Chronic intracellular Ca$^{2+}$ buffering shapes Ca$^{2+}$ oscillations in developing spinal interneurons

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ABSTRACT

During the development of spinal cord, the maturation of neuronal circuits is a complex process, involving genetic and epigenetic mechanisms cooperating for the maturation of motor control (Jessell, 2000; Kiehn, 2006). Variations in the concentration of intracellular Ca$^{2+}$ are crucial signals in this process of maturation; in fact, Ca$^{2+}$ signals may lead to the emergence of specific neuronal phenotypes or guide the formation of cellular connectivity.

The organotypic cultures of embryonic mouse spinal cord represent an ideal experimental approach to study the maturation and physiology of the individual neurons and spinal networks. In fact, this experimental model reproduces in vitro the heterogeneous populations of cells, the three dimensional connections between these cells and the basic cytoarchitecture of the spinal cord observed in in vivo development (Avossa et al., 2003).

In this experimental model, three different types of Ca$^{2+}$ activity have been identified and characterized: waves, bursts and oscillations (Fabbro et al., 2007; Sibilla et al., 2009). These Ca$^{2+}$ signals are all generated by ventral interneurons, but each of them shows a specific pattern of expression during development and has different underlying mechanisms.

In this thesis, I focused my attention on the most peculiar of these Ca$^{2+}$ signals: the electrical activity-independent Ca$^{2+}$ oscillations. The main aim of my thesis was to better clarify the mechanisms underlying the generation and role of Ca$^{2+}$ oscillations in spinal neurons, by investigating the effects of pharmacological manipulation of intracellular Ca$^{2+}$ buffering on Ca$^{2+}$ oscillations behavior, neuronal biophysical properties and neuronal network activity in organotypic spinal cultures. To this aim, I treated spinal cord slices with two different intracellular Ca$^{2+}$ buffers, BAPTA-AM and EGTA-AM, and I monitored their impact using both Ca$^{2+}$-imaging and single cell patch-clamp techniques.

Initially, I investigated the effects on Ca$^{2+}$ oscillations induced by both chronic and acute treatment with BAPTA-AM. For the first time I described a change in the activity of oscillating neurons. In particular, after chronic incubation with BAPTA-AM, I
reported a significant increase in the number of neurons recruited to generate $\text{Ca}^{2+}$ oscillations, which was accompanied by a modulation of oscillations kinetic. $\text{Ca}^{2+}$ oscillations recorded after chronic incubation with BAPTA-AM maintained their peculiar features (Fabbro et al., 2007; Sibilla et al., 2009), in particular their $\text{Ca}^{2+}$-dependence, thus supporting the idea that the BAPTA-induced oscillations represent an amplification of the true oscillations phenomena, amplified by a prolonged intracellular $\text{Ca}^{2+}$ buffering. Despite a potentiating effect of chronic BAPTA-AM treatment on $\text{Ca}^{2+}$ oscillations, its acute application completely blocked $\text{Ca}^{2+}$ oscillations in all neurons.

The next step was to verify whether the observed effects could be related to changes in the biophysical properties of neurons or in neuronal network electrical activity. By patch clamp experiments I showed that the chronic BAPTA-AM treatment induces a significant enhancement in the frequency of heterogeneous (GABA-glycine and AMPA mediated) spontaneous post-synaptic currents (PSCs) when compared to untreated cultures. Neuronal membrane capacitance and input resistance were comparable to those of control neurons, thus confirming neuronal health.

As the reported results pointed to an increased excitability at the level of single neuron, I analyzed the impact of BAPTA treatment on the functional expression of a family of channels extremely important in the regulation of neuronal excitability: voltage-gated $\text{K}^{+}$ channels. I observed the presence of a significant increase in the amplitude of $\text{K}^{+}$ currents ($I_K$) in slices chronically treated with BAPTA-AM. To analyze the type of $I_K$ involved I separated the different $I_K$ components ($\text{Ca}^{2+}$-dependent $-I_{K(Ca)}$, transient $-I_{K(A)}$ and delayed-rectifier $-I_{K(DR)}$), demonstrating that, in BAPTA-AM treated cultures, the $I_{K(Ca)}$ and $I_{K(A)}$ components were similar to control cultures. Conversely, I found a potentiation of $I_{K(DR)}$, i.e. an increase in its maximal current amplitude. Furthermore, I found that acute application of BAPTA-AM partially reduces the magnitude of total $I_K$. Action potentials are other critical players reflecting neuronal excitability. Chronic BAPTA-AM treatment did not affect action potential kinetic; however, I found that BAPTA-treated neurons show a different distribution profile of excitability, with a widening of the population of ventral spinal interneurons displaying a tonic firing pattern and a decrease in the one showing an adapting firing behavior.

To explore the specificity of BAPTA-AM effects I employed another intracellular $\text{Ca}^{2+}$ chelator: EGTA-AM. I reported, as a consequence of a chronic EGTA-AM treatment of spinal neurons, an increase in the population of oscillating neurons (similarly to BAPTA treatment), but, without changes in oscillations kinetic. However, the study of synaptic
activity in EGTA-AM treated slices did not reveal any change in the frequency or kinetic of spontaneous or miniature PSCs. Interestingly, in contrast to BAPTA treatment, EGTA-AM had no effect on $I_K$.

Overall, the results reported in this thesis show, on one hand, a specific effect of BAPTA-AM on $K^+$; most importantly, on the other hand, they support the needing of a correct intracellular $Ca^{2+}$ homeostasis for the genesis of $Ca^{2+}$ oscillations and indicate the presence of a homeostatic adaptation as a rebound effect of chronic manipulation of intracellular $Ca^{2+}$.
Durante lo sviluppo del midollo spinale la maturazione dei circuiti neuronali è un processo complesso, che coinvolge meccanismi genetici ed epigenetici che promuovono la maturazione del controllo motorio (Jessell, 2000; Kiehn, 2006). Variazioni della concentrazione di Ca$^{2+}$ intracellulare sono un segnale cruciale in questo processo di maturazione; infatti, i segnali al Ca$^{2+}$ possono portare all’emergere di specifici fenotipi neuronalici o guidare la formazione della connettività cellulare.

Le colture organotipiche di midollo spinale di embrioni di topo rappresentano un approccio sperimentale ideale per studiare la maturazione e la fisiologia sia dei singoli neuroni spinali che dei networks sinaptici. Infatti, questo modello sperimentale riproduce in vitro l’eterogeneità della popolazione neuronale, le relazioni anatomico-funzionali tra queste cellule e la citoarchitettura del midollo spinale, osservati nello sviluppo in vivo (Avossa et al., 2003).

In questo modello sperimentale, tre diversi tipi di segnali al Ca$^{2+}$ sono stati identificati e caratterizzati; onde, bursts e oscillazioni (Fabbro et al., 2007; Sibilla et al., 2009). Questi segnali al Ca$^{2+}$ sono tutti prodotti da interneuroni ventrali, ma ciascuno mostra uno schema di espressione spazio-temporale specifico durante lo sviluppo e riconosce meccanismi diversi di generazione.

Nel mio progetto di tesi ho focalizzato l’attenzione sul più peculiare di questi segnali al Ca$^{2+}$: le oscillazioni. Lo scopo principale del mio progetto di tesi è stato meglio chiarire i meccanismi alla base della generazione ed il ruolo delle oscillazioni al Ca$^{2+}$ nei neuroni spinali, attraverso lo studio degli effetti della manipolazione farmacologica del buffering del Ca$^{2+}$ intracellulare sul comportamento delle oscillazioni, sulle proprietà biofisiche dei neuroni e sull’attività della rete neurale di colture organotipiche spinali. A tal fine, ho trattato fettine di midollo spinale con due diversi chelanti intracellularari del Ca$^{2+}$, BAPTA-AM e EGTA-AM, e, successivamente, ho monitorato gli effetti sul normale sviluppo utilizzando sia la tecnica del Ca$^{2+}$ imaging che quella del patch clamp a singola cellula.

Inizialmente ho investigato gli effetti sulle oscillazioni al Ca$^{2+}$ indotti da trattamenti sia cronici che acuti con BAPTA-AM. Per la prima volta ho descritto un cambiamento...
nell’attività dei neuroni oscillanti. In particolare, dopo l’incubazione cronica con BAPTA-AM, ho documentato un significativo aumento del numero di neuroni in grado di generare oscillazioni al Ca$^{2+}$, il quale è stato accompagnato da una modulazione delle cinetica delle oscillazioni. Le oscillazioni al Ca$^{2+}$ registrate dopo trattamento cronico con BAPTA-AM hanno mantenuto le loro peculiari caratteristiche (Fabbro et al., 2007; Sibilla et al., 2009), in particolare la loro dipendenza dal Ca$^{2+}$ extracellulare, consentendo di sostenere l’idea che le oscillazioni BAPTA-indotte rappresentino lo stesso fenomeno di quelle spontanee amplificato dal prolungato aumento del buffering del Ca$^{2+}$ intracellulare. A differenza dell’effetto potenzante del trattamento cronico con BAPTA-AM sulle oscillazioni, la sua applicazione acuta ha indotto un bloccato completo delle oscillazioni in tutti i neuroni che presentavano questo tipo di attività. Il passo successivo è stato chiedersi se questi cambiamenti potessero essere in qualche modo legati a variazioni delle proprietà intrinseche dei neuroni o nell’attività elettrica della rete neuronale. Ho riportato, con la tecnica del patch clamp, che il trattamento cronico con BAPTA-AM induce un significativo incremento della frequenza delle correnti spontanee post-sinaptiche eterogenee (GABA-glicina ed AMPA mediate) rispetto al controllo. La capacità di membrana e la resistenza di input erano, invece, comparabili a quelle dei neuroni di controllo, confermando il buono stato di salute neuronale. Poiché i risultati precedenti hanno indicato potenzialmente un aumento di eccitabilità a livello del singolo neurone, ho analizzato l’impatto del trattamento con BAPTA-AM sull’espressione funzionale di una famiglia di canali, i canali al K$^+$ voltaggio dipendenti, molto rilevante nella regolazione dell’eccitabilità neuronale. Ho documentato la presenza di un significativo aumento di ampiezza delle correnti al K$^+$ (I_k) in fettine cronicamente trattate con BAPTA-AM. Per analizzare quale tipo di I_k contribuisse in maniera specifica a questo aumento di ampiezza ho separato le diverse componenti di I_k (Ca$^{2+}$-dipendenti - I_k(Ca) - , transienti - I_k(A) - e delayed-rectifier - I_k(DR) -), dimostrando che in colture cronicamente trattate con il BAPTA-AM le componenti I_k(Ca) e I_k(A) hanno ampiezze simili a quelle di controllo. Viceversa, ho notato un potenziamento funzionale delle I_k(DR), ossia un aumento dell’ampiezza massima della corrente. Inoltre, ho osservato che l’applicazione acuta del BAPTA-AM riduce parzialmente l’ampiezza delle I_k. Poiché anche i potenziali d’azione hanno risentito della diversa eccitabilità neuronale, ho analizzato le proprietà di firing di questi neuroni. Il trattamento con il BAPTA-AM
non ha alterato la cinetica dei potenziali d’azione, tuttavia, ho riscontrato che neuroni trattati con BAPTA-AM hanno mostrato un diverso profilo di distribuzione dell’eccitabilità, con un aumento dei neuroni ventrali che mostrano un comportamento di firing di tipo “tonic” ed una reciproca riduzione di quelli “adapting”.

Al fine di esplorare la specificità degli effetti del BAPTA-AM ho utilizzato un altro chelante intracellulare del Ca$^{2+}$: l’EGTA-AM. Ho riportato, come conseguenza al trattamento cronico con EGTA-AM dei neuroni spinali, un aumento di neuroni in grado di generare oscillazioni (in maniera analoga a quanto riportato con il BAPTA-AM), non riscontrando tuttavia nessuna variazione della cinetica delle oscillazioni stesse. Tuttavia, lo studio dell’attività sinaptica nelle fettine cronicamente trattate con l’EGTA-AM non ha rilevato alcun aumento di frequenza o cambiamento di cinetica nelle correnti post-sinaptiche sia spontanee che in miniatura. È interessante notare che, a differenza del trattamento con il BAPTA-AM, l’EGTA-AM non sembra avere avuto alcun effetto diretto sulle $I_K$.

