific meaningful stimulus.

it is likely that parameters, which affect the net behavioural outcome in the previous experiment include the TS number and the trials. As shown before (fig. 13), the test stimulus number affects the spontaneous recovery time of the response. Furthermore the repetition of ten trials increases the habituation of the run down phase as well as the plateau-like level (fig. 16). The next experiment shows how these two variables affect the MVR learning kinetics and also that habituation and sensitization operate as separate phenomena, in continuous competition against each other. An increasing number of TS (fig. 33) have been delivered before the CS. If the CS was delivered every 15 TS (fig. 33A), MVR showed sensitization not only in the run down phase but also in the plateau-like level which is higher than the previous one. When the CS was given every 30 TS (fig. 33B), the first series after the CS was equal to the previous one (fig. 33A) but from the second one, a different behaviour of the response appeared. The sensitization effects became weaker and weaker during the trial while the depression effect was stronger. At the end of the experiment, the response amplitude was lower than before. The learning kinetic was completely modified by the number of stimuli when 50 TS were used before the CS (fig. 33C). In the last series after the last CS, the response amplitude increased during the first 25 stimuli and then it began to decrease. Usually the rate and the degree of sensitization are proportional to the conditioning stimulus intensity and frequency. This experiment shows that the repetition of the habituation and sensitization series affects both the fast run down phase and the plateau level as well. The habituation process continues its action after the CS and the CS itself is not able to immediately reverse the response depression. The previous balance (fig. 33A) between habituation and sensitization changes in the last experiment (fig. 33C) in favour of habituation because of two variables. The first is the increasing number of TS which increase the depression effect rate and the response amplitude falls down to the control value before the next CS. The second variable is a consequence of the first: the lower number of CS delivered during the trial. The importance of this last variable

could be clearer if the response amplitude of the last experiment (33C) is compared to the previous one (fig. 33A). Indeed the response did not reach the maximum value of before (250% of fig. 33A vs. 150% of fig. 33C) after the CS and furthermore the response amplitude reached the control value before that the next CS began its action.

The TS number is a parameter to control habitation and sensitization processes. Furthermore habituation seems to be responsible for the changes in the behaviour of MVR. If it is the correct interpretation of the data, it would be possible to use 30 stimuli with a greater TS number in the trial to have the same behaviour shown by

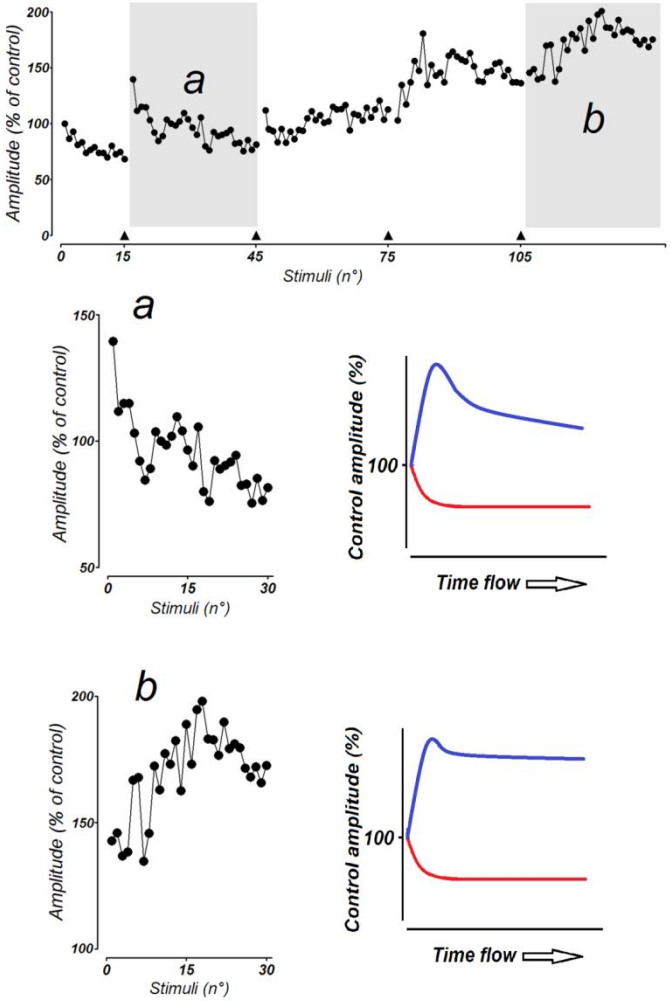


Fig. 34

Habituation process affects sensitization of the response. Habituation and sensitization are different processes which compete against each other. A trial in which CS (0, 5 s, 500 µs, 30 T, 70 Hz) has been delivered every 30 test stimuli (50 µs, 1.6 T, 1 Hz) except for the first series in order to compared this experiment to the one in fig 18. The sensitization effect of the CS after the first presentation **(a)** is instantaneous and the reflex reaches the maximum amplitude soon after the CS and then it decreases. Instead the lasts 30 responses after the CS **(b)** show a different kinetic in which the peak of sensitization is reached after 15 s and then it starts to decrease. The sensitization process becomes stronger with the repetition of the CS and its effects lasts less in the first series than in the others. On the other side habituation is stronger during the first series and then it begins to be overlapped by sensitization.

the response with 50 stimuli (the amplitude of the reflex started increasing and then decreasing). The experiment in fig. 34 shows a trial of 135 TS in which the CS was delivered every 30 test stimuli (the first series of habituation has only 15 in order to compare this data with that in fig. 32 and 33). The shape of the response amplitude changed completely from the first series (a in fig. 34) to the last one (b in fig. 34). The bottoms of the graphs in fig. 34 show the second series (a) and the last one (b), expanded in size. In the second series, sensitization was stronger than habituation only in the first part while the facilitatory effect continued to be present for the entire last series. In this last series the response reached the greater amplitude after more than 20 test stimuli and the start to decrease. The habituation process exerts its effects on sensitization (as shown in fig. 32) but the higher repetition of the CS in this experiment evokes a longer term facilitatory effect as well. If the response amplitude is compared between the second series and the last one, there is an increase in the response amplitude little by little in the experiment, suggesting that the sensitization effects are increasing with the repetition of the CS. In this instance, the induction of depression precedes that of facilitation (TS are delivered before the CS) and the habituation expression divides the neural signal before the subsequent multiplicative effect of facilitation (now acting on a smaller signal than at the beginning of the trial). If the multiplicative effect of sensitization is reduced because of the habituation expression, the sensitization effect needs a longer stimulation time or a greater number of repetitions to reach the greatest effect.

Increasing the number of TS before the CS seems to decrease the neural signal multiplied by sensitization after the CS. So if the action of depression reduces the signal before the action of facilitation, facilitation should not be able to reach the same amplitude of before (fig. 34) when the number of TS is further increased. The experiment in fig. 35 shows a trial of 200 TS in which the CS has been delivered every 50 stimuli (TS and CS are the same as the experiment in fig. 33). The CS has been delivered four times (as in the experiment of fig. 34) but with a greater number of TS in between (a greater habituation process). Habituation exerts its cumulative effects

on the sensitization process and the response amplitude did not reach the amplitude level of the one in fig. 34. By the way, there is a general sensitization effect which increases the value of the response amplitude from the control but it is not as great as that one in the previous experiment. The response amplitude after every CS increases in size but after about 25 TS it decreases again to the control value, sometimes less. Probably, the number of the TS before the CS and their features (intensity, frequency, time of stimulation etc.) evoked a depression effect stronger than the facilitatory effect elicited by the CS. The rate of sensitization is strongly influenced by the features of the CS (fig. 21 and 22) and by features of the TS (fig. 23 and 24) as well as the effects of depression. In the experiments shown before, the balance of the response was set by the number of the TS, whose intensity and frequency increased the depression effect (pure habituation process) more than the facilitatory