Complessivamente i risultati riportati in questa tesi se, da una parte, mostrano un effetto inibitorio specifico del BAPTA-AM sui canali al K$^+$, dall’altra supportano l’idea che il normale controllo dell’omeostasi intracellulare del Ca$^{2+}$ è indispensabile nella genesi delle oscillazioni e validano la presenza di un adattamento omeostatico come recupero dalla manipolazione cronica del Ca$^{2+}$ intracellulare.
INTRODUCTION

The spinal cord is a long and cylindrical structure of nervous tissue that extends caudally from the brain and, together with it, is part of the Central Nervous System (CNS). The spinal cord is the main connection between the brain and the rest of the body; it gathers information from the body and sends it to the brain, it also allows the brain to send orders and messages to the muscles and glands. According to its rostrocaudal location the spinal cord can be divided into four parts: cervical, thoracic, lumbar and sacral, each of which comprise several segments.

A transversal section of the adult spinal cord shows white matter (containing myelinated and unmyelinated nerve fibers) in the periphery, gray matter (mainly containing cell bodies of neurons and glial cells) inside and a tiny central canal filled with cerebrospinal fluid (CSF). The gray matter shaped like a “butterfly” is made up of the dorsal horns, which comprises of sensory nuclei that receive and process incoming somatosensory information, and the ventral horns, which comprises motor neurons that innervate muscles.

The neuronal circuits in the adult mammalian spinal cord can generate rhythmic oscillatory activity that is transformed into locomotive commands, such as walking and breathing, which are necessary for animals to survive (Marder and Rehm, 2005). This motor program depends upon activity of central pattern generators (CPGs), which are a complex, distributed spinal neuronal network that can endogenously produce rhythmic patterned outputs (Marder and Calabrese, 1996; Kiehn and Butt, 2003). The concept of CPGs is based on experiments showing that neuronal spinal networks are capable of producing rhythmic locomotive movements when the spinal cord is isolated from the brain and sensory inputs (Sherrington, 1910; Brown, 1911; Rossignol, 1996; Kiehn et al., 1997). In healthy conditions, the CPGs interact with proprioceptive feedback and receives descending signals from the higher-level (supra-spinal) centers. The higher centers, in turn, may select and initiate the appropriate motor programs from the repertoire of the spinal cord. The descending commands from supra-spinal centers to spinal interneurons are automatically integrated into the current state of proprioceptive and exteroceptive information (McCrea, 1996). Therefore, locomotion results from a
complex interplay between the CPGs, descending signals and multiple feedback and feed forward modulatory signals (Nistri et al., 2006; Taccola and Nistri, 2006). Various models of CPGs operation assume as crucial the role of a class of spinal interneurons (located ventrally to the central canal), that via commissural interneurons distribute synaptic inputs to left and right motor pools of the hind-limb muscles (Kiehn et al., 2000; Grillner and Wallén, 2002; Kiehn and Kullander, 2004). The synaptic nature of these interneuronal connections has been shown to be excitatory, mediated by glutamate, and inhibitory, mostly mediated by glycine or combined glycine/GABA. In particular, the interplay between the excitation and inhibition is crucial for the segmental left–right alternation and in segmental and inter-segmental crossed motor synergies during locomotion (Kiehn et al., 1997; Hochman and Schmidt, 1998; Kiehn, 2006).

Interneurons are probably the most important modulating cell types in the spinal cord, in fact, they integrate supraspinal commands and sensory inputs and control the excitation or inhibition of spinal motor neurons. Hence, the fine motor control mostly depends on the integrity of spinal interneuronal networks (Nógrádi and Vrbová, 2000). Although interneurons have an important role in the generation of motor patterns, little is known about their identity, function and role in the formation of the network to which they pertain.

**Diversification and differentiation of spinal neurons**

The spinal cord is assembled with remarkable precision during embryonic development, the selectivity inherent in its formation helps to establish functional spinal circuits, which are needed to integrate inputs originated from the higher centres or the periphery and to generate and maintain rhythmic motor outputs, including repetitive alternation of left-right and extensor-flexor muscle contractions as key motor output behaviours. Recent studies have begun to address the important question of how diversification programs established during development control the emergence of functionally distinct neuronal subpopulations required to support these tasks. They highlight the importance of genetic programs and time of neurogenesis in setting up a spatial matrix in which
terminally differentiated neuronal subpopulations are interconnected in highly precise patterns (Jessell, 2000; Arber, 2012).

Spinal cord neurons are derived from local progenitors. Spinal progenitor cells are arrayed at conserved dorsoventral positions along the midline and proliferate to give rise to postmitotic neurons during temporally restricted periods. Early action of ventral sonic hedgehog (Shh) and dorsal bone morphogenetic protein (BMP) signalling sources leads to spatial subdivision of progenitor domain territory along the dorsoventral axis (Jessell, 2000; Arber, 2012).

In particular, the main signalling involved in the differentiation of motor neurons and ventral interneurons is the response to the gradient of the protein Shh, secreted by the notochord and floor plate. In fact, ectopic expression of Shh in vivo and in vitro can induce the differentiation of motoneurons and ventral interneurons. Conversely, elimination of Shh signalling by antibody blockade in vitro or through gene targeting in mice prevents the differentiation of motor neurons and most classes of ventral interneurons (Jessell, 2000; Martí and Bovolenta, 2002). Several studies provide evidence that a group of homeodomain proteins express by ventral progenitor cells, might sense graded Shh signalling (Briscoe et al., 1999; Pierani et al., 1999; Briscoe et al., 2000). These homeodomain factors fall into two classes (class I and class II proteins), identified by their expression pattern and their Shh regulation modality. Shh mediates the repression of class I homeodomain proteins (Pax7, Dbx1, Dbx2, Irx3 and Pax6) and the induction of expression of class II proteins (Nkx6.1 and Nkx2.2) at different threshold concentrations. This mechanism allows Shh to control neuronal fate and the establishing of different progenitor populations defined by the expression of Pax6 and Nkx2.2, in addition each progenitor domain generates a distinct class of postmitotic neurons (Briscoe et al., 2000) (Figure 1). These five ventral distinct populations of spinal neurons (V0–V3, VMN), can first be identified at about embryonic day 10 (E10). By E13, they begin to migrate toward their settling position which they reach before birth and where they remain postnatally.
Figure 1. Three phases of Shh-mediated ventral neural patterning. a) Shh mediates the repression of class I homeodomain proteins (Pax7, Dbx1, Dbx2, Irx3 and Pax6) at different threshold concentrations and the induction of expression of class II proteins (Nkx6.1 and Nkx2.2) at different threshold concentrations. Class I and class II proteins that abut a common progenitor domain boundary have similar Shh concentration thresholds for repression and activation of protein expression, respectively. Shh signalling defines five progenitor domains in the ventral neural tube. b) The pairs of homeodomain proteins that abut a common progenitor domain boundary (Pax6 and Nkx2.2; Dbx2 and Nkx6.1) repress each other’s expression. c) The relationship between neural progenitor (p) domains and the positions at which post-mitotic neurons are generated along the dorsoventral axis of the ventral spinal cord (Jessell, 2000).

The following sections report a brief summary of the works that have characterized the differentiation and the electrophysiological and anatomical properties of each of the ventral spinal neurons classes in mammals as well as their specific activity pattern and function during locomotion.

VO interneurons

These cells are generated between E10 and E13 from progenitors located at the dorsal–ventral boundary of the developing neural tube that express Dbx1 (Pierani et al., 1999). VO interneurons are derived from Dbx1-expressing progenitors and make up a diverse set of mostly commissural neurons, present in all spinal segments (Pierani et al., 1999), including excitatory (approximately one-third) and inhibitory (approximately two-thirds) populations (Lanuza et al., 2004). A recent study demonstrates diversification of VO interneurons into multiple, molecularly distinct, subclasses. Initially, the population was divided into the dorsally located (V0d), and ventrally located (V0v) cells. Both of
these subpopulations express the homeodomain protein Dbx1; however the V0v cells can be distinguished by postmitotic expression of the homeodomain protein Evx1 (Moran-Rivard et al., 2001). The majority of both the V0d and V0v subpopulations extend their axons commissurally (Moran-Rivard et al., 2001; Pierani et al., 2001). Recently, a third subpopulation of V0 cells has been identified (V0c), which express the transcription factor Pitx2 (Zagoraiou et al., 2009). These neurons are a subset of the V0v cells in fact, they transiently express Evx1 but, differently to V0v, they are cholinergic (V0c). Interestingly, these neurons share few characteristics with other members of the V0 population as they project their axons ipsilaterally and provide excitatory input onto motoneurons (Zagoraiou et al., 2009) (Figure 2).

![Diagram of V0 neuronal subpopulations](modified from Arber, 2012)

**Figure 2.** Diversification of V0 neuronal subpopulation in the spinal cord. Assigned function by genetic perturbation experiments is show below (modified from Arber, 2012).

The primary role for the V0 populations is to establish alternating left–right motor activity during locomotion (Lanuza et al., 2004), in fact the Dbx1 mutant mice, which lack all three subpopulations of V0 interneurons, exhibit frequent episodes of co-contraction between contralateral motor neurons. Interestingly, selective perturbation of cholinergic neurotransmission in V0c neurons, by eliminating choline acetyltransferase (ChAT) induces selective behavioural defects in task-dependent motor performance during swimming but not basic locomotion (Zagoraiou et al., 2009).
**V1 interneurons**

V1 interneurons are defined by the expression of the transcription factor Engrailed-1 (En-1). They are inhibitory and they send their axons along an ipsilateral trajectory and project rostrally for one to two segments. Anatomical studies have shown that Renshaw cells (Wenner et al., 1998; Saueressing et al., 1999; Wenner et al., 2000; ), Ia inhibitory interneurons (Alvarez et al., 2005) and several uncharacterized subpopulations derive from this progenitor (Figure 3).

![Figure 3](image)

**Figure 3.** Diversification of V1 neuronal subpopulation in the spinal cord.Assigned function by genetic perturbation experiments is show below (modified from Arber, 2012).

Inactivation of En1 gene in mice, for example, affects locomotive speed (Gosgnach et al., 2006), but it is difficult to predict how coincident elimination of Renshaw cells, a fraction of Ia inhibitory interneurons and a handful of other populations is comparable to unique perturbation of any one V1 subpopulation alone.

**V2 interneurons**

V2 interneurons, which are originated from Lhx3 progenitors, can be divided into multiple subtypes, each having unique characteristics. Ipsilaterally projecting excitatory V2a neurons express the transcription factor Chx10 (Peng et al., 2007; Crone et al., 2008), commissural projections inhibitory V2b mark by the expression of Gata2 and Gata3 (Ericson et al., 1997; Zhou et al., 2000; Karunaratne et al., 2002; Smith et al., 2002; Lundfald et al., 2007) and V2c interneurons positive for the transcription factor Sox1 (Panayi et al., 2010). Notch signalling through the regulation of the transcriptional
cofactor Lmo4 tilts the balance between V2a-V2b subtypes and contributes to diversification (Del Barrio et al., 2007; Lee et al., 2008; Joshi et al., 2009) (Figure 4).

Figure 4. Diversification of V2 neuronal subpopulation in the spinal cord. Assigned function by genetic perturbation experiments is show below (modified from Arber, 2012).

V2a interneurons are involved in left-right coordination in fact, selective ablation of these neurons, using transgenic methods, leads to deficits in right-left alternation of locomotion especially at higher locomotor speeds (Crone et al., 2009). Anatomical tracing studies reveal a direct excitatory input of V2a interneurons onto commissural interneurons that drive left-right alternation (Crone et al., 2008).

V3 interneurons
Little is known about diversification of excitatory and predominantly (=85%) controlaterally projecting V3 interneurons (Zhang et al., 2008). Postmitotically these cells express the transcription factor Sim1 (Briscoe et al., 1999; Goulding et al., 2002) and, before birth, they reach dorso-medial and ventro-medial regions of the spinal cord (Figure 5).
V3-derived interneurons are necessary for a robust and organized locomotive rhythm during walking, meaning that some intrinsic, measurable properties of the rhythm are kept invariant. In a transgenic mouse line, in which the V3 cells were selectively silenced, there is a marked irregularity of the locomotive rhythm, in which step-cycle period and burst duration varied from step to step and asymmetry was seen between ventral roots on the left and right sides of the spinal cord (Zhang et al., 2008).

**Hb9 neurons**

A homeodomain transcription factor, Hb9, is expressed by embryonic motor neurons (Arber et al., 1999) and a small population of interneurons (Hinckley et al., 2005; Wilson et al., 2005). Differently from motor neurons, which are placed in the ventrolateral spinal cord, Hb9 interneurons are located adjacent to the ventral commissure from cervical to midlumbar spinal cord segments (Hinckley et al., 2005; Wilson et al., 2005; Ziskind-Conhaim et al., 2010). Hb9 interneurons receive monosynaptic input from low threshold sensory afferents (Hinckley et al., 2010), they are glutamatergic, synaptically interconnected and project their axons ipsilaterally (Hinckley et al., 2005). It has been demonstrated that Hb9 interneurons are rhythmically active during locomotor-like ventral root discharges (Hinckley et al., 2005). However their role remains poorly described because, differently from other ventral interneuron populations, fictive locomotion cannot be elicited in the absence of Hb9 as it is also expressed in all motor neurons.
Spinal interneurons could be also diversified by combinatorial expression pattern of Ca$^{2+}$ binding proteins (CBPs) and by their position in the ventral horns. Calbindin is a good marker for Renshaw cells in the ventral spinal cord (Arvidsson et al., 1992; Sanna et al., 1993; Carr et al., 1994; Alvarez et al., 1999; Geiman et al., 2000), moreover the different expression of parvalbumin and calretinin allows to distinguish two different populations of V1 interneurons: the first one expresses calbindin and/or parvalbumin but little calretinin, the second does not express CBPs and is usually located in a more dorsal position.

**Role of electrical activity in the development of neural spinal networks**

A distinct feature of the nervous system is the intricate network of synaptic connections among neurons of diverse phenotypes. In a previous section, we have seen that several crucial events take place before the correct network connections are formed. At first, neural stem cells proliferate to generate neurons. Then, these new born neurons migrate to final target regions, where they complete their differentiation process. So initial connections are formed largely through molecular mechanisms that depend on intrinsic developmental programs. Although genetic instructions have a great importance, it is now widely accepted that electrical activity plays a central role in the development of the nervous system (Goodman and Shatz, 1993; Cline, 2003). There are two main forms of electric activity in the nervous system: spontaneous activity and experience-driven activity (Moody, 1998; Moody and Bosma, 2005). Usually spontaneous activity is detected at early stages of embryonic development, it is independent of sensory inputs or motor outputs and it is widespread; while experience-driven activity occurs later in development, during postnatal stages. Both types of activity regulate the maturation and refinement of neural circuits. In particular, spontaneous activity is thought to guide large rearrangements in the nervous circuits, while experience-driven activity contributes to the refinement and consolidates these circuits into mature forms (Penn and Shatz, 1999; Zhang and Poo, 2001).

In the spinal cord, as well as in other CNS areas, spontaneous activity usually comprises recurring episodes emerging more as a population behavior, due to the firing of large amounts of neurons, rather than the outcome of specific and localized rhythm
generating networks. This activity is characterized by bursts of action potentials rapidly spreading over the whole spinal segments (O’Donovan et al., 1998; Nakayama et al., 2001; Hanson and Landmesser, 2003; Ren and Greer, 2003; Yvert et al., 2004). These early forms of activity influence developmental events such as neuronal differentiation, synaptic connections, establishment of neurotransmitters phenotype and neuronal migration (Holliday and Spitzer 1990; Gu et al., 1994; Moody; 1998; Moody and Bosma, 2005). During spinal cord maturation this synchronous activity is replaced by mature locomotive patterns (Bate, 1999; Nishimaru and Kudo, 2000; Wolpaw and Tennissen, 2001).

Spontaneous activity in the developing spinal cord is accompanied, at the appropriate developmental stage, by contraction of the muscles innervated by the active neurons (Grillner et al., 1998; Rekling and Feldman, 1998; Tabak et al., 2000). The expression of spontaneous motility in most vertebrates develops in a rostral to caudal way, initially as relatively small random movement and proceeding to highly patterned motility with alternating flexor and extensor motor output as well as activity that alternates on the two sides of the body.

In both rat and mouse spinal cord development, spontaneous rhythmic activity can be observed early in embryogenesis (Nishimaru et al., 1996; Suzue, 1996; Branchereau et al., 2002; Hanson and Landmesser, 2003; Ren and Greer, 2003), before the innervation of muscles is complete (E11-14), when motoneurons are still migrating and extending axons (Nishimaru et al., 1996; Milner and Landmesser, 1999; Branchereau et al., 2000; Hanson and Landmesser, 2004). Episodic spontaneous activity is observed from E12–18; however, by E18.5 the pattern becomes variable (Branchereau et al., 2002). During the perinatal period, episodes of spontaneous activity can lead to occasional bouts of coordinated locomotor-like activity being expressed (Bonnot et al., 1998; Whelan et al., 2000). Moreover, in the rodent spinal cord maturation of spontaneous motility shifts during development from the cervical to the lumbar region: the motor activity at early stages is more expressed near the head (70% of the movements) whereas, while the cord matures, the activity in the lumbar segments becomes predominant (Moody and Bosma, 2005).

Thus, in mammals, the networks that drive rhythmic motor neuron activity are formed in the spinal cord at early stages of CNS maturation. These primitive networks are maintained at a later stage of development to adapt and perform the complex locomotor behaviors (Sillar et al., 1997) via subsequent functional changes.
A question arises: how does spontaneous activity influence the development of spinal circuits?

The available evidence suggests that spontaneous activity in the spinal cord is a network phenomenon that does not depend on pacemaker neurons. Two general features of developing networks are crucial for the expression of spontaneous activity. The first is the hyperexcitability nature of the network and the second is the presence of activity-dependent depression of neuronal and network excitability. It has been proposed that the conjunction of these two characteristics is responsible for the expression of spontaneous activity in the developing spinal cord (O’Donovan and Chub 1997; O’Donovan and Chub, 1998).

During development, the classical inhibitory neurotransmitters GABA and glycine can depolarize spinal neurons (Obata et al., 1978; Cherubini et al., 1991; Wu et al., 1992; Sernagor et al., 1995; Nishimaru et al., 1996; Leinekugel et al., 1997; Fischer et al., 1998; Milner and Landmesser, 1999; Hanson and Landmesser, 2006). It is assumed that this occurs because the intracellular Cl⁻ concentration of developing spinal neurons is maintained at a higher concentration than in the adult (Wu et al., 1992). Thus, the absence of conventional hyperpolarizing inhibition results in the high excitability of developing spinal networks. This increased excitability induces a temporary reduction of the efficacy of synaptic connections as a function of activity (Fedirchuk et al., 1999; Tabak et al., 2000; Chub and O’Donovan, 2001; Marchetti et al., 2005; Chub et al., 2006): immediately after an episode of spontaneous activity the majority of neurons are depolarized, so the ongoing synaptic excitation within the network is not powerful enough to trigger another event. As the network recovers from the previous event the ongoing synaptic excitation increases in efficacy, until the neurons reciprocally excite each other enough to trigger another network-encompassing event. In this scenario, the long interval between events is due to the relatively slow re-accumulation of Cl⁻ in neuron dendrites via Cl⁻ transporters (Chub and O’Donovan, 2001; Marchetti et al., 2005; Chub et al., 2006). Evidence for a reduction in the excitatory drive is provided by the reduction of the size of GABA_A mediated postsynaptic currents following a network event. In addition, blockade of the chloride-accumulating transporter NKCC1 (in the presence of ionotropic glutamate receptor antagonists, so that excitatory glutamate transmission is also absent) blocks spontaneous network activity during development (Marchetti et al., 2005), indicating that lowering levels of intracellular Cl⁻ reduces the excitability of the network.
In the last few years, several studies have tried to understand how spontaneous activity can influence the spinal cord development. Several evidences show that the developmental instructions brought by spontaneous activity are transduced by a cascade of events beginning with the entry of Ca\(^{2+}\) ions, through voltage-gated Ca\(^{2+}\) channels, leading to transient elevation in the cytoplasmic Ca\(^{2+}\) concentration in fetal spinal neurons (Holliday and Spitzer, 1990; Wong et al., 1995; Moody, 1998). In fact, Ca\(^{2+}\) channels blockers repress activity-dependent developmental events (Komuro and Rakic, 1992; Linsdell and Moody, 1994; Moody, 1998; ), while artificially reproducing Ca\(^{2+}\) transient can rescue activity-deprived cells (Gu and Spitzer, 1995; Moody, 1998). Ca\(^{2+}\) plays an important role in regulating a great variety of cellular processes: proliferation, gene transcription, contraction and secretion. This versatility emerges from the use of an extensive molecular repertoire of signaling components, which include a Ca\(^{2+}\) signaling toolkit that can be assembled in combinations to create signals with widely different spatial and temporal profile. The intracellular Ca\(^{2+}\) is carefully controlled from the cell through specific Ca\(^{2+}\) ON/OFF mechanisms which recruits different array of channels, pumps and exchanger. The Ca\(^{2+}\) flowing through these channels constitute the elementary event of Ca\(^{2+}\) signaling. Ca\(^{2+}\) can act within milliseconds in highly localized regions or it can act much more slowly as a global wave that spreads the signal throughout the cell and between coupled neurons, passing through gap junctions, to coordinate the activities of whole tissues or organs (Bootman et al., 2001). However, the cells have to handle Ca\(^{2+}\) with care, in fact exceeding its normal and spatial boundaries can result in cell death through apoptosis (Berridge, 1998; Berridge et al., 2000; Bootman et al., 2001). For this reason, various pumps and exchangers, such as for instance mitochondrial membrane Ca\(^{2+}\) ATPase and Na\(^+/\) Ca\(^{2+}\) exchange, are responsible for returning the elevated levels of Ca\(^{2+}\) back to the resting state.
Figure 6. Diagram of the wide variety of developmental events triggered by spontaneous activity. The blue boxes at the top indicate events that are not linked to the influx of Ca\(^{2+}\) during activity, but rather directly to changes in membrane potential or increases in [Na]. Red dashed lines and arrows indicate negative-feedback loops. Green dashed lines and arrows indicate positive-feedback loops (Moody and Bosma, 2005).

A good example of how the different combinations of ON/OFF mechanism can induce a wide range of process is represented by neurons. In fact, Ca\(^{2+}\) ions in these cells have a crucial role in the reception and transmission of signals, in the regulation of neuronal excitability as well as the cellular changes that underlie learning and memory (Figure 6). Moreover in neurons Ca\(^{2+}\) signaling toolkit can be mixed and matched to create a wide range of spatial and temporal signals. They can generate Ca\(^{2+}\) signals that are restricted in tiny volumes of spines or larger signals that spread over many dendrites, perhaps reaching the soma and axon (Bootman et al., 2001).
Ca\textsuperscript{2+} signalling in spinal cord development

Ca\textsuperscript{2+} ions act as a second messenger inside the cell to mediate a wide spectrum of cellular functions. At their resting state, cells maintain a baseline of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) at approximately 100-150 nM through Ca\textsuperscript{2+} homeostasis mechanisms, [Ca\textsuperscript{2+}]i is much lower than the extracellular one (1.5-1.8 mM). This basal concentration is crucial for cells to respond effectively to various Ca\textsuperscript{2+} signals elicited by extracellular stimuli or membrane depolarizations that often reach a concentration of several hundred nanomolar to a few micromolar (Clapham, 1995; Berridge et al., 2003). Ca\textsuperscript{2+} signals are often amplified by further Ca\textsuperscript{2+} release from internal stores, such amplification depends on the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mediated by the ryanodine and IP3-sensitive channels in the membrane of the internal stores (Berridge et al., 2000). The Ca\textsuperscript{2+} ion diffuses very slowly in the cytoplasm (diffusion coefficient at 10 \( \mu \text{m}^2 \text{s}^{-1} \)) (al-Baldawi and Abercrombie, 1995; Murthy et al., 2000; Nakatani et al., 2002) owing to the abundant immobile cytoplasmic Ca\textsuperscript{2+}-binding proteins (Blaustein, 1988; Baimbridge et al., 1992). Therefore, Ca\textsuperscript{2+} signals are typically localized by limited diffusion but may become global when substantial internal release is involved. Limited activation of Ca\textsuperscript{2+} channels in the plasma membrane or in the ER can result in high [Ca\textsuperscript{2+}]i only near the channels, creating a microdomain of [Ca\textsuperscript{2+}]i elevation or Ca\textsuperscript{2+} “sparks.” The spatiotemporal properties of cytoplasmic Ca\textsuperscript{2+} signals depend on the number and extent of activation of Ca\textsuperscript{2+} channels in both the plasma membrane and the membrane of internal stores (Figure 7).
Figure 7. Intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) increases through Ca$^{2+}$ influx from extracellular pools through various channels (voltage-, ligand- or concentration-gated channels). Ca$^{2+}$ concentration can also increase through release from endoplasmic reticulum stores through the ryanodine (RyR) and inositol-1,4,5-trisphosphate receptors (Ins(1,4,5)P$_3$R), or from mitochondrial stores through the mitochondrial Na$^+$/Ca$^{2+}$ exchange (MNCX) and mitochondrial pores opened during mitochondrial permeability transition (MPT). Counterbalancing mechanisms fight to halt Ca$^{2+}$ concentration increase in the cytoplasm. The plasma membrane Ca$^{2+}$ pump (PMCA), Na$^+$/Ca$^{2+}$ exchange (NCX), sarco-endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA), mitochondrial membrane Ca$^{2+}$ ATPase (MMCA) and relaxed specificity channels (Uniporter) function to restore normal Ca$^{2+}$ levels. Ca$^{2+}$-binding proteins in the cytoplasm and in the endoplasmic reticulum offer additional Ca$^{2+}$ buffering capacity (Syntichaki and Tavernarakis, 2003).