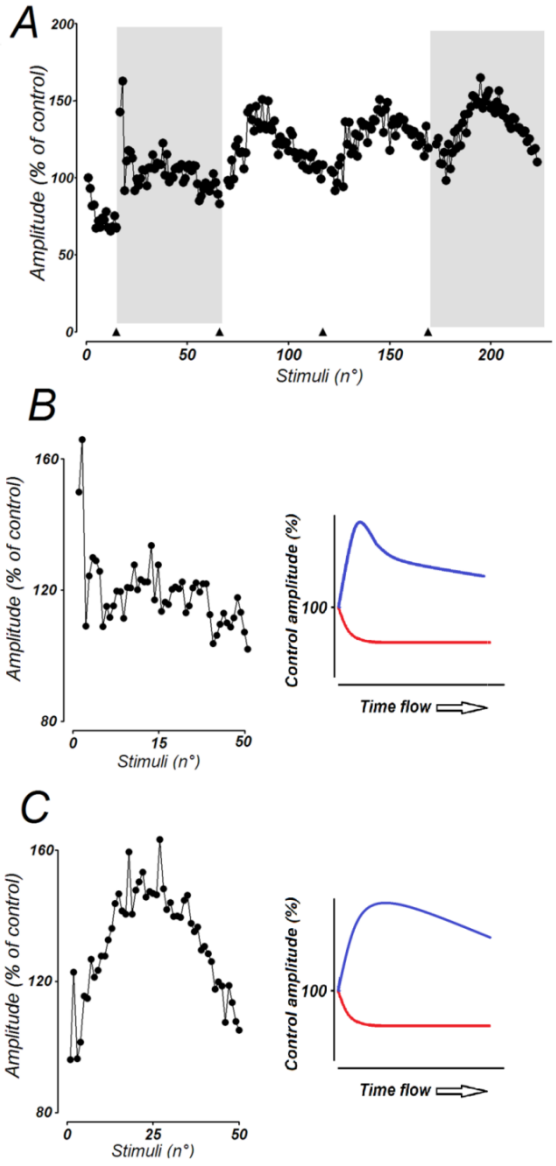


Fig. 35

Habituation of dishabituation. The dishabituatory effect tended to wane when the CS was repeated. The same experiment as in fig 8D. A conditioning stimulus was delivered every 15 test stimuli. The responses are greatly potentiated for a brief period, but after a while the effect of the CS on the response was attenuated or habituated.

one.

The shape of the final output from the motorneurones could be modified by increasing or decreasing the test stimuli number (it means increasing or decreasing the depression action because the test stimulus was low and evokes only a small monosynaptic response) or by increasing or decreasing the CS number (which will increase or decrease the facilitation effects). The balance between habituation and sensitization could be modified using other parameters of the conditioning or the test stimulus. When the CS intensity is decreased the competition balance is moved in favor of habituation. In the experiment in fig. 36 (which is the same of fig. 19D) the test stimulus was given by an electrode and the CS was delivered by another electrode on the same root, using the same intensity of the test stimulus but with a higher frequency and every 15 s. When the CS was delivered, the TS was withheld. In this instance, the expression of depression precedes that of facilitation (the response was depressed before being sensitized) and depression divides a neural signal such that a subsequent multiplicative effect of facilitation (now acting on a signal smaller than that at the beginning of the trial) will be reduce in efficacy. In contrast to parallel expression, the experiment shows that in this case dishabituation, or facilitation, is not the disruption of habituation but rather a separate facilitatory process superimposed upon the habituated response. Facilitation under this condition is purely

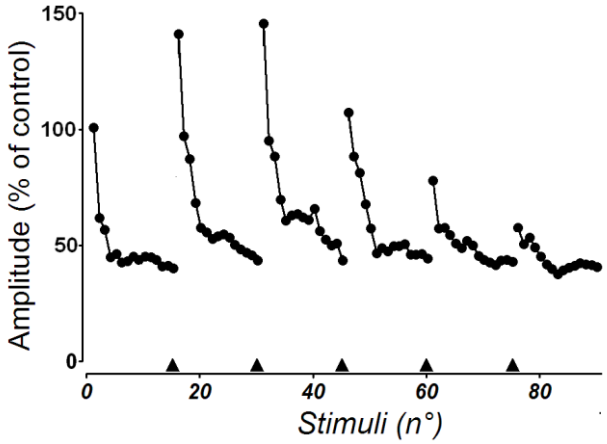


Fig. 36

Balance between habituation and sensitization. The competition between depression and facilitation occurs within the network. **A)** when the CS (0, 5 s, 500 µs, 30 T, 70 Hz) is delivered every 50 stimulus tests (50 µs, 1.6 T, 1 Hz), the habituation and sensitization processes interact in a complex manner; **B)** and **C)** are the 2th and the 5th series (grey colour) of A).

"restorative "in that the process does not prevent the initial signal decrement but only tries to affect some recovery after the fact. Furthermore not only a smaller signal is multiplied by a constant facilitation but a progressive smaller signal is multiplied by a progressive smaller facilitation.

The incremental effect of the sensitization can become much stronger and can reach a saturation state when the balance between sensitization and habituation is turned to the advantage of facilitation. Some experiments were performed in order to see whether sensitization can reach a state of saturation, meaning that further CS will not evoke further increases of the response amplitude and the habituation process is too weak to decrease the amplitude

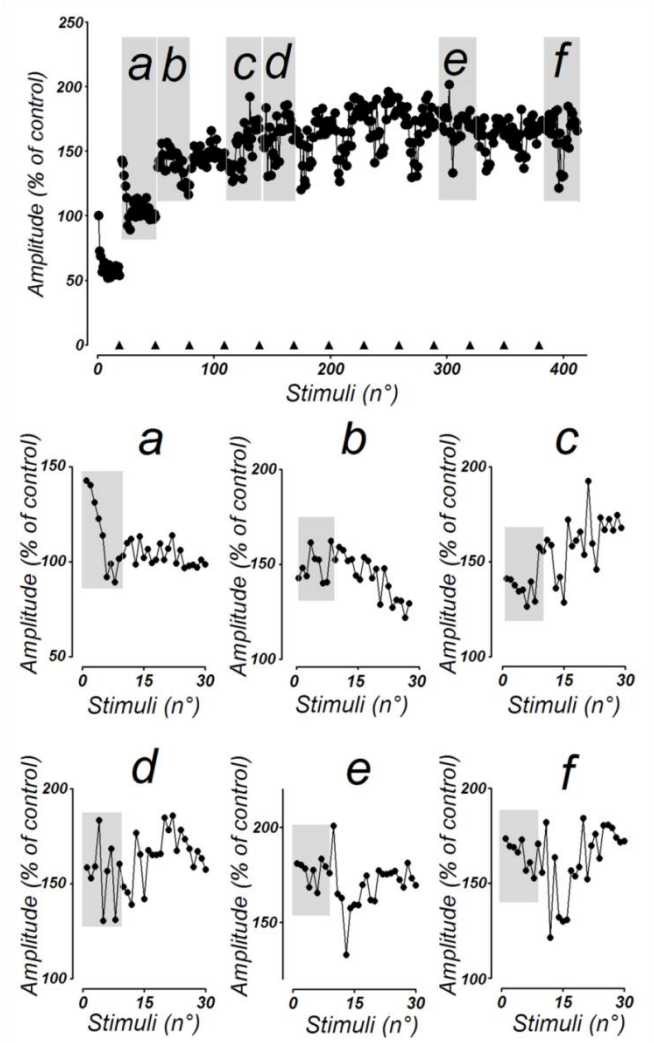


Fig. 37

Habituation may be replaced by enduring sensitization. The balance between facilitation and depression could be modified by changing the features of the test stimulus. In the upper graph, using the same CS (0, 5 s, 500 µs, 30 T, 70 Hz) but a test stimulus with lower intensity 1.2 T (50 µs, 1 Hz), the response shows the depression during the first series but then the habituation process disappears soon after the CS (black triangles) and the response was sensitized for the rest of the trial. The small letters in the graph indicate some examples of the series during the trial. Note how the kinetic of the behaviour changed during the series. The last two series (e and f) show a depression process which did not withstand sensitization. Furthermore the incremental effect of sensitization reached the greatest value on the response and then it did not increase more.

response. Fig. 37 shows a trial in which a CS (the same features of that one used in the experiment in fig. 32, 33 and 34) has been delivered every 30 TS for 400 s. The habituation process was clearly affecting the response amplitude (a in fig. 37), decreasing it soon after the first CS and further bringing it down to a value below the control value. The second CS (b in fig. 37) evoked a longer-lasting sensitization in which facilitation withstands depression, leading to a decline in amplitude only after 15 stimuli. From the 4^{th} CS (c in fig. 37) the sensitization effects were greater than the habituation ones and the response amplitude increased throughout the series. After the 5^{th} CS (d in fig. 37), the response amplitude was stable and it did not show depression. Apparently the habituation process did not affect the reflex anymore while the incremental effect of sensitization reached a saturated effect. Indeed the features of the TS (number, intensity and frequency) and the features of the CS (intensity, frequency, duration of the train, etc.) moved the balance in favour of sensitization. The facilitation process was stronger than depression. The depression was still present but it was pretty weak, compared to sensitization.