In neurons, transient elevations of [Ca$^{2+}$]$_i$ convey and convert different patterns of spontaneous activity into distinct biochemical events to properly modulate different neuronal functions. In fact, changes in [Ca$^{2+}$]$_i$ with different spatial and temporal characteristics can generate even opposite cellular responses due to various downstream targets that are modulated by intracellular Ca$^{2+}$ signals. In many cases the resulting
transient increases in \([\text{Ca}^{2+}]_i\) is linked to the expression of specific genes (Dolmetsch et al., 1998; Greer and Greenberg, 2008). In other cases, \(\text{Ca}^{2+}\) activates cytoskeletal elements or exocytosis to carry out its developmental roles (Watt et al., 2000; Moody and Bosma, 2005).

In developing neurons transient elevations of \([\text{Ca}^{2+}]_i\) contribute to the specification of neuronal subtype through the regulation of axon path finding, dendritic growth and arborisation and specification of neurotransmitter subtype. Increases in \([\text{Ca}^{2+}]_i\) levels, resulting from membrane depolarization, affect the frequency and stability of contacts because they may regulate filopodial motility, which influences the establishment of synaptic contacts at the level of axonal growth cone, or the formation of postsynaptic dendrites (Lendvai et al., 2000). Moreover neuronal activity generated by growing axons can trigger the secretion of neurotransmitters from growth cones, allowing the onset of synaptic activity between growth cones and target neurons (Xie and Poo, 1986).

The development of a functional ventral spinal network comprises intrinsic genetic programs and epigenetic mechanisms (Jessell, 2000; Kiehn, 2006). Among epigenetic mechanisms, transitory elevation of \([\text{Ca}^{2+}]_i\) levels have been implicated in the regulation of various stages of neuronal development including: proliferation, migration, differentiation, formation of neuronal connectivity and survival (Komuro and Rakic, 1992; Komuro and Rakic, 1993; Gu and Spitzer; 1997; Berridge et al., 2000; Spitzer et al., 2000; Spitzer, 2002).

Embryonic spinal neurons usually express widespread synchronous \(\text{Ca}^{2+}\) transients, both in culture and in the intact spinal cord. Based on kinetic properties, these fluctuations are distinguished as two types of activity: fast \(\text{Ca}^{2+}\) bursts (Kudo et al., 1991; Branchereau et al., 2000; Hanson and Landmesser, 2003) and slow \(\text{Ca}^{2+}\) waves (Gu et al., 1994; Spitzer, 1994; Gu and Spitzer; 1995; Gu and Spitzer; 1997). Bursting activity consists in synchronous firing of large number of neurons, during bursts of neuronal activity, the \(\text{Ca}^{2+}\) that enters from the outside is taken up by the internal store. Whereas, \(\text{Ca}^{2+}\) waves initiate in random area and spread to neighbouring cells through the gap junctions. Mechanisms to explain the propagation of the wave are all based on a positive feedback process, in which the \(\text{Ca}^{2+}\) increase at the initial site diffuses and activates closet stores, which release more \(\text{Ca}^{2+}\) further outwards to hit additional stores (Berridge and Dupont, 1994).
This collective synchronous activity shown during spinal network maturation is assumed to play an essential role in establishing appropriate connections, because they drive (mostly via transient Ca^{2+} elevations) network refinement and synaptic consolidation (Feller, 1999).

Manipulation of intracellular Ca^{2+}

In the previous sections we have seen that Ca^{2+} signals play a significant role in nervous system development, involved in neural induction, proliferation, migration, axon guidance and growth cone morphology (Gu et al., 1994; Komuro and Rakic, 1996; Leclerc et al., 1997; Weissman et al., 2004; Jacques-Fricke et al., 2006). Several studies tried to alter Ca^{2+} regulation, using synthetic exogenous Ca^{2+}-chelators, to better understand the influence of Ca^{2+} ions on neuronal maturation (Hirai et al., 1999; Ramakers et al., 2001; Ringler et al. 2008).

Nowadays, there are a lot of organic Ca^{2+} buffers, everyone with a typical binding kinetic and binding affinity. Such chelators, usually also bind other di- and trivalent cations, which will interfere with Ca^{2+} binding. Of particular importance are Mg^{2+} ions, because there are present in cells at millimolar concentration. The first Ca^{2+} buffers to be described was ethylenediaminetetraacetic acid (EDTA), which binds Ca^{2+} or Mg^{2+} via four carboxylate and two amine groups (Kaplan and Ellis-Davies, 1988) (Figure 8). However, the main Ca^{2+} buffers used in biological experiments are ethylene glycol-bis(2-aminoethylether)-N,N′,N″,N‴-tetraacetic acid (EGTA) and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N‴-tetraacetic acid (BAPTA) because they bind Ca^{2+} with high selectivity over Mg^{2+} (Tsien, 1980; Pethig et al., 1989) and with a 1:1 stoichiometry. The structure of BAPTA is similar to that of EGTA, but differs in the switch from the aliphatic amino groups of EGTA to the aromatic amino groups in BAPTA (Figure 8). This is an important feature because the aliphatic amines of EGTA have pK_{a} values between 8.5 and 9.5, whereas in BAPTA they are between 6 and 6.3 (Martel and Smith, 1974; Tsien, 1980). The lower pK_{a} of the BAPTA aromatic amines makes this chelator less sensitive to pH and kinetically faster than EGTA.
Figure 8. Structure of Ca\textsuperscript{2+} chelators commonly used in biological experiments.

All these Ca\textsuperscript{2+} buffers are unable to cross lipid membranes due to their nature, making necessary the use of physical methods, such as including the chelators in the patch pipette, or chemical methods, like using acetoxyethyl ester (AM) derivatives which make the buffers lipophylic, to load them inside the cell.

The loading of these compounds into neurons is reversible, because they are metabolized and actively extruded (by organic anion transport mechanisms) from the cell (Di Virgilio et al., 1990; Munsch and Deitmer, 1995; Ouanounou et al., 1996). These features make Ca\textsuperscript{2+} buffers ideal for reversibly manipulating cytoplasmatic Ca\textsuperscript{2+} buffering in neurons.
The physiological effects of exogenous Ca\textsuperscript{2+} buffers are well characterized, including their presynaptic effects on attenuating neurotransmitter release (Adler et al., 1991; Niesen et al., 1991; Fredholm and Hu, 1993; Roberts, 1993; Robitaille et al., 1993; Winslow et al., 1994; Tymianski et al., 1994b; Ouanounou et al., 1996; Spigelman et al., 1996), postsynaptic effects on neuronal membrane excitability (Lancaster and Nicoll, 1987; Kohr and Mody, 1991; Schwindt et al., 1992) and Ca\textsuperscript{2+} homeostasis (Neher and Augustine, 1992; Zhou and Neher, 1993; Tymianski et al., 1994a).

Others studies show that the increasing of Ca\textsuperscript{2+} buffering capacity of neurons reduces the neurotoxicity due to endogenous synaptic overactivity; the Ca\textsuperscript{2+} chelators may act postsynaptically by reducing cytosolic Ca\textsuperscript{2+} increases in neurons (Tymianski et al., 1993; Tymianski et al., 1994a) and/or presynaptically by attenuating synaptic transmitter release (Adler et al., 1991; Niesen et al., 1991; Tymianski et al., 1994b; Ouanounou et al., 1996; Spigelman et al., 1996).

In this study I investigated the changes induced by chronic and acute intracellular Ca\textsuperscript{2+} manipulation on Ca\textsuperscript{2+} oscillations behaviour, neuronal network activity and neuronal biophysical properties in organotypic cultures developed from embryonic mouse spinal cord. These cultures are a useful model for studies requiring long term survival of the preparation, such as studies that involve chronic incubation with drugs or toxins, because they mimic some important features of spinal segment development in vivo (Avossa et al., 2003; Rosato-Siri et al., 2004; Furlan et al., 2005; Furlan et al., 2007; Sibilla et al., 2009).

**Organotypic culture**

Embryonic organotypic cultures represent an ideal experimental approach to investigate neuronal maturation and physiology of the spinal cord. This experimental model provides flexible access to specific neural circuits and maintains their basic properties, allowing them to reproduce most of their natural network activities. In fact, organotypic slices reproduce in vitro the heterogeneous populations of cells, the three dimensional connections between these cells, the basic cytoarchitecture of the spinal cord, including
the dorsal-ventral orientation of the spinal segments, the spatial-temporal regulation of neuronal and non-neuronal markers and the complex electrophysiological pathways of network dynamics found in vivo (Streit et al., 1991; Streit, 1993; Ballerini and Galante, 1998; Ballerini et al., 1999; Galante et al., 2000; Galante et al., 2001; Rosato-Siri et al., 2002; Avossa et al., 2003) (Figure 9).

Figure 9. Immunocytochemistry of organotypic cultures with the anti-NF-H antibody SMI32. A) Organotypic culture after 8 DIV: the black arrows indicate the SMI32-positive processes exiting bilaterally from the ventral part of the slice. Note that cell body staining is not very apparent at this stage. B) Organotypic culture after 14 DIV: motoneurons are located near to the ventral fissure whereas, DRG neurons are positioned laterally to the slice. Note the extent of neuronal processes exiting from the slice. C) Organotypic culture after 21 DIV: motoneurons and DRG neurons are both located in the ventral region (Avossa et al., 2003).

Furthermore, within organotypic cultures, the distribution of extracellular signalling molecules may be replicated more faithfully since their local concentration may be elevated in the intercellular spaces within the tissue, whereas in dissociated cultures these molecules may diffuse away freely once released. Although they cannot exactly mimic the in vivo situation because the inter-segmental connections of the spinal cord are lost, organotypic cultures may offer a useful compromise between dissociated cultures and in vivo models.

Since organotypic slices can be maintained in vitro for several weeks, they allow to evaluate long-term effects of acute experimental manipulations, as well as the effects of chronic treatments on neuronal network function (Tasker et al., 1992; Vornov et al., 1994; Newell et al., 1995; Strasser and Fischer, 1995). In contrast, acute slice preparations can be maintained for a maximum of approximately 12 h in vitro; although the duration of experiments must be kept short. Another greater advantage is the relative easiness by which a drug (Müller et al., 1993) or toxin (Rimvall et al., 1987; Vornov et
can be applied to the slice. Moreover, this culture may offer the opportunity to investigate the mechanisms underlying the lesion (Stoppini et al., 1993) and regeneration of neuronal pathways (Muller et al., 1994; Heimrich et al., 1996), alteration in gene expression by viral vectors (Bergold et al., 1993) and, as in this model system the heterogeneous cell populations found in vivo are maintained, it allows to study the interaction between cell types and the spinal cord development (Gähwiler et al., 1997; Galante et al., 2000; Galante et al., 2001; Rosato-Siri et al., 2002). In addition, organotypic slices permit the combination of sophisticated techniques such as electrophysiology, fluorescence imaging, pharmacology, molecular biology etc. In the spinal cord organotypic slice cultures the morphology (Delfs, 1989), electrophysiology (Ballerini and Galante, 1998; Ballerini et al., 1999; Rosato-Siri et al., 2004; Furlan et al., 2005; Furlan et al., 2007), development and distribution of various spinal cord glial cells and neurons, including motoneurons, interneurons and muscle fibres (Barber et al., 1993; Phelps, 1996; Avossa et al., 2003) have been extensively studied. In particular, it has been shown that the differentiation of astrocytes and oligodendrocytes proceeds in parallel with the differentiation of motoneurons and the formation of neuromuscular junction (Avossa et al., 2003).

One peculiar feature expressed during development of the entire spinal cord, maintained in the organotypic spinal slices, is the generation of spontaneous activity. This activity is typically organized into synchronous bursts or episodes, characterized by simultaneous activation at different spinal levels and separated by quiescent periods (Kudo et al., 1991; O’Donovan et al., 1998; Feller, 1999; O’Donovan, 1999). This spontaneous synchronous activity tends to disappear upon maturation of embryonic spinal networks. These changes have also been shown to occur in co-cultured organotypic spinal cord and muscle explants (Rosato-Siri et al., 2004). In particular, it has been reported that at an early stage of in vitro development (1 week of in vitro growth), ventral spinal interneurons generate spontaneous bursts of electrical activity and that this activity directly drives motoneuronal firing; this activity drastically decreases as in vitro development proceeds (2 and 3 weeks of in vitro growth) and, when still present, it is not correlated with muscular contraction (Rosato-Siri et al., 2004). This activity and its evolution in older cultures was similar in frequency, duration and dependence on glutamatergic synaptic transmission to those described in utero (Branchereau et al., 2002; Hanson and Landmesser, 2003; Whelan, 2003).
Another important feature expressed during spinal circuit development, observed also in organotypic spinal culture, is the switch of GABAergic interneurons from excitatory to inhibitory (Wu et al., 1992; Branchereau et al., 2002; Rosato-Siri et al., 2004; Furlan et al., 2007). In fact, during early stages of motor network maturation in vitro, the pharmacological block of both GABA and glycine receptors did not appear to limit burst duration or frequency; while at later stages of motor network maturation in vitro, GABA and glycine receptor activity appears to have an inhibitory role as, their pharmacological block invariably induced synchronous bursting in preparations that did not burst spontaneously (Rosato-Siri et al., 2004). This temporal pattern is associated with a differential spatial distribution of GAD-labelled neurons, with positive neurons becoming increasingly restricted to the dorsal horn regions at later stages of development in vitro, as it was observed in vivo (Avossa et al., 2003).

In the ventral area of developing organotypic slice cultures five different types of ventral interneurons can be identified on the basis of their discharge patterns (Prescott and Koninck, 2002; Szucs et al., 2003; Theiss and Heckman, 2005; Lu et al., 2006; Furlan et al., 2007): a) ‘tonic’ cells, that continuously fired APs without apparent accommodation; b) ‘adapting’ cells, that discharged an early burst of APs followed by adaptation; c) ‘delay’ cells, that generated APs after a lag; d) ‘irregular’ cells without discernible discharge patterns; e) ‘transient’ cells, that generated a single AP only (Furlan et al., 2007). These five neuronal classes show a different distribution during the spinal cord development, in particular, the firing properties of the majority of ventral recorded cells changed from ‘adapting’ at early embryonic ages to ‘tonic’ later in development (Furlan et al., 2007) (Figure 10). It is noteworthy that a similar classification of firing pattern of ventral spinal interneurons has been reported for newborn spinal cord preparations, indicating that the observed differences were not caused by culturing conditions (Szucs et al., 2003; Theiss and Heckman, 2005). It is interesting to note that firing properties changes occurred in coincidence with the critical transformation of spontaneous activity from bursting to sporadic discharges (Whelan, 2003; Rosato-Siri et al., 2004). Furthermore, electrophysiological and immunocytochemical results strongly suggest that the ‘adapting’ cell type at early embryonic stages of development could be mainly identified as the GABAergic phenotype (Furlan et al., 2007).
Figure 10. Discharge patterns of ventral interneurons at 7 and 14 DIV. A) At 7 DIV depolarizing current steps of 500 ms duration and 0.1 – 0.2 nA amplitude induced different discharge patterns that identified four different populations of ventral spinal interneurons: tonic, adapting, delay, and irregular. B) At 14 DIV depolarizing commands allowed to identify four ventral spinal interneurons categories: tonic, adapting, delay, and transient. C and D) The Histograms show the distribution (expressed as percentage) of each cell type at 7 DIV (C) and 14 DIV (D) (Furlan et al., 2007).

In the maturation of motor circuit, the neuronal population usually expresses different spontaneous rhythmic Ca^{2+} transients that depend on the embryonic age tested.
At early developmental stages these Ca\(^{2+}\) dynamics have been correlated with waves or bursts of electrical activity (Momose-Sato et al., 2005; O’Donovan et al., 2005; Momose-Sato et al., 2007). These spontaneous Ca\(^{2+}\) transients are also reported in organotypic spinal culture, where they were typically synchronous and originated from widespread propagating waves that became organized into large-scale rhythmic bursts (Sibilla et al., 2009) (Figure 11).

**Figure 11.** Spontaneous waves and bursts observed in organotypic spinal cord cultures during the first week of *in vitro* growth. A) Representative examples of synchronous and repetitive Ca\(^{2+}\) waves generated in the ventral area at 8 DIV. Note that waves were fully suppressed by 6-cyano-2,3-dihydroxy-7-nitroquinoxaline acid (CNQX 5 µM). Inset: expanded record of a series of waves. B) Regular and synchronous Ca\(^{2+}\) bursts generated from ventral spinal interneurons at 9 DIV. Bursts were completely blocked in the presence of tetrodotoxin (TTX 1 µM). Inset: expanded record of a series of bursts (modified from Sibilla et al, 2009).

During maturation, in organotypic spinal culture differently to *in vivo* models or other *ex vivo* culture, synchronous Ca\(^{2+}\) rises invading the entire spinal region disappear. In fact, the majority of slices display regular Ca\(^{2+}\) oscillations which are generated by few neurons (~10%) in restricted ventral areas (Fabbro et al., 2007; Sibilla et al., 2009). Moreover, contrary to those detected in waves and bursts, the oscillations are completely asynchronous and independent from action potential or synaptic activity (Fabbro et al., 2007; Sibilla et al., 2009). Transient synchronization could be achieved
by exposing the ventral areas to exogenous depolarizing stimuli (Fabbro et al., 2007; Sibilla et al., 2009).

Pharmacological dissection of Ca\(^{2+}\) oscillations shows their dependence of multiple Ca\(^{2+}\) stores and source with different contribution in distinct neurons even in the same ventral region (Fabbro et al., 2007; Sibilla et al., 2009). In particular, Ca\(^{2+}\) oscillations are not completely removed by thapsigargin (sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase blocker) or Ca\(^{2+}\)-free solution displaying a heterogeneous behaviour, whereas they are strongly dependent on mitochondrial Ca\(^{2+}\) buffering ability as shown by their complete inhibition by CCCP (that specifically collapses the mitochondrial electrochemical gradient) or by CGP (mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger blocker) (Fabbro et al., 2007; Sibilla et al., 2009) (Figure 12).

In this study I focused on Ca\(^{2+}\) oscillations because, as shown above, they have distinctive features compared with other Ca\(^{2+}\) signals. In particular, I investigated the changes induced by acute and chronic treatment with different intracellular Ca\(^{2+}\) chelators, in the kinetic of this intracellular Ca\(^{2+}\) signals, in the number of oscillating neurons and slices involved and in the contribution of different intracellular Ca\(^{2+}\) stores and sources.
Figure 12. Complex Ca\(^{2+}\) sources for oscillations at 2 weeks. A and B) The response to Ca\(^{2+}\)-free solution was bimodal, Ca\(^{2+}\) oscillations were completely removed (traces in A) or not (traces in B). C and D) Thapsigargin (5 µM, 10 min) blocks Ca\(^{2+}\) oscillations (traces in C) but does not completely remove Ca\(^{2+}\) transients (traces in D). It is interesting to note the clear increase in baseline. E) Ca\(^{2+}\) oscillations are suppresses by the mitochondrial protonophore CCCP (2 µM, 3 min). B) mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger blocker, CGP (30 µM, 10 min), completely inhibits oscillations in all oscillating neurons (modified from Sibilla et al., 2009).
Fluorescent indicators and Ca\textsuperscript{2+} imaging

Neuronal network activities exhibit complex but apparently precise spatio-temporal patterns. Initial studies on the organizational structure of neuronal circuits were based primarily on anatomical observations. However, to study the role of different populations of neurons in generating network activity, we need to have a dynamic vision of the network. This can be achieved at the cellular level using for example single-cell patch-clamp recordings. However, every circuit in the brain is composed of a myriad of functional microcircuits that are at work at multiple temporal and spatial scales. Recording from a single cell is similar to recording from a single voice in a chorus: it might sing with the others, only with voices of similar tone, or even alone (Cossart et al., 2005). To characterize fully the dynamics of a network, larger scale recordings that describe the temporal relation between different assemblies need to be performed. The imaging technique allows simultaneous recording from many neurons.

Optical techniques are becoming an increasingly attractive alternative method because of their apparent non-invasive nature and ease of use. One area in which optical techniques have largely replaced other methods is the measurement of intracellular ion concentrations with fluorescent indicators. Traditional measurement, based on ion sensitive electrodes, show some problems arising from the fact that energy must be extracted from a system (cell) in order to determine the measurement process, thus perturbing the system to be measured. In contrast to this idea with optical techniques (such as fluorescence and absorbance measurements) the experimenter actually supplies energy to examine the system, by supplying photons to the system and recording the results of the interaction of the photons with the system. Of course, there are also disadvantages due to this interaction. In fact, photons will interact with any molecule that has absorbance in the wavelength range of the photon, thus molecules that are of no interest to the experimenter may also produce a signal that will contaminate the experimental record (cell autofluorescence). Moreover, it is usually necessary to introduce special molecules into the cell so that specific cell functions can be examined and the introduction of these probes will perturb the system. The amount of material that is being examined with the photons may be small so that there will be quantal limitations in the signal to noise ratio that can be achieved.

(All refs. from Cannel and Thomas, 1994).
General principles of fluorescence

Fluorescence techniques are now established as one of the standard methods of intracellular ions concentration measurement. However, other older optical methods of intracellular ion concentration measurement have been developed. The first one involved the measurement of dye absorbance (metallochromic indicators) but unfortunately these indicators may not be used to investigate large invertebrate cells and muscle fibres. In the past few years the photoproteins (e.g. aequorin) that emit photons were also used in response to the oxidation of a bound prosthetic group, this catalysis is regulated by Ca$^{2+}$ ions. This method is used to measure intracellular Ca$^{2+}$ concentrations, but it is quite difficult to introduce the photoproteins into the cell.

Nowadays the fluorescent indicators are the most common method of investigation used; these dyes are molecules whose optical properties (absorbance or fluorescence) change with parameters of cellular activity. Available indicators include those sensitive to membrane potential (Vm), to cAMP, to intracellular pH (Rink et al., 1982), to Ca$^{2+}$ concentration (Blinks et al., 1982) and to concentrations of other ions (H$^+$, Na$^+$, K$^+$, Mg$^{2+}$, Zn$^{2+}$ and Cl$^-$). The fluorescent probe (fluorophore) is designed to respond to a specific stimulus. The process responsible for the fluorescence of fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in Figure 13.

![Jablonski diagram](image)

**Figure 13.** Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. The labelled stages 1, 2 and 3 are explained in the text.
A photon of energy ($h\nu_{EX}$) is supplied by an external source such as an incandescent lamp or laser and absorbed by the fluorophore, creating an excited singlet state ($S_1'$). This state exists for a finite time (typically 1–10 nanoseconds). One of the primary modes of decay involves emitting a second photon ($h\nu_{EM}$); since some of the first photon's energy is converted to vibrations, the second emitted photon typically has less energy than the first, and thus a longer wavelength (stokes shift). Not all the molecules initially excited by absorption ($S_1$) return to the ground state ($S_0$) by fluorescence emission. Alternatively, the photon emitted may be reabsorbed or the excited state may be quenched by collision with another molecule. Therefore, the number of emitted photons are somewhat less than the number of absorbed photons and the ratio between them is called the quantum efficiency (modern fluorochromes have quantum efficiencies of about 0.3). The entire fluorescence process is cyclical, the same fluorophore can be repeatedly excited and detected, unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching). The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques.

Fluorescent molecules are characterized by their excitation spectrum, which measures the relative affinity of absorbing a photon as a function of wavelength, and by their emission spectrum, which measures the relative probability of emitting a photon at each wavelength. Another important characteristic of fluorescent molecules is their quantum yield, which measures the fraction of the time that an absorbed photon gives rise to an emitted photon.