DISCUSSION AND CONCLUSIONS

The monosynaptic reflex in *in vitro* spinal cord of rat exhibits the nine parametric features of behavioural habituation, indicated by Rankin and colleagues[26]. Furthermore it has been shown that the characteristics of the TS and the CS affected the depression and facilitation processes which could interact in a very complex manner. The induction of habituation and sensitization could give rise to many different learning kinetics of the MVR, well explained by the dual process theory of learning of Thompson and Spencer (1966).

Habituation is a behavioural phenomenon defined by the parametric relations between response decrement and stimulus/training variables. It has been widely documented in research with various organisms and central processes underlain habituation must to be inferred (Thompson et all., 1970). Indeed a decrement to repeated stimulation at a synapse is termed habituation if it exhibits properties consistent with the behavioural definition of habituation (Kandel et all., 1973).

HABITUATION IN *IN VITRO* SPINAL CORD OF RAT

Habituation can be defined as a response decrement depicted by a negative exponential function, in result to repeated presentation of a stimulus (criterion 1). The monosynaptic response in *in vitro* preparation was depressed by the repeated presentation of the stimulus and it recovery when the stimulus was withheld (fig. 12). The depression kinetic was an exponential decrement which presented a fast run down phase during the first six stimuli and then it reached a plateau like level, maintained until the end of the stimulation. We have found that the reflex showed no depression as the interval between successive stimuli reached 30 s. In other studies the monosynaptic pathway showed a substantial depression of the response amplitude after the stimulation of Ia afferents (using short-interval double-pulses [83] or moderate rates of repetitive stimulation down to 0.1 Hz [80]) but their data showed a shorter time course of habituation development. On the other hand our data are consistent with other studies on the monosynaptic response in spinalized [91] and *in*

vitro[92] spinal cord of rats. The depression kinetic of the MVR could be also compared to the kinetics showed for other in vitro preparation as the embryonic chick spinal cord preparation (Lee& O'Donovan, 1991) and the mouse preparation (Yan Li et all., 2001). Same kinetic of response depression has been found in other in vivo experiments, included the tail reflex of rat in response to tapping (fig. 37). Experiments on animals, in which the central nervous system was left intact, showed a depression substantially similar in nature, although in this case the depression was less severe and the amplitude variation was large [91]. Thus, it is clear that the low frequency depression in the monosynaptic reflex of the *in vitro* rat spinal cord, was scarcely influenced by the loss of supraspinal input due to the cord section.

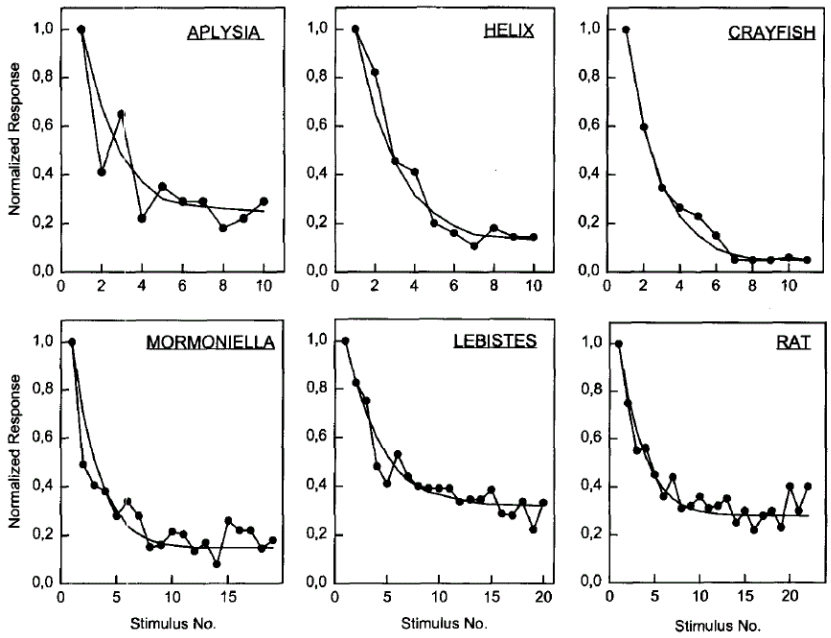


Fig. 37

Case of habituation. Aplysia: the siphon withdrawal reflex (redrawn from Carew and Kandel, 1973). Helix: tentacle withdrawals in response to repeated touches of the tentacle (Christoffersen, 1992c). Crayfish: the tail-flip reflex (redrawn after Krasne and Woodsmall, 1969). Mormoniella: male courting behaviour (after Barrass, 1961). Lebistes: escape swimming in response to a repeated shadow (redrawn from Russell, 1967). Rat: the tail reflex in response to tapping (data from Lehner, 1941). Dots show normalized experimental response values and the smooth curve represents equation [1] (see materials and methods).

Parameters of the stimulus

The response depression was affected by the parameters of the stimulus, as postulated by Rankin and colleagues (2009). The number of the stimulus (criterion n° 2) and the stimulus intensity (criterion n°4) affected, respectively, the recovery (fig. 13) and the run down/plateau level of the response (fig. 14). The number of the stimuli affected the recovery of the response in a direct way, the greater the number, the longer the recovery to the control value. In most cases, where the time course of recovery after a single habituating session has been described, the same pattern has been observed: a fast phase of recovery followed by a slow phase (Hinde,1960; Rankin 1989). In our experiment probably it could be seen in the 50 stimuli trial (white circle in fig. 13). The value of the response was about 55% of the initial control amplitude, soon after the end of the habituation trial. After 5 stimuli (a little bit more than 2 minutes) the recovery of the reflex reached 90%. Afterwards the reflex amplitude reached the 100% of the initial control value after further 14 stimuli (7 minutes). It seems probable that the two distinct time domains of fast and slow recovery represent the disappearance of short-term habituation followed by dissipation and long-term habituation. The same results are present in many studies. For instance, on a slower time scale, the recovery from habituation of the tail-flip response of crayfish also demonstrates the two phases (Krasne and Woodsmall, 1969). It has to be noted that a single session of stimuli will normally produce a limited amount of long-term habituation, and this would explain the slow recovery (see fig. 16 and the paragraph about potentiation of habituation for further and stronger evidences).

The stimulus frequency (criterion n°3) affected both the decrement of the response and the recovery phase (fig. 15 in results). Indeed the depression intensified increasing the stimulation frequency and this could reduce the response amplitude down to about 20% of its maximal value during 40 Hz stimulation. The facilitation and potentiation which are normally observed at the first stage of high-frequency activation in the *in vivo* adult spinal cord preparations of cat [124,125] could

not be detected at any of the tested stimulation frequencies in the in *vitro* spinal cord of rat when the stimulus intensity was so low to activate only the monosynaptic response. Our data are consistent with the ones, showed by Lev-Tov and Pinco (1992) in *in vitro* neonatal spinal cord of rat in which no facilitation process has been seen. Curiously in our experiment, the 10 Hz stimulation (fig. 15D, white triangles) decreased the response without facilitation but when the frequency was changed to the control value in order to measure the spontaneous recovery, the reflex recovered immediately. Furthermore it exceeded the control amplitude for about 5 minutes, decreasing to the control value later on. The stimulation frequencies do not change the number of DR afferents activated by the stimulus [80,126] and the increase in amplitude has been seen only using stimulation frequency higher than 4 Hz. Perhaps the frequency-dependent facilitation could be present during the depression phase but it was hidden by the stronger habituation process. During the habituation of a response, the decrease has been widely considered the changes provoked by the experience (the repetition of the stimuli) while the recovery of a habituated response has been considered the memory of the experience [28]. The link between behaviour and neuronal/circuit properties (as stated by Kandel, 1973) would allow us to assume that the recovery phase from habituation represents the disappearance of habituation (*in vivo* habituation) and analogously, the recovery from depression could represent the dissipation of depression (*in vitro* habituation, Schilhab and Christoffersen, 1996). If we assume this paradigm, the facilitation process evoked by the stimulation frequency could be seen not during the decrease of the response but during its recovery. The facilitation process could be present during the depression process but was hidden by it. It reappeared when the source of depression was weaker (the control recording used 1 min. interstimulus interval) and the facilitation processwas still present.