Ion sensitive probes are made by attaching groups that bind ions to the fluorescence part of the molecule, the most common ion dyes have a Ca$^{2+}$ coordination site based on the BAPTA or EGTA molecules conjugated with various fluorophores. These fluorescent dyes bind only one Ca$^{2+}$ ion, with high selectivity over Mg$^{2+}$ in the physiological range and with relative insensitivity to H$^+$. The Ca$^{2+}$ fluorescent molecules can be used to observe the changes in intracellular parameters because the binding of inorganic ions to dyes, forming ion-dye complexes, changes their spectral properties in response to the binding of Ca$^{2+}$ ions (Tsien 1980; Grynkiewicz et al., 1985; Minta et al., 1989). The more common spectral changes comprise a shift in the excitation or emission spectra or a modification in the quantum efficiency. For instance, the binding of Ca$^{2+}$ to the dye quin-2 increases its fluorescent emission. However, this fluorophore does not allow a
Because it is difficult to relate the absolute fluorescence directly with \(Ca^{2+}\) concentration since the overall magnitude of the signals is also dependent on the concentration of indicator, the volume of the cell (or path length which is being illuminated), the intensity of the illumination, the properties of the detection system and finally the cell autofluorescence.

Therefore other intracellular \(Ca^{2+}\) dyes have been developed that shift their emission or excitation spectrum; for example in the fura-2 the excitation spectrum is modulated by \(Ca^{2+}\) binding; in the absence of \(Ca^{2+}\), the dye absorbs ultraviolet light mainly at wavelengths around 380nm, when \(Ca^{2+}\) ions are bound to the molecule, the excitation peak shifts to 340nm with the position of the emission spectrum poorly affected (Gryniewicz et al., 1985). \(Ca^{2+}\) has a similar dual-wavelength effect on indo-1 except that is the emission spectrum which is sensitive to \(Ca^{2+}\) binding, in fact the peak emission wavelength of indo-1 is 482nm while with a \(Ca^{2+}\) molecule bound it is 398nm (Gryniewicz et al., 1985). The dyes that have dual emission or excitation spectrum are called ratio-metric. Ratio-metric methods are based on the evaluation of the ratio between the fluorescence intensities at two different wavelengths. The main advantage of using ratio-metric dyes over single wavelength probes is that the ratio signal allows to determine the concentration of intracellular \(Ca^{2+}\) ions independently of all artefacts.

To select the ideal probe it is important to evaluate 1) the affinity and selectivity for the target ions; 2) the dissociation constant of the dye (Kd) that suggests the ion concentrations range at which the dye is suitable. The desired indicators must have a proper Kd compatible with the ion concentration range of interest. Optimum ion concentration range that an indicator can reliably measure is between 0.1 x Kd and 10 x Kd. If the ion concentration is too low, there will be almost no change in fluorescence with a change in ion concentration, leading to a very poor signal to noise ratio. If the ion concentration is too high, almost all the dye will be bound and there will be no measurable change if the concentration of ions rises further.

**Fundamentals of \(Ca^{2+}\) dyes**

In the last few years strong improvements have been made in the field of \(Ca^{2+}\) dyes. The development of \(Ca^{2+}\) sensitive probes has revolutionized techniques for the
measurement of intracellular Ca\(^{2+}\) concentrations. With these dyes, it has become possible to analyse the variations of intracellular Ca\(^{2+}\) from a wide variety of cell types in a much less invasive fashion compared to earlier techniques. The Ca\(^{2+}\) dyes are formed by fluorescent products of intracellular Ca\(^{2+}\) chelators (such as BAPTA or EGTA) and have excitation spectra in ultraviolet light (UV) field. These Ca\(^{2+}\) indicators can be divided into high affinity dyes (like quin-2, fura-2, indo-2), middle affinity dyes (such as fura-4F, fura-5F, fura-6F) and low affinity dyes (as fura-FF, mag-fura2).

**Quin-2**: is the first generation of Ca\(^{2+}\) probes (Tsien *et al.*, 1982; Tsien and Pozzant, 1989). As a derivative from EGTA, Quin-2 forms complexes with Ca\(^{2+}\) ions at the molar ratio of 1:1, when excited at 340nm an increased emission intensity peaking (about 6 times) at 490nm is observed. Quin-2, as the other single wavelength Ca\(^{2+}\) probes, is usually excellent for tracking the kinetics of changes in Ca\(^{2+}\) concentration, but it is much more complicated for collecting absolute data about Ca\(^{2+}\) concentration that is related to the fluorescence F by:

\[
[Ca^{2+}] = K_d \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}
\]

where \(F_{\text{min}}\) and \(F_{\text{max}}\) are the fluorescence levels at zero and saturating ion concentrations, respectively, and \(K_d\) is the dissociation of the ion-indicator complex (Grynkiewicz *et al*, 1985). Therefore, the increasing fluorescence is the only parameter that indicates the presence of intracellular Ca\(^{2+}\), but it is not a real value because it can depend on other factors, for instance the concentration of the probe, the intensity of the light and so on.

**Fura-2**: developed by Grynkiewicz and colleagues (1985), it is a second-generation Ca\(^{2+}\) indicator based on BAPTA. Fura-2 inherits its high affinity for Ca\(^{2+}\) and selectivity over Mg\(^{2+}\) from the chelator, but has also the ability to fluoresce when excited with UV light. The excitation spectrum for fura-2 changes, depending on whether or not it is bound to Ca\(^{2+}\), whereas the emission peak is constant (510nm) (**Figure 14**).
Figure 14. Excitation spectra for fura-2 with emission monitored at 510 nm. Note that with illumination at 340 nm the fluorescence increases with increasing Ca\textsuperscript{2+}, while at 380 nm the fluorescence decreases with increasing Ca\textsuperscript{2+}.

If fura-2 is bound to Ca\textsuperscript{2+} then the excitation maximum is around 340 nm, if fura-2 is not bound to Ca\textsuperscript{2+} then the excitation maximum is about 380 nm. The ratio of fluorescence measured at 340 nm to that measured at 380 nm is therefore related to the free Ca\textsuperscript{2+} concentration by the analogous equation:

\[
[\text{Ca}^{2+}] = \frac{K_i S (R-R_{\text{min}})}{(R_{\text{max}}-R)}
\]

where \(R_{\text{min}}\) is the ratio at two wavelengths at zero ion concentration, \(R_{\text{max}}\) is the ratio at saturating ion concentration, \(R\) is the ratio of the measurement, \(K_i\) is the constant unifying instrumental parameters and Kd of the fluorophore for Ca\textsuperscript{2+}, and \(S\) is a scaling factor given by the ratio between \(R_{\text{min}}/R_{\text{max}}\) (Gryniewicz et al, 1985).

The use of the ratio automatically eliminates confounding variables, such as variable dye concentration and cell thickness, making Fura-2 one of the most appreciated tools to quantify Ca\textsuperscript{2+} levels.
A typical property of all the indicators that undergo either excitation or emission shift is the presence of an isosbestic point, where the fluorescence is just due to the indicator concentration and is not correlated with Ca$_{2+}$ concentration. The isosbestic point is only present when two species are in equilibrium (in our case Ca$_{2+}$ bound and free form of Fura-2). This point appears at 360nm for Fura-2 (Figure 15).

Moreover Fura-2 shows a greater affinity for Ca$_{2+}$ than Quin-2, thus making Fura-2 a better Ca$_{2+}$ dye than Quin-2, as a lower dye concentration is needed, while still providing a good fluorescence intensity.

**Fluo-3:** was developed by Tsien (1989) for use with visible-light excitation sources (488nm). Fluo-3 is essentially not fluorescent unless bound to Ca$_{2+}$ and exhibits an emission peak at 525nm. The most important properties of Fluo-3 are a very large fluorescence intensity increase (40 times) in response to Ca$_{2+}$ binding, and a high Kd (400nm), which gives the possibility to measure large increases in the Ca$_{2+}$ concentration (Figure 16).
An exciting new development in the use of fluorescent probes for biological studies has been the development of naturally fluorescent proteins as fluorescent probes. One of the first engineered probes was camgaroo-1, which contains a calmodulin domain at position 145 of YFP (yellow fluorescent protein). Ca\(^{2+}\) binding can lead to a sevenfold increase in fluorescence of the probe. The most powerful feature of camgaroo-1 is the fact that that living, unstained samples can be observed. However, calmodulin has a high interaction with proteins in the cytoplasm that causes a progressive partial inhibition and consequently a decrease in the expected fluorescence. During recent years to work around this problem, Ca\(^{2+}\) sensors using other Ca\(^{2+}\)-sensitive proteins have been explored. For instance, troponin C, that binds Ca\(^{2+}\) in the skeletal and cardiac muscle, interacts less with other cytoplasmic proteins and this property improves the fluorescent responses (Heim and Griesbeck, 2004; Griesbeck, 2004; Knopfel, 2006; Mank et al., 2006).

**Dye-loading procedures**

Most chemical fluorescent indicators are charged molecules, therefore they are cell impermeant. Indicators can be introduced into cells by direct injection through a
microelectrode (Cannell et al., 1987; Cannell et al., 1988) or patch electrode (Almers and Neher, 1985; Neher and Almers, 1986; Oheim et al., 1998). Once inside the cell, the fluorescent indicators generally remain there for a reasonable period, allowing making stable recordings. Even if there is some loss of indicator over time, this can be compensated by the ratiometric measurement method (see above). However, there are cases in which indicator loss is a problem, which can however be reduced by injecting the indicator in a dextran-linked form. Linkage of the indicators to the dextran polymer does not in general seem to significantly affect their properties, however it inhibits cellular transport of the indicator. The advantage of these loading procedures is that it is possible to load the charged form of the indicator directly into the cytosol, in a form which cannot penetrate into sub-cellular compartments, such as the mitochondria or sarco-endoplasmic reticulum. However, both of these techniques are invasive and requires specialized instruments and practice. In addition, the number of cells that can be loaded with probes is limited.

There is an elegant way for introducing dyes through the plasma membrane barrier using chemically modified precursor probes (acetoxymethyl -AM- ester forms) which penetrate the plasma membrane easily. The AM ester form is non-invasive and is the most popular method for loading fluorescent ion indicators into cells (Tsien, 1981). These esters mask negative charges, thus making the probe molecule hydrophobic enough to be membrane permeant. Once inside the cell, non-specific esterases, present in almost all cell types, hydrolyze the esters yielding the active form of the indicator, which is therefore trapped and accumulated in the cell. The use of Ca\textsuperscript{2+} AM ester indicators allows not only to load small cells, such as blood platelets, but it also provides a very robust method for imaging the activity of large populations of cells simultaneously. However, the ester loading technique is not completely without problems. For instance, the loaded amount cannot be directly controlled and there is a risk of excessive loading. This may then result in additional buffering of [Ca\textsuperscript{2+}], which affects [Ca\textsuperscript{2+}], measurements as well as Ca\textsuperscript{2+}-dependent cellular signalling. The additional buffering depends both on the affinity of the indicator and on the ratio between the concentration of the indicator and the free concentration of the target, an issue which is critical particularly for ions which have a low intracellular concentration, such as Ca\textsuperscript{2+} ions. The indicator can also be trapped within some intracellular organelles and, for this reason, it cannot homogeneously distributed throughout the cell. The degree of compartmentalization is dependent on numerous factors (e.g., the loading
condition, cell type, and type of indicator) (Cobbold and Rink, 1987; Malgaroli et al., 1987; Roe et al., 1990). The same indicator can also be sequestered within different organelles in different types of cells. For example, it has been demonstrated that fura 2 accumulates well within mitochondria of endothelial cells (Steinberg et al., 1987), lysosomes of fibroblasts (Malgaroli et al., 1987) and secretary granules of rat mast cells (Almers and Neher, 1985). This feature depends on the fact that there are several mechanisms of dye sequestration (Malgaroli et al., 1987; Di Virgilio et al., 1989). The sequestration of some indicators is partially mediated by organic anion transport systems (Di Virgilio et al., 1990). In addition, incomplete hydrolyzation of some of the indicators in the cytosol allows the ester-derived indicator to cross organelle membranes into the intracellular compartments, which can have substantial esterase activity themselves (Lukács and Kapus, 1987; Gunter et al., 1988) (Figure 17). Because the mechanisms of both transport to organelles and hydrolysis depend on biochemical phenomena, compartmentalization and cytosolic dye concentration are strongly influenced by temperature. Accordingly, by changing the loading and/or hydrolyzing temperature, compartmentalization can be modulated (Roe et al., 1990; Minamikawa et al., 1997).
Figure 17. Introduction of esterified fluorescent Ca$^{2+}$ indicators into cells. The fluorescent indicator, in this example fura-2, is presented to cells as the acetoxymethyl ester (fura-2-AM). This relatively lipid soluble chemical permeates into the cytoplasm of cells, where esterases cleave the acetoxymethyl ester groups, liberating the Ca$^{2+}$ binding moiety (fura-2). Fura-2 then reversibly combines with Ca$^{2+}$ and, when excited alternatively with light of wavelengths 340 and 380 nm, gives rise to fluorescence at $\sim$500 nm. Potential complications with the use of these fluorescent dyes, indicated in this figure by ?, are uptake into intracellular organelles and leak of dye from the cells (George et al., 2006).