Short term and long term effects of habituation

The effect of multiple series of stimulus repetitions and spontaneous recoveries results in a response decrement which becomes successively more rapid and/or

more pronounced (criterion 3, potentiation of habituation). Experiment in fig. 16 showed the effects of ten habituation trails on the monosynaptic response compared to a single trails session. The first response was lower than that one in the single trial. Besides the plateau like level of ten trials was reached faster than that one of the single trial and was lower in amplitude (potentiation of habituation). Furthermore the effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level (criterion n°6). Again in fig. 16 the interval between trials was 1 minute, just sufficient for a complete recovery. The repetition of trials affected the decrement of the response and the recovery as well. Indeed the effect of stimulation beyond asymptotic levels can alter subsequent behaviour, for example, by delaying the onset of spontaneous recovery (Rankin and Broster, 1992) or increasing the recovery time. The long term retention of habituation is one of the most important features to distinguish habituation from other forms of depression. For instance such continuous decrements over repeated trials are not a characteristic of neuronal refractoriness [58].

Besides from another experiment, it has been showed that the stimuli number directly affected the recovery of the response in one session: the greater the stimuli number, the longer the recovery (fig. 13 in results). In this experiment, the two phases which usually characterize the response recovery, have been difficult to see because of the gradual increasing of the reflex amplitude. On the contrary, after ten habituation trials (grey squares, fig.16) the reflex amplitude showed clearly the two phases, separated by a sudden change in reflex amplitude. A first faster phase in which the value increased from 50% to 55% of the control amplitude in about 4 minutes. A second slower phase, in which the reflex amplitude suddenly jumped from 55% to 75% of the control amplitude in one minute and then reached the 100% in more than 20 minutes. It seems that the long-term habituation after ten sessions increased the slower phase. Indeed, the first phase was similar between the two experiments (fig. 13 and fig .16) while the second phase of fig. 5 was much slower and presented a response much greater than the previous one in fig. 2. It seems

probable that the two distinct time domains of fast and slow recovery represent the disappearance of short-term habituation (present also in fig. 13 with the same number of responses) followed by dissipation of the long-term habituation[127].

Lastly some studies, which investigated the effects of the number of the stimuli and the interval between trials on long-term habituation, suggest that the effect on the learning kinetic could be due to the interval between trials while the delay of the recovery could be due to the stimuli number per each trial. For instance, if intervals between sessions are kept short or eliminated, the amount of habituation produced by a session is drastically reduced. This was observed by Hinde (1954), in an in vivo study of the mobbing response of chaffinches, demonstrating the inefficiency of "massed stimulation" compared with stimulation with 24-hr intervals. Again, results from Aplysia confirm this inefficiency of massed sessions in producing habituation as observed in the siphon withdrawal reflex after four training sessions either spaced by 24 hr or massed (Carew et al., 1972). In our model is not possible use this long experiment time to study the formation of long term memory but, likely, the number of stimuli affects the recovery but also the next decrement (see below).

Stimulus generalization

In the intact organism, a common elements theory of stimulus generalization (criterion n°7) must include first-order afferent fibers, interneurons and motoneurons as potential common elements. Thus, behavioural studies of tactile stimulus generalization typically involve stimulation of adjacent skin regions, which may activate common afferent fibers [41]. In the simplified model of the *in vitro* spinal cord described here, the possibility to use different dorsal roots, which could be stimulated only with an electrical stimulus, could help to understand if habituation occurs when fibres, common to different segments, are stimulated. The degree to which this generalization occurs depends on how similar are the two stimuli (Mackintosh 1974) but, at the same time, failure to discriminate between a trained stimulus versus a novel one could result in an inappropriate response. In our

experiments (fig. 17), the repeated activation of the Ia fibres in one root evoked depression not only in the monosynaptic response of the homologue ventral root but also in other segments. Each root can evoke the monosynaptic response only from the homologue ventral root and not from another one. Anyway the activation of L1 dorsal root can affect the MVR_{L3}, probably throughout common fibres (via interneurons). Furthermore habituation occurs using a combination of a root from the thoracic level with a root of the lumbar level, although with a lower degree of habituation. These results are expected. Indeed stimulus generalization (even within the same sensory modality like in our experiment) is consistent with the idea that habituation is happening centrally rather than in primary sensory afferents [26]. Generalization of habituation has not been found in monosynaptic system as in the withdrawal reflex of Aplysia [31] or in monosynaptic reflex of frog activated from lateral column [32] but it has been found in polysynaptic reflex of mammalian models, such as the flexion reflex of cat spinal cord[34]. In this study, the changes in excitability of the first order afferent neurones have been ruled out for spinal habituation and sensitization [34]. Additionally the alterations in excitability of motoneurons have been ruled out as well [128]. In our experiments the excitability of Ia fibres did not change during the experiment, as the effectiveness of the stimulus. The experiments in adult spinal cord of spinalized cat [128] and our experiments, with roots from different segments of the spinal cord, suggest that the plasticity site underlain transfer of habituation from one segment to the other one could be occurred, mainly, via interneurons.

Age on habituation

Many new cases of habituating reflexes have been described in the last twenty years [127,129-132] and a new feature has been added: the age-dependent habituation. A study of habituation in a mechanosensory reflex of Caenorhabditis elegans has showed that habituation is an age-dependent form of learning (Beck and Rankin, 1992, Rankin et all 1989). Older worms showed faster and greater habituation of reversal responses at trains of taps than did young ones. The lower rate of

habituation in young worms was associated with (and probably caused by) a higher rate of recovery from habituation in young compared with old specimens. A similar impediment with age of the long-term retention of habituation also has been observed in Aplysia [133]. On the contrary in our experiments, younger rats showed a faster and greater habituation degree and a faster recovery, compared to the older ones (fig. 18). This contradictory results could be explained, taking into account the differences in the experiment conditions. First of all, the age-dependent habituation has been investigated only in invertebrate models (Aplysia, Caernobitis Elegans, Crabs) and there is no study about the effect of aging on mammalian models of habituation. Furthermore the rat spinal cord exhibits functional immaturity at birth in comparison to other invertebrate models [134] but changes rapidly during the first two post-natal week [126]. The neurones are still not myelinated and the latencies of the ventral root reflex components decreased as the age of the rat increases from 7 and 14 days [135]. Besides the depression effect of habituation in our model probably is not completely due to the central plasticity, distinctive of this kind of learning, but probably to the immature neurotransmitter release system as well [136]. Still the differences in speed of the learning kinetic could be due to changing property of the motoneurons [137]. For instance the rheobase in motoneurons increases more than five times during postnatal development [126,138]. Lastly It is now well accepted that the effectiveness of Ia-motoneuron connections can be modulated presynaptically by last-order GABAergic interneurons (reviewed in [112]) and their activation could play a rule in mammalians habituation [139]. This system in not mature at the birth and needs two weeks to reach the final adult state of inhibition which is usually seen in adult rats. From a behavioural point of view, younger rats could take advantages by their faster and greater habituation and faster recovery, compared to the older ones. Indeed the number of new stimuli for new born animals should be higher compared to the older ones. The possibility to discriminate faster between meaningless stimuli and important ones could be an important advantage for young animals. Although the memory of the learning quickly wanes (which exactly is the situation of human infants) in young animals compared to the old ones.