An additional potential problem is that complete hydrolysis results in the formation of cytotoxic compound byproducts, such as formaldehyde and acetic acid, although serious toxicity problems have not been reported.

Despite the above-mentioned problems, the use of the AM-ester form of the indicator remains the best route to take when cells cannot be loaded by an electrode or patch pipette and when there is the needing to load many cells at the same time, as adequate experimental controls and handling expertise can overcome the limitations of this technique. Therefore, for my thesis work I chose fura-2-AM as a tool to investigate the behavior of large populations of neurons in complex tissues,
**K⁺ channels**

In this thesis work one set of experiments was targeted at investigating the changes in the K⁺ conductance, as a result of intracellular Ca²⁺ manipulation; a brief introduction on the main families of K⁺ channels and their properties is therefore provided.

K⁺ channels are the widest family of ion channels. K⁺ channels are present in all living cells, where they are associated with the regulation of many important physiological functions like neuronal and cardiac repolarization, insulin release, smooth muscle relaxation and hormone secretion (Rudy, 1988; Hille, 2001).

K⁺ channels structure is characterized by four subunits associated to form homotetrameric channels (made up by four identical α subunits) or heterotetrameric channels (made up by three α and one β subunits). K⁺ channels are grouped into families based on their structure as well as physiological criteria. These channels are classified into three main families namely 2TM, 4TM and 6TM based on the number of transmembrane (TM) domains present in the subunits (Jenkinson, 2006, Figure 18).

**Figure 18.** Schematic representation of the structural classification of K⁺ channel domains. A) Six transmembrane domains are composed of six transmembrane segments (S1-S6) and one conducting pore (P) between S5 and S6, with a voltage sensor (positively charged amino acid residues) located at S4. The inset shows the general assembly of a homotetrameric K⁺ channel. B) Two trans-membrane domains are characterized by two trans-membrane segments (M1 and M2) with a P-loop in between. C) Four trans-membrane domains have four transmembranes segments (M1-M4) with two P-loops. (modified from Shieh et al., 2000).
Six trans-membrane domains channels
The six trans-membrane domains channels are composed of homo or hetero-tetramers subunits each containing six trans-membrane (S1-S6) segments and one pore loop (P) between S5 and S6, with a voltage sensor located at S4. These channels include: delayed rectifier $K^+$ channel ($K_{\text{DR}}$), transient $K^+$ channel ($K_A$), $Ca^{2+}$-activated $K^+$ channels ($K_{\text{Ca}}$).

The delayed rectifier $K^+$ channels ($K_{\text{DR}}$) are activated by depolarization and conduct a sustained outward $K^+$ current (Rudy, 1988), with fast activation kinetics (Hille, 2001), which contributes to the falling phase of the action potential and the fast afterhyperpolarization (fAHP) (Barrett et al., 1980; Schwindt and Crill, 1981; Hounsgaard et al., 1988; Mosfeldt Laursen and Rekling, 1989; Nishimura et al., 1989; Takahashi, 1990; Viana et al., 1993; Chandler et al., 1994).

The transient $K^+$ channels ($K_A$), activate when a cell is depolarized after a period of hyperpolarization (Rudy, 1988; Hille, 2001), generate a rapidly inactivating outward $K^+$ current that contributes to action potential repolarization (Adams et al., 1982; MacDermott and Weight, 1982; Pennefather et al., 1985).

The $Ca^{2+}$-activated $K^+$ channels ($K_{\text{Ca}}$) are activated by the presence of $Ca^{2+}$ ions and are classified based on their conductance into SK (small conductance), IK (intermediate conductance) and BK (large conductance) (Blatz and Magleby, 1987; Romey an Lazdunsky, 1984; Pennefather et al., 1985; Blatz and Magleby, 1986), which also differ in their voltage dependence and pharmacology. Two peptide toxins are useful for dissecting $K_{\text{Ca}}$ into its BK and SK components: apamin blocks SK channels (Hugues et al., 1982) and charybdotoxin blocks some BK channels (Miller et al., 1985). The opening of SK channels has almost no voltage dependence, while BK channels are voltage sensitive. $K_{\text{Ca}}$ give rise to fAHP in neurons when the action potential is associated with a large $Ca^{2+}$ influx.

Two trans-membrane domains channels
The members of this family of $K^+$ channels are composed by four subunits (Yang et al., 1995) each containing two trans-membrane segment (M1 and M2) and a pore loop (P) in between (Ho et al., 1993; Kubo et al., 1993). This family comprises the inward rectifier $K^+$ channels ($K_{\text{ir}}$), with a conductance which increases under hyperpolarization and decreases under depolarization (Hille, 2001). $K_{\text{ir}}$ family comprises ATP-sensitive $K^+$ channels, hetero-octamers made
up by four inward rectifiers subunits, which form the ion conducting pore, and four peripheral sulfonylurea receptors as regulatory subunits. ATP-sensitive K\(^+\) channels are inhibited by intracellular ATP and form a link between energy metabolism and electrical activity of the cells (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997).

**Four trans-membrane domains channels**

Four trans-membrane domains channels have an unusual structure with four putative trans-membrane domains (M1-M4) and two pore domains (P1 and P2) (Ketchum et al., 1995; Lesage et al., 1996). They represent perhaps the most abundant class of K\(^+\) channels, with more than 50 distinct members in C. elegans (Wang et al., 1999) and 14 in mammals (Patel and Honoré, 2001). These channels are constitutively open or possess high basal activation that gives rise to “leak” K\(^+\) current. The well-known role of this K\(^+\) current is to stabilize the negative resting membrane potential and counterbalance depolarization.
METHODS

Preparation of spinal cord cultures

In the present work organotypic cocultures of spinal cord and dorsal root ganglia were obtained with the roller tube technique, which allows spreading and flattening of the tissue, as it was reported by Gähwiler (Gähwiler, 1981).

The following short paragraphs describe in details the main steps of the culturing procedure.

Explantation

Organotypic slice cultures were obtained from mouse embryos at days 12-13 of gestation (E12 - E13). Pregnant mice were sacrificed, then embryos were extracted by caesarean section leaved in their amniotic sacs, and put into a Petri dish containing ice-cold Gey’s balanced salt solution (GBSS; in mM: 1,5 CaCl₂; 5 KCl; 0,22 KH₂PO₄; 1 MgCl₂, 0,3 MgSO₄, 137 NaCl, 2,7 NaHCO₃, 0,8 Na₂HPO₄, 5,6 Glucose; pH 7,4; osmolarity 296 mOsm). From this point the dissection was performed under a laminar flow hood in sterile conditions and under microscope (Olympus SZ40) control. Through a small cut in the amniotic sac embryos were freed from their amniotic sac and put into a new Petri dish with fresh GBSS. They were decapitated and their legs, tails and abdomen cut away (Figure 1A). The backs of the embryos were cut into 275 μm thick transverse slices with a tissue chopper (McIlwain) (Figure 1B) and put into a Petri dish with fresh GBSS. Slices were chosen from the low thoracic and high lumbar levels. They were cleaned from the surrounding tissue to leave only spinal cord with the two dorsal root ganglia (DRG) attached (Figure 1C) and then transferred into a new Petri dish with GBSS and maintained at +4°C for one hour.

Embedding and incubation

After that, single slices were placed in a drop (20 μl) of chicken plasma (Rockland) put on a glass coverslip. Plasma clotting was induced by addition of a drop (30 μl) of thrombin (Sigma-Aldrich, Italy) (Figure 1D and Figure 1E). After 30-45 min, to allow
stabilization of the clot, the coverslips were placed into plastic tubes (Nunc) filled with 1 mL of fresh medium (Figure 1F; see composition below) and immediately put in a roller drum (Figure 1H), which rotates at approximately 120 rph, at 37 °C with 5% CO₂. Rotation is essential for feeding, aeration and flattening of the cultures. In fact, slices are submerged in medium during half a turn and covered with only a film of medium during the other half.

**Figure 1.** Preparation of organotypic cultures from embryonic mouse spinal cord. (A) Isolation of spinal cords from mouse fetuses. (B) Slices are cut with tissue chopper. (C) Dissection of spinal cord slices after cut. DRGs still remain attached to the slices. (D) The spinal cord/DRG slices are cleaned from surrounding tissue. (E) The slices are fixed on glass coverslips, which are then inserted into plastic tubes, with the culture medium (F) and (G). (H) The tubes were kept in a roller drum (Adapted from Delf et al., 1989).

Prior to use glass coverslips (12x24 mm, thickness 1.2 mm, Vitromed) were treated as follows: inserted into a teflon holder, submerged in hydrochloric acid 0.5 N for 24 h, then repetitively washed with distilled water and leaved in absolute alcohol for 30 min. Then coverslip are dried and sterilized at +100°C in an oven over night.
Medium and medium change
The standard medium consists of: 67% Dulbecco’s modified Eagle’s medium (Invitrogen, Italy), 25% fetal bovine serum (FBS, Invitrogen), 8% sterile water, 5 ng/mL nerve growth factor (NGF, Almone, Israel) diluted in Hanks and BSA (1 mg/mL). The pH is 7.35 and osmolarity 300 mOsm.
During the first six days in vitro (DIV) the medium (B) contains a higher concentration of NGF (20 ng/mL), which is needed for a good development of cells and culture. After this period the old medium is replaced by a fresh standard one (Medium C), with the addition of a mixture of antimitotics (Cytosine arabinoside, Fluoro-deoxyuridine, uridine, Sigma), which allows to prevent the exaggerated growth of non-neuronal cells. After 24h this antimitotics-enriched medium is replaced by a fresh standard one (Medium A), which is then replaced weekly. The compositions of the three types of medium are shown in the following (Table1).

<table>
<thead>
<tr>
<th>Components</th>
<th>Medium 1</th>
<th>Medium 2</th>
<th>Medium 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-MEM</td>
<td>67%</td>
<td>67%</td>
<td>67%</td>
</tr>
<tr>
<td>FBS</td>
<td>25%</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>8%</td>
<td>8%</td>
<td>8%</td>
</tr>
<tr>
<td>NGF</td>
<td>20 ng/ml</td>
<td>5 ng/ml</td>
<td>5 ng/ml</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Solution 100X Antimitotics</td>
<td>/</td>
<td>10 μM</td>
<td>/</td>
</tr>
</tbody>
</table>

Table 1. Shows the quantity of single components.

Spreading and flattening of slices
While growing in vitro, slices become spread and flattened, features which are essential for visualization of single neurons. In general, the spreading degree depends on many factors like the rotation speed, the origin of the explanted tissue and the initial thickness of the slices. In fact, soon after the dissection, slices are too thick to allow the visualization of single cells. A fast rotation of the tubes allows the plasma coagulum and the damaged cells on top of the slices to be gradually washed, with a better access to individual neurons for electrophysiological recordings. The roller-tube technique assures the preservation of the organotypic organization of the tissue while facilitating
the identification of single cells. Moreover, being a long-term preparation, the tissue can completely recover from the dissection trauma and easily adapt to the new *in vitro* conditions. For all these reasons, the roller-tube technique is widely used to obtain organotypic slice cultures.

**Morphology of spinal slices**

Organotypic cultures are an *in vitro* preparation that preserves the original structure of the spinal cord segment. After two weeks in culture the slice appears as in figure 2; the orientation of the spinal cord is clearly marked by the dorsal root fibers and by the ventral fissure (Figure 2, red arrow) which allows distinguishing ventral horns (Figure 2, black square).

![Figure 2](image)

**Figure 2.** Bright field picture at 5x magnification of the slice after two weeks of *in vitro* growth. Dorsal and ventral areas are clearly distinguishable by localizing the dorsal root ganglia and the dorsal root fibers which growth (indicated by blue arrows) to innervate other structures and the ventral fissure (indicates by red arrow) which marks the ventral horns region (indicates by black square).