The effect of aging on habituation shows the sensitive to differences in how an information is encoded, across the same organism in development. Using same frequency and intensity, the stimulus was able to elicit different learning kinetics in preparations of different ages, meaning that it has been elicited a different behaviour. Furthermore even within the same preparation, the way in which a stimulus (stimulus intensity, frequency, temporal relationship, etc.) elicits a behaviour is affected by the amount of experience (criterion 3 or 6) or by preparedness (the general state of the system, criterion 19 and 20)[140]. Because each learning mechanism may be governed by a different set of principles, and because each may depend on a different set of neurobiological mechanisms, it must be attentive when a model is used to study plasticity induced by learning.

INTRINSIC AND EXTRINSIC SENSITIZATION

Usually dishabituation represent a special instance of sensitization in which the facilitation is simply superimposed on a habituated response level[36]. Sensitization (or dishabituation) has been used to distinguish habituation from other forms of synaptic depression, as muscle fatigue (Criterion 8,[26]). Dishabituation could be induced by repeated application of the primary or intrinsic stimulus (stimulus in the same side and of the same modality of the stimulus which evokes habituation) and it has been called intrinsic sensitization [39,41]. In *in vitro* spinal cord of rat the intrinsic sensitization (or dishabituation) has been tested on the MVR, using a brief high frequency train of TS which potentiated the responses soon after its application (fig. 19C). The response amplitude exceeded the control value of about 50% and then was depressed again. The TS elicited both the depression and the facilitation processes, depending by the frequency of the stimulation used. Such intrinsic sensitization has been used in many studies about sensitization, like in rat acoustic startle response[44] and in the monosynaptic ventral root reflex of the in vitro frog spinal cord[32]. Besides the intrinsic sensitization has been used also to induce wind up or central sensitization in mammalian spinal cord [70,141,142]. The property of the stimulus to elicit

both the opposite processes is one the most important point in theory of the dual processes postulated by Thompson and colleagues (1966, see below).

When sensitization process is activated by a strong, noxious (and so qualitatively different) conditioning stimulus, it has been called extrinsic sensitization. The monosynaptic response in *in vitro* rat spinal cord was greatly potentiated when a high voltage, high frequency train (CS) was delivered to another segment, different from that one where the TS was applied (fig. 20). The response exceeded about 150% of the control value and then was depressed again. The possibility to use a strong, noxious stimulus to induce sensitization revealed a difference between intrinsic and extrinsic sensitization in our model. The primary stimulus was never able to induce a recovery of the reflex as great as the secondary (or extrinsic) stimulus did. Nevertheless the repetition of the high frequency train (CS) evoked a smaller amount of response recovery (fig. 19D). This phenomenon has been called habituation of dishabituation (criterion 9). Interestingly, when the extrinsic sensitization was induced, the facilitatory effect tended to wane during the repetition of the CS, although a higher number of CS repetition was necessary. The same phenomenon, the habituation of both intrinsic and extrinsic sensitization, has been found by Thompson in the flexion reflex of the spinal cat [33,34,57] but not in Aplysia or other invertebrate models.

Stimulus parameters determined how great the sensitization effects are. Higher stimulus frequency and intensity encode novelty or risk for the organism, eliciting stronger sensitization process to increase rapidly a response. The CS used for dishabituation was the TS at higher frequency (fig.19) while the CS for sensitization was a train at high frequency and intensity (fig.20). The sensitization effects have been increased by the use of both the two parameters (intensity and frequency). Probably to induce sensitization process, the intensity of the stimulus is the most important parameter. Indeed the possibility of a stimulus to activate nociceptors is judged important by the organism on the bases of just its initial strength. Then the frequency give information about the novelty of the stimulus. Its repetition decreases

its informational value (novelty) and the depression of the response was promoted, at least for low frequencies, when the intensity was low (able to activate only Ia fibres).

The response recovery is related to the general state of the system which can increase or decrease the response indirectly (for instance inducing extrinsic sensitization) and to the state of the pathway, responsible of the behaviour (in our model the monosynaptic pathway between sensorial neurones and motoneurons). This second variable has been investigated in our model (fig. 12 and 13). The parameters of the TS can affect the general degree of sensitization and so the final output from the motoneurons. As the intensity of the stimulus was increased, the rate of recovery decreased, comparing the value of the reflex before and after the CS (fig. 12). When the recovery has been compared to the maximum response of the reflex (4 T), the recovery of the reflex reached the same amplitude for all the intensities used (about 50 % of the maximum control amplitude). It suggests that the smallest response has been potentiated by the CS more than the greatest one. Furthermore the sensitization effects of the CS are greater when the frequency of the TS is low (2 Hz) rather than high (40 Hz) (fig. 13). Probably the effects of sensitization are always the same, increase all the responses of the system in a branch specific way (Clark and Kandel 1984). Nevertheless its action could be highlighted when the response is elicited with a TS parameters, completely different from the CS, which are able to evoke only depression.

Ia interneurons and the flexion reflex afferents (FRA)

The monosynaptic excitation of homonymous and synergistic hindlimb motoneurons, following activation of group Ia muscle spindle afferents, is considered to be a fundamental component of postural regulation (e. g. Eccles et al. 1957). Accordingly, muscle stretch during movements activates group Ia afferents which, by monosynaptically exciting motoneurons, produces muscle contraction to counter muscle lengthening (see Lundberg, 1969). At the same time, monosynaptic reflex is

not an inflexible unit of behaviour, but rather, they are highly modifiable, particularly during more complex behaviour [143,144]. In addition, its amplitude is a function of the motor task being performed [144,145] (Garrett et al. 1984; Llewellyn et al. 1990; Morin et al. 1982; Moritani et al. 1990). In our experiment, the L3 ventral root response showed an increase in amplitude when a strong, noxious stimulation was provided to L1 dorsal root. On the contrary, when the reflex was elicited on an extensor root and the CS was delivered from a flexor root, the response showed a different behaviour. Indeed the usual rise, induced by extrinsic sensitization, was not present and an additional response decrement has been seen soon after the CS (fig. 26). Then the response began to rise to the plateau-like level, until the next CS, which still depressed the response (fig. 26A and B). Lastly when the intensity of the CS was increased, the additional depression was greater as well as the amplitude reached before the next CS (fig .26). it is well known that the group Ia afferent fibres, after entering the spinal cord, gives off a collateral branch that synapses on an interneuron. This interneuron, in turn, synapses on the antagonist α-motoneuron, exerting an inhibitory action (reciprocal inhibition). It causes an IPSP in the α-motoneuron that is ultimately manifested as a relaxation of the antagonist muscle (Schomburg & Steffens, 1986). Furthermore noxious stimulation induce limb withdrawal reflexes that have evolved to minimise tissue damage (Sherrington, 1910), based on response in which all flexor muscles, ipsilateral to the stimulus, were excited and all extensors inhibited. The Flexor Reflex Afferents (FRA) system is a multisensorial feedback system, which uses common segmental interneuronal systems and ascending pathways to channel information from a variety of receptors that are activated during movement (Lundberg, 1979; Lundberg, Malmgren & Schomburg, 1987c). The protective result of these afferents is obvious; it quickly removes the part of the body from the vicinity of the offending object by contracting the appropriate muscles, usually flexors, and relaxing extensor muscles (using the pathway of the reciprocal inhibition). Nociceptive afferents with a wide group of non-nociceptive afferents of different origin (low to medium threshold cutaneous receptors, medium to high threshold muscle endings, joint receptors) may activate

the FRA system, although some of them only under particular conditions (Eccles & Lundberg, 1959; Schomburg & Steffens, 1986). In our experiments, the strong stimulation was able to activate pain fibres, which probably, elicited the flexion reflex. The activation of the flexion reflex suddenly decreased the reflex amplitude (the additional depression) and not long after, the response was affected by the general arousal of the system, elicited by the CS. The learning kinetic showed by the monosynaptic reflex, probably, is due to its recruitment in the more complex activity of the flexion reflex, deactivating the extensor motoneurons and enhancing the flexor motoneurons activity. When the flexion reflex terminated its protective rule, the facilitation process appeared again but this time with a different time course (probably induced by the residual action of the flexion reflex and the complex interaction between facilitation and habituation).

The CS from a flexor root was able to increase the depression on the response habituation of an extensor root, probably activating the flexion reflex and the action of the reciprocal inhibition. When the TS on the extensor root was increased enough to counteract the action of the flexion reflex, the additional depression of the reflex disappeared (fig. 27). As said before, the flexion reflex is activated not only by painful stimuli but by other afferents as well. Furthermore Schouenborg and colleagues showed that the recruitment of muscles into hind limb withdrawal reflexes in the rat is organised such that noxious stimuli activate those muscles that are best situated to move the limb directly away from the stimulus (Schouenborg & Kalliomaki, 1990; Schouenborg et al. 1994) and inhibit those muscles that would produce a movement towards the stimulus (Weng & Schouenborg, 1996). The withdrawal reflexes are tailored to produce the most appropriate movement according the site at which the stimulus is applied, which could require extensors to act as the primary movers. This type of organisation has also been seen in rabbit (Clarke et al. 1989), man (Andersen et al. 1999) and cat (Levinsson et al. 1999a). In our experiments, the use of a strong CS on a flexor root and a strong TS on an extensor root, probably, activated two different pathways, in competition each other, counteracting their action. The

possibility of the flexion reflex to evoked the most appropriate response to a painful stimulus, depending the site in which the painful stimuli is applied, can explain why an extensor reflex could be sensitize either by a flexor root, with an additional depression and a delayed facilitation, and by an extensor root, with an instantaneous facilitation (fig. 28). The difference between this two sensitization is the peak of the response amplitude. Indeed while the flexor sensitization allowed to reach the greatest amplitude before the next CS (fig. 28A), the flexor sensitization elicited the maximum response soon after the CS (fig. 28B)

In presence of habituation and sensitization ?

Likely habituation is a term denoting many processes. In animals without a central nervous system such as Aplysia or "spinal" subjects whose spinal cord has been severed from the brain, habituation is a relatively simple phenomenon involving processes local to sensory systems (Squire & Kandel, 1999). One should not expect the habituation observed in more complex behaviours to involve exactly the same mechanisms as those responsible for comparable behavioural effects in more simply reflexive responses.

First of all it is important to be sure that the depression of the response is the consequence of a learning process as behavioural habituation. Indeed a depression of the response could have different underlain causes and most of them are not a learning process. Our experiments indicates habituation as underlain mechanism of response depression because it was possible to exclude other causes.

1. Effectiveness of the stimulus: The decrease of the MVR to the repeated stimulation of the dorsal root could not be due to the effectiveness of the stimulus itself. It has been showed the recordings of the antidromic volley of the dorsal root activation and the ventral root response during the entire experiment (fig. 11A and B). If the response depression were due to the decreased stimulus effectiveness, the dorsal root recordings should also show the same decline in activation while it did not happen

(upper traces on fig. 11A). The activation of the Ia fibres were stable during the entire experiment and the oscillation around the control value was less than the 10 %.

2. Sensory adaptation or motor fatigue. The *in vitro* spinal cord preparation presents some advantages to distinguish the different mechanism underlain motor fatigue or depression process. Indeed there are no receptors to activate the afferent pathways (no muscle spindles or nociceptors). The ventral roots are not connected to muscles because the preparation is composed by only the CNS, without any peripheral component. Furthermore the distinction between sensory adaptation/motor fatigue and habituation could be done testing dishabituation (the response still occurs and exceed the control value after the CS, fig. 19s) and/or frequency-dependent spontaneous recovery (more rapid recovery following stimulation delivered at a high frequency than to stimulation delivered at a lower frequency, fig. 15D) on the studied response. For this last parameter, it has to be said that Thompson and Spencer (1966) noted that the length of time necessary to observe spontaneous recovery was extremely variable even for responses that were known to recover. Thus, although the reversibility of habituation to a particular stimulus is crucial to distinguishing it from non-specific decrements in response, spontaneous recovery is not in itself sufficiently reliable to establish habituation.
3. Neurotransmitter depletion. Lastly the monosynaptic response decrement was not due to neurotransmitter depletion. Indeed the MVR showed the dishabituation process(fig. 19), an instantaneously recovery of the superimposed habituation response. It suggests that further neurotransmitter was present at the synapses level between sensorial neurons and motoneurons. Besides the intensity-dependent depression of the monosynaptic response showed that the higher the stimulus intensity, the lower the depression degree (fig. 14). A higher stimulation

of the reflex should evoke a greater activation of a single motoneurons and so an augmentation of neurotransmitter release. It means that the more the motoneurons are activated, the greater the release of neurotransmitter from the Ia fibres. If it was the cause of the response decrement in the *in vitro* preparation, the increase in stimulus intensity would bring to higher degree of response depression faster and faster as the stimulus intensity increases. Instead the response decrement was enhanced by the decreasing of the stimulus intensity suggesting that the depletion of neurotransmitter did not underlie the habituation process.

Sensitization is a simple increase in response to stimulation and this effect can theoretically occur at any point from nociceptors in peripheral tissues to brain areas which specifically respond to nociceptive inputs. In our model, this process spans from sensorial neuron, via dorsal root ganglia, to the spinal cord in which there are neurones responding specifically to touch stimuli or noxious stimuli or both (Wagman and Price, 1969; Price et al., 1976; Dong et al., 1978; Lamour et al., 1982; Craig et al., 1994). We showed that dishabituation (or intrinsic sensitization) could be produced by the same stimulus which elicited depression (changing just the frequency). Thompson and colleagues stated that dishabituation process is not the disruption of habituation but rather a separate facilitatory process superimposed on the habituated response (1966). In our model the facilitatory process was activated also when the CS was provided by a different segment. In contrast to habituation, which is defined as a stimulus specific process, (Castellucci and Kandel 1974), sensitization is a heterosynaptic modulation of the motoneurons output, via interneurons (Groves and Thompson, 1970), defined branch specific. Our experiments of generalization of sensitization indicates that this process is a general arousal of the system, at least for tested segments. When this “modulatory system” is activated, spreads its general action. It takes the control and coordinates the monosynaptic responses of different segments to shape a final adaptive behaviour (flexor and extensor conditioning).

THE DUAL PROCESS THEORY : A COMPETITION FOR THE FINAL BEHAVIOUR

Habituation and sensitization are two types of non-associative learning, whose effects consist in a change of behaviours. The dual process theory assumes that different types of underlain neural processes are responsible for increases and decreases in responsiveness of stimulation. The habituation process elicits a depression of the neural signal while the sensitization process evokes a facilitation of the signal. These two processes are not mutually exclusive but rather both they may be activated at the same time. The behavioural outcome depends on which underlying process is stronger, indicating that they compete each other to control the final behaviour [37].

The underlying processes that suppress and facilitate the response are called habituation and sensitization as well. Usually it is thought that the decrease of the response (the habituation effect) is a direct mirror image of the habituation process and the sensitization effect is the reflection of the sensitization process. In fact, both the habituation and sensitization effects are the net result of sensitization and habituation processes. The distinction between the effects (an observable behaviour) and the underlying processes is very important. One single observable effect can depend by more than one single underlain process. In order to understand the dual process theory and its implications in our experiments it is important to distinguish the behavioural effects from the underlain processes.

The balance between these two opposite processes could be set by different variables, one of them is the stimulus intensity. In their attempts to characterize the neural analogues of dual process learning in cat, Groves and colleagues studied the flexion reflex in cat, elicited with an electrical stimulation of the skin or the cutaneous nerve [37]. The polysynaptic circuit underlain the reflex remained incompletely understood but they were able to record from interneurons and indicated three type of interneurons whose activity change in different ways as leaning proceeded [41]. The non-plastic interneurons, whose activity did not change during learning process. The H type interneurons, named after their tendency to depress (Habituation) and the S

type interneurons, which showed an increase and a decrease of the activity, closely to the changes in the muscles response. It is well known that the flexion reflex could be evoked using different types of fibres (as tactile or Aδ fibres, see the previous section on Ia interneurons and FRA) and, probably, they were not able to elicited only depression or facilitation because the same stimulus activated different type of fibres. In our experiments when a TS, able to activated only Ia fibres, have been used, it was never seen the facilitatory process to precede or to follow habituation process on the MVR amplitude (fig. 3A). The absence of an increase in response, when a low intensity TS was used, does not exclude the possibility that a stronger habituation process masked a very weak facilitatory process. On the contrary when a strong TS has been used (so able to activate not only Ia fibres, as the stimulus which elicits the flexion reflex), the intensity of the TS determine an increase of the stimuli number to reach the plateau level (graph on the left in fig. 30). In this last experiment the habituation process was much slower (for instance, compared to fig. 14A) but apparently no facilitation process was present. When the frequency was changed to 4 Hz (the intensity was 5 T as before), an temporary increase of the response amplitude was seen (middle graph of fig. 30). The facilitatory process was unmasked by the higher frequency , probably because the balance between the two opposite processes changed and the sensitization process became stronger than habituation and was able to affect the net behavioural outcome. Undoubtedly the facilitatory process was present when the frequency was 1 Hz as well (a greater stimuli number was necessary to reach the plateau level). Maybe it was not as strong as habituation process and it was not able to show its effects directly on the net behavioural outcome.

Habituation on the sensory side and sensitization on the motor side

Opposite to the flexion reflex and the experiments of Thompson and colleagues, in our experiments it was possible to elicit “pure” habituation process with a low intensity/low frequency TS and a “pure” sensitization process with high-voltage/high-frequency CS (fig. 31). Probably pure habituation and sensitization process are purely

hypothetical concepts. Indeed in our conditions (e.g., electrical stimulation of the root to evoke the H-reflex is unlikely to reflect isolated recruitment of the group Ia afferents[146], difference between fibres activated by the stretch reflex and the H reflex[121] or the different ages of the rats with overlapping activation threshold for different fibres[113]) is not possible to be sure to elicit only one process. it is better to say that pure habituation process means that the strongest depression and the weakest facilitation possible have been elicited (the opposite for pure sensitization). Anyway the opportunity to use a monosynaptic pathway to induce depression and a polysynaptic system to induce extrinsic sensitization clarifies the dual process theory.

Our experiments is an additional proof of the theory of Thompson (but also of many other scientists) who called the stimulus which induces learning as the *primary stimulus* and the corresponding stimulus/response pathway as the *primary pathway*. Further any collateral pathways that is indirectly influenced (e.g., through heterosynaptic or presynaptic modulation) by the primary pathway is termed *secondary pathway* and the corresponding driving stimulus *secondary stimulus*. In our model the primary stimulus was the TS and the primary pathway the monosynaptic circuit. On the contrary the secondary stimulus was the CS and the secondary pathway all the interneurons activated from another segment, during extrinsic sensitization. furthermore the depression process was always induced (and at least stronger) when the primary pathway was activated by the primary stimulus (fig. 14 and 15) while it was weaker when was activated by a secondary stimulus (generalization of habituation). Sensitization was present when a CS at high frequency (dishabituation or primary sensitization, fig. 19) was delivery on the primary pathway but its effects could be probably due to interneurons (and so generated indirectly). The secondary stimulus (the CS from another root) induced the extrinsic sensitization which affects the responses in a unspecific way.

These results suggest a hypothesis. The reflex habituation could be primarily due to a depression in the primary pathway, meaning that the neural mechanism underlain habituation could be mainly homosynaptic while sensitization process

could be mainly due to a heterosynaptic pathway. It is consistent with more of the studies on invertebrate [26,28,48] and vertebrate[41,92,95,147], although same other studies on vertebrate indicate an increase of the inhibitory transmission[139,148,149] or others mechanisms [150], as a cause of habituation. It is to be said that same our preliminary data on the effects of picrotoxin and strychnine on habituation (not showed) suggest that GABAergic interneurons could play a rule in the habituation process of the in vitro spinal cord of rat. The initial absence of facilitation and the higher habituation degree when a low intensity/low-frequency TS was delivered could be due to an inhibitory action. Indeed the response amplitude showed a small facilitation and a higher value of the amplitude at the plateau-like level when the blockers have been added to the bath. Anyway, before any other further investigations, these results suggest the hypothesis that habituation could be mainly related to the sensory side of the reflex arc (homosynaptic depression or presynaptic modulation) while sensitization process could be related to the motor side, meaning that probably depression is a process which occurs in the sensorial neuron while facilitation acts on the motoneurons throughout interneurons. Similar conclusions have been reached by Davis and colleagues, working on the acoustic startle reflex of the rat[151]. They characterized the circuit and suggested that sensitization affect the behavioural outcome working as a modulatory system, acting heterosynaptically and in a locus downstream of at least one depressing locus[151]. The same conclusions have been reached by other studies on vertebrate reflexes [59,60] and are generally true for invertebrate [61].

Facilitation process showed a decrease in effects when the CS was repeated as in the flexion reflex in cat[37]. It seems that stimulus intensity is more important for sensitization process than for habituation while stimulus frequency is the most important parameter to induce response decrement. Indeed sensitization process quickly increases the response to a stimulus whose value is judged high on the basis of its initial novelty or strength. Habituation process serves mainly to decrease the response to a stimulus whose informational value has decrease as a results of its

irrelevant repetition. When the high intensity stimulus is repeated, its novelty is decreased and a depression of sensitization rate starts It could indicate that habituation acts on facilitation and that all the stimuli could evoke depression of the signal when they are repeated.

Interactions between depression and facilitation: shaping the outcome

Depression is dividing the neural signal by some factors while sensitization is multiplying it. The effects of their action are a change of the final response amplitude. This two processes could interact in a very complex manner. Indeed the depression and facilitation processes underlain the behavioural effects are controlled by many parameters. In our model it has been possible distinguish the TS which evoked only depression process from the stimulus which induced intrinsic or extrinsic sensitization (the CS). Modifying stimuli parameters (number, frequency, intensity, stimulus interval between series), it was possible to control the induction and expression of the two processes and to shape the learning kinetic of the response.

When habituation was induced before intrinsic sensitization (low intensity/high frequency CS) and their effects were evaluated in only one series, the net behaviour was the expression of these two processes which worked in series (before depression, then facilitation and again depression). The response showed an exponential decrement, then a fast and temporary recovery soon after the CS delivery and, lastly, it waned again (fig. 19C). Depression's expression preceded sensitization's expression. It means that depression divided the neural signal such that the subsequent multiplicative effect of facilitation will be reduced in efficacy. The neural signal was smaller after the action of depression and facilitation acted on a signal smaller than that one at the origin of the circuit (before depression). In this conditions, facilitation is purely restorative because the process did not prevent the initial signal decrement but only tried to effect some recovery after the decrement.

Both the two processes are expected to decay with the passage of time without stimulation. The loss of habituation process with time results in recovery of the

response (spontaneous recovery, fig. 13) while the temporal decay of sensitization results in a decrease of the elicited response, down to its normal non-arousal level (fig. 19C and 20B). When interval between series does not allow to dissipate the previous depression or facilitation effects, the memory of habituation and sensitization processes can affect the future learning kinetic. For instance when the effects depression and intrinsic sensitization on learning kinetics were evaluated in more than one series, the response amplitude did not show a more severe decrement. Rather it showed a continuous decrease of the recovery rate after the CS (habituation of dishabituation, fig. 19D). Indeed, differently from the previous experiment (fig. 19C), during habituation of dishabituation a smaller signal (depression acts continuously and its effects in more than one series) was multiplied by a progressively smaller facilitation. Facilitation rate was maximum during the first CS delivery and then CS effect decreased and facilitation tended to wane. The different learning kinetic could be explained by the different competition between the two processes. The induction of habituation and sensitization was serial as before (stimulus to induce habituation, then sensitization and, finally, habituation again) while their expression was serial as well as parallel. The effects of habituation were suddenly expressed in the first series (serial expression). They waned before the next CS was delivered but they are not completely decay (remember the effect of depression on spontaneous recovery). The effects of depression were added to the next series and the depression process become stronger as the series went on (parallel expression). On the contrary when an high intensity stimulus was repeated, its novelty was decreased and a depression of the CS-response overlapped with facilitation. Sensitization process was activated by a modulatory system[151] and its output showed a decrement, causing sensitization to wane. As stated before, habituation serves primarily to decrease responsiveness to a stimulus, weak or strong, that experience has proven to be inconsequential, meaning that habituation, and so depression, is more heavily influenced by the repetition of the stimulus than by the intensity. Depression not only compete with facilitation to determine the final

behavioural outcome but, in this particular case, depression also work to reduce the induction of facilitation.

The stimulus intensity strongly influences the rate of sensitization which can vary substantially. When habituation and extrinsic sensitization (high voltage/high frequency CS) were induced and the response amplitude was evaluated in more than one series, the response showed a general increase of the amplitude (21C). The depression appeared firstly, then a fast and great recovery developed, increasing the response value more than twice compared to the control. To conclude, the next depression was not able to depress the response to the previous plateau-like level. The capacity of sensitization to withstand habituation increased with stronger inputs and its effects are a general increase of the response. Furthermore the effect of a new CS were added to the one of the previous CS. Anyway depression was also present and its action depressed the response soon after the CS delivery. Moreover its action became stronger as the experiment went on. When the balance between facilitation and depression is in favour of habituation, facilitation induced by intrinsic or extrinsic sensitization tends to wane[33] and it causes the continuous depression of the response.

The repetitive stimulation results in a habituation of the elicited response. However if the parameters of the TS and CS were set to move the balance in favour of facilitation, habituation could be replaced by enduring sensitization. The response amplitude could be potentiated and the depression process apparently disappeared (fig. 29). The learning kinetic of the response amplitude shown a general increase after the CS delivery but the depression process after the first 100 TS was not able to induce depression of the response. It seems that the monosynaptic circuit increased its responsiveness to noxious stimulation as the stimulation proceeded. The increase of the response amplitude is mediated by the modulatory system, activated by the noxious stimulation (the CS) but between one CS and the next, the amplitude of the response did not decrease anymore.it seems that the responsiveness to innocuous stimuli was enhanced. It well known that in the spinal cord, repeated stimulation (at

constant strength) of dorsal root afferents, including nociceptive C fibres, can elicit a progressive increase in the number of action potentials generated by motoneurons and interneurons (Sivilotti et al., 1993; Thompson et al., 1993b; Russo and Hounsgaard, 1994; Baranauskas and Nistri, 1996). Interestingly, they usually are investigated to understand the cellular mechanisms of hyperalgesia and allodinia in spinal cord (Woolf et al., 1992; Thompson et al., 1993b; Sivilotti and Woolf, 1994). The windup phenomenon is the most convenient model of sensitization investigated at single cell level (Woolf, 1983; Woolf and Wall, 1986; Thompson et al., 1990). It has been demonstrated that the stimulation typically employed to induce windup (1 Hz for 20±30 sec, very similar to the stimulation in our experiments) is accompanied by short lasting sensitization (3±5 min), increasing the responsiveness of spinal neurones (Woolf and Wall, 1986).). It indicates an activity-dependent increases in excitability of nociceptive relay neurones at the superficial (lamina I) or deeper (lamina V) dorsal horn of the spinal cord (Craig AD, pain mechanisms: labelled lines versus convergence in central processing). In recent years other forms of central sensitization have been found which involve activity-dependent increases in synaptic efficacy. Such non-associative and input-dependent hypersensivity mechanisms of hyperalgesia are in perfect agreement with the notion of extrinsic sensitization and the link between mechanisms of learning/memory and hyperalgesia/allodinia is getting stronger and stronger(Central sensitization and pain: do pain and memory share similar mechanisms) (learning and memory in pain pathways).

IMPLICATION OF HABITUATION AND SENSITIZATION IN MORE COMPLEX FORMS OF LEARNING

Lorenz (1965) suggested that habituation is likely to be the phylogenetically oldest process for modifying an organism's behaviour. The universality of habituation and sensitization implies that these processes, however it may be explained, are fundamental to the adjustment of all organisms in their respective environments and that it has not altered radically through evolution. As Humphrey (1933) emphasized, the pervasiveness of habituation as an "elementary conservative phenomenon

common to living systems of many different grades of complexity, belonging to them qua self-conserving systems however they may be organized" justifies an inquiry into the nature of the phenomenon without giving a precise description of all the processes concerned. Clearly, the capacity to ignore only those stimuli that are irrelevant and to channel behaviour into organized and directed actions in response to meaningful stimuli is necessary to conserve energy and focus behaviour.

When we talk about habituation and sensitization, we could think this non associative types of learning as basic mechanisms of more complex form of learning. Indeed most studies of habituation examine the waning of a reflexive response (e.g., a leg flexion[33]), suggesting that habituation and sensitization can be observed in ''voluntary" or ''goal-directed" behaviours, not just in stimulus-elicited, reflexive, behaviours. Furthermore sensitization and habituation can occur to biologically meaningful stimuli that are needed for the survival of the animal or the species (e.g., food, water).

In the paradigm of operant conditioning a reinforcer is a stimulus that increases the frequency of the response that it follows (i.e., behaviour is altered by its consequences). A reinforcer is demonstrated only if the strengthening or maintenance effect occurs. Sensitization and habituation occur to the sensory properties of reinforcers when those reinforcers are presented repeatedly or for a prolonged time. For instance the rate of operant responding supported by a reinforcer (e.g., food, water) often changes systematically within experimental sessions. Rate of responding changes even when the conditions of reinforcement are held constant across the session. Rate of operant responding often increases to a peak and then decreases (McSweeney, 1992; McSweeney, Weatherly, & Swindell, 1996a). Habituation to the sensory properties of food contributes to satiety for food (e.g., Epstein, Rodefer, Wisniewski, & Caggiula, 1992; Swithers & Hall, 1994). This led McSweeney and colleagues to the hypothesis that sensitization and habituation to the sensory properties of the reinforcer alter the effectiveness of that reinforcer in controlling behaviour. According to this idea, both sensitization and habituation

occur to reinforcers throughout the session as those reinforcers are repeatedly presented. Sensitization dominates during the first few reinforcer presentations. It increases the effectiveness of the reinforcer and is primarily responsible for the early-session increases in operant responding. Habituation dominates later, after several reinforcers have been presented. It decreases the effectiveness of the reinforce and is primarily responsible for the late-session decreases in responding.

This hypothesis take place when some scientists use the Thompson and Spencer list of habituation properties to define habituation and sensitization in a reinforce. The within-session decreases in response rate take a typical negative exponential form (e.g., McSweeney, Hinson, & Cannon, 1996). Spontaneous recovery occurs between experimental sessions (e.g., Aoyama & McSweeney, 2001a; McSweeney & Johnson, 1994; Murphy, McSweeney, Kowal, McDonald, & Wiediger, 2006). The decreases in response rates are faster when stimuli are presented at faster than at slower rates (e.g., McSweeney, 1992). The decreases may be steeper for less, than for more intense, stimuli (Melville, Rue, Rybiski, & Weatherly, 1997). Longterm habituation occurs (e.g., McSweeney, 1992; McSweeney, Weatherly, & Swindell, 1995b). Dishabituators also lose their ability to restore habituated responding with their repeated presentation (Murphy et al., 2006).

In conclusion habituation and sensitization (or better depression and facilitation) might provide a general-process contributor to the regulation of many goal-directed behaviours. According to this explanation, each motivated behaviour is maintained by a reinforcer (e.g., food for eating; the drug for drug taking; money for money seeking). The behaviour stops when habituation decrease the reinforcer so that the reinforcer no longer supports behaviour directed towards it. The behaviour resumes when spontaneous recovery sufficiently restores the effectiveness of the reinforcer so that it again supports behaviour. It should be noted that this model does not argue that habituation is the sole regulator of all goal-directed behaviour. Instead, the model argues that habituation and sensitization are one of several variables that could contribute to the regulation of many motivated behaviours.

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