Different cellular phenotypes are present and develop within organotypic spinal cultures. Indeed, this preparation can be employed to monitor the development of motor neurons, interneurons, muscle fibers and dorsal root ganglion cells (Rosato-Siri *et al.*, 2004; Avossa *et al.*, 2003).
Motoneurons are generally identified from their localization and morphology. In fact, immunocytochemical studies (Streit et al., 1991; Avossa et al., 2003), using SMI32 (marker for projecting neurons, DRG neurons and motoneurons) and ChAT-ir (marker of cholinergic neurons, motoneurons) show that they are localized on both sides of the ventral fissure and they are characterized by large, triangle-shaped soma (>25 μm) and a multipolar neuritic arborization.

Another important class of spinal cells is represented by the ventral pre-motor interneurons which are involved in the generation of rhythmic motor patterns (Rosato-Siri et al., 2002; Galante et al., 2001; Galante et al., 2000; Ballerini et al., 1999; Ballerini and Galante, 1998). In organotypic spinal slices interneurons can be visually identified on the basis of specific morphological criteria. In fact, they have a cell body diameter of less than 20 μm and they are monopolar or bipolar cells, characterized by a widespread dendritic arborisation (Ballerini and Galante, 1998; Ballerini et al., 1999; Avossa et al., 2003; Furlan et al., 2005; Furlan et al., 2007). As emerged from intracellular electrophysiological recordings, in organotypic cultures ventral interneurons generate spontaneous synaptic activity with characteristic temporal patterns (Rosato-Siri et al., 2004; Ballerini et al., 1999; Ballerini and Galante, 1998).

During development DRG cells flatten into monolayers, symmetrically located at both sides of the spinal slice, and extend long neurites (Spenger et al., 1991). DRG neurons are easily identified by their morphology, characterized by a polygonal profile and a large cell body (40-50 μm diameter) with one or two large processes emanating from it (Avossa et al., 2003). These cells never display spontaneous synaptic activity although they spontaneously generate action potentials (Galante et al., 2000).

**Ca\(^{2+}\)-imaging**

Organotypic slices grown *in vitro* for 2 weeks (*in vitro*, DIV) were incubated in the recording solution (see below) integrated with 0.5% Bovine Serum Albumin (BSA, Sigma-Aldrich, Italy) and loaded at room temperature (RT; 22 ± 2°C) with Fura-2-AM (2 - 5 μM final concentration; Sigma-Aldrich, Italy). Fluorescent indicators are susceptible to oxidation and bleaching during storage and rapidly lose activity if exposed to light and air at room temperature. For this reason, I prepared the
Fura-2-AM, in dry DMSO, in aliquots each of which contained the amount of indicator usually required for a single experiment. The aliquots were kept frozen at 20°C and repeated freeze/thawing cycles were avoided. After 45 minutes the Fura-containing loading solution was removed and the slices were washed with, and kept in, the recording solution for 45 minutes to allow complete de-esterification of the dye. A single Fura-2-loaded slice was then placed in a recording chamber mounted on an inverted microscope (Eclipse TE 200, Nikon, Japan), where it was perfused with the recording solution at 5 ml/min. Videomicroscopy and Ca\(^{2+}\)-imaging measurements were carried on at RT. The Fura-2 loaded cultures were observed with a 40X objective (1.8 NA, Nikon, Japan). All the recordings were taken from a small (125 μm x 95 μm) visual field located in the ventral area. Slices were excited at wavelengths of 340 and 380 nm with a monochromator device equipped with integrated light source (Polychrome IV, Till Photonics). Excitation light was separated from the light emitted from the sample using a 395 nm dichroic mirror. Images of emitted fluorescence >510 nm were acquired continuously for 1500 s as a maximum (500 ms integration time for frame) by a cooled slow-scan interline transfer camera (IMAGO CCD camera, Till Photonics) and simultaneously displayed on a color monitor. This protocol minimized photo-bleaching as confirmed by robust responses produced by 100 mM KCl (Carlo Erba, Italy) pulse application at the end of the recording session. Camera was operated on 4 x 4 pixel binning mode. The imaging system was controlled by an integrating imaging software package (TILLvisION, Till Photonics) using a personal computer. Video frames were then digitized, integrated and processed to convert fluorescence data into Ca\(^{2+}\) maps by computing a ratio of 340/380 nm excitation wavelength values (ΔR; integrating imaging software package, TILLvisION, Till Photonics, Figure 3).
I recorded Ca\textsuperscript{2+} signals from selected ventral areas, in which I visualized neuronal cell bodies, clearly identified by their shape and size in bright field microscopy (Sibilla et al., 2009; Fabbro et al., 2007). Ca\textsuperscript{2+} oscillations were indeed recorded from ventrally located spinal neurons (<20 μm somatic diameter), which fulfilled the criteria for interneuronal identification on the basis of their round shape and were located in close proximity (20-300 μm) to the ventral fissure (Sibilla et al., 2009; Fabbro et al., 2007; Ballerini et al., 1999; Ballerini and Galante, 1998; Spenger et al., 1991; Streit et al., 1991). As previously shown, these cells are clearly distinguishable from other neurons with the typical morphology of motoneurons as well as from DRG neurons (Sibilla et al., 2009; Fabbro et al., 2007). In this thesis I decided to define as interneuron any neuron (in the ventral region) that was clearly distinguishable from motor neurons. This category, thus, includes projection neurons even if their ultimate target was confined to the slice preparation and whose full maturation is lacking in the embryonic spinal cord.
since axons have not yet reached their targets (Eide and Glover, 1995). Therefore, in accordance with this convention, developmental studies usually use the term “interneuron” for all non-motor neurons (Nissen et al., 2005).

For each experiment, 2 regions/slice were analyzed and 8±5 oscillating interneurons were selected (usually focused in the most superficial plane) to investigate changes in intracellular Ca\(^{2+}\) concentration. Signals were colored spots in clear correspondence to previously identified cell bodies and were analyzed by limiting the area of interest over the cell body, excluding the background (see example in Figure 3). Interneurons were visible in pseudo-colors from blue to red, corresponding to increasing scale of Ca\(^{2+}\) concentrations.

**Electrophysiological recordings**

Electrophysiological experiments were carried out using the same type of recording chamber used in Ca\(^{2+}\) imaging experiments, with a constant perfusion with standard Krebs solution at 5 ml/min flow. All experiments were carried on at RT.

Electrophysiological data were obtained from visually identified ventral spinal interneurons by performing voltage or current-clamp recordings in the whole-cell configuration.

Individual neurons were patch clamped with micropipettes pulled from thin-wall borosilicate glass capillaries (Clark) by mean of a two steps puller (List-Medical); tip resistances were 4-7 MΩ when filled with the intracellular solution (see below).

During experiments data were digitized online at 10 kHz using an A/D converter (Digidata 1322A or 1440A, Axon Instruments), filtered at 2 KHz and stored for further analysis with the pCLAMP software (Axon Instruments, version 10.2).

**Patch clamp whole cell recordings: voltage clamp**

To record currents an Axopatch 1-D (Axon Instruments) was used allowing clamp of the membrane potential at constant values. Neurons were kept at a holding potential (Vh) of -56 mV (not corrected for junction potential). After the formation of a 1-4 GΩ seal, the fast capacitance transient due to the pipette was cancelled and after breaking through the membrane no series resistance compensation was adopted, since its value
was usually below 15 MΩ (12 ± 4 MΩ) and no distortion in synaptic events was observed. Cell input resistance and membrane capacitance were assessed after the establishment of whole-cell configuration and during the experiment by evaluating the response to hyperpolarizing steps. Voltage clamp mode was used to analyze spontaneous and miniature PSCs. Different K⁺ currents were recorded, employing different voltage protocols (see Results), in the presence of tetrodotoxin (TTX, 1 µM) in the bath solution to block Na⁺ currents.

**Patch clamp whole cell recordings: current clamp**

Current was injected in the cell by means of a Multiclamp-700-B (Axon Instruments) amplifier to set the resting membrane potential around –60 mV. Resistance was balanced in the bridge mode and capacitive transients were minimized by negative capacitance compensation. Current clamp mode was used to monitor spontaneous spiking activity. Action potential discharge patterns were investigated by delivering depolarizing current steps of variable intensity (0.1 nA - 0.2 nA, 500 ms duration) while keeping the cells at -60 mV resting potential with steady intracellular current injection.

**Solutions**

**Extracellular solutions**
While recording, cultures were continuously perfused with the standard Krebs extracellular solution containing (mM):

- 152 NaCl
- 5 KCl
- 2 CaCl₂
- 1 MgCl₂
- 10 HEPES
- 10 glucose

pH adjusted to 7.4 with NaOH (Carlo Erba, Italy); osmolarity was 310 mOsm.
Modified Ca\(^{2+}\)-free solution consisted in the same recording solution except for (in mM): 0 CaCl\(_2\), 3 MgCl\(_2\) and 5 EGTA (Carlo Erba, Italy).

**Intracellular solution**

Micropipettes were filled with an intracellular solution, whose composition was (mM):
- 120 K gluconate
- 20 KCl
- 10 HEPES
- 10 EGTA
- 2 MgCl\(_2\)
- 2 Na\(_2\)ATP

pH adjusted to 7.35 with KOH (Carlo Erba, Italy); osmolarity 295 mOsm.

**Drugs**

Drugs were bath applied through the perfusion solution. Stock solution were made usually at concentration 100 higher than the final ones and stored at -20 °C. The final concentration was obtained adding an amount of the stock solution to standard Krebs.

The effects of drugs started to be detectable after about 30 s from the application, however they were measured at steady state (5 min).

The following drugs were used:

- Tetrodotoxin (TTX; Latoxan, France) 1 μM to block Na\(^+\) channels
- 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (acetoxyethyl ester) (BAPTA-AM; Sigma-Aldrich, Italy) 30 μM to chelate intracellular Ca\(^{2+}\)
- Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (acetoxyethyl ester) (EGTA-AM; Sigma-Aldrich, Italy) 20 μM to buffer intracellular Ca\(^{2+}\)
Chronic treatments

For experiments involving chronic treatments, organotypic cultures were incubated in medium containing either 30 μM BAPTA-AM or 20 μM EGTA-AM. After 24 hours for BAPTA-AM and 15 hours for EGTA-AM, the medium was replaced with fresh one without any intracellular Ca\(^{2+}\) chelator for 1-9 hours before recordings. This variable washout period did not introduce significant changes to the effects induced by chronic treatment. Control cultures, namely untreated cultures, were subjected to the same medium changes without addition of any Ca\(^{2+}\) buffers. Treated and untreated cultures coming from the same culture group are called “sister cultures”.

Data analysis

Results are presented as mean ± S.E.; n is the number of neurons, if not otherwise indicated.

Intracellular Ca\(^{2+}\) oscillations (i.e. signal rises over baseline), expressed as ΔR, were considered significant if they exceeded 5 times the S.D. of the baseline noise. Each event was also visually inspected to exclude artifacts. Repetitive signal period was measured as the time interval between the onsets of two subsequent events. Episode duration was defined as the time from its onset to the 90% of its return to baseline. After obtaining the average values for period and duration from each cell in a slice, data were pooled for all slices recorded under the same experimental conditions and averaged for further comparison. Since the amplitude of Ca\(^{2+}\) transients was highly variable, I did not consider the absolute value of amplitudes as a parameter for the characterization of Ca\(^{2+}\) signals.

Membrane capacitance and input resistance were measured both in voltage and current clamp configurations at resting potential (-58 and -60 mV). In voltage clamp mode, cell was stimulated with a 100 ms lasting hyperpolarizing stimulus (-10mV), then, in the recording, area below capacitative transients was measured and normalized for voltage transient amplitude to calculate cellular capacitance; input resistance was obtained through Ohm’s law, by measuring the amplitude of steady state current generated by voltage transient. In current clamp configuration, a 100 ms lasting 0.01 nA negative
current injection was performed and the resulting voltage transient was recorded. Input resistance was valued by means of Ohm’s law and capacitance was calculated as ratio between constant of decay of voltage transient (fitted with a monoexponential function) and input resistance.

Single spontaneous and miniature synaptic events were detected by use of the AxoGraph X (Axograph Scientific) event detection program on an Apple Computer workstation. On average, 200-300 PSCs were analyzed from each cell in order to obtain mean kinetic and amplitude parameters. From the average of these events, I measured the rise time, calculated from 10 to 90% peak amplitude, the peak amplitude and the value of decay (expressed as τ) by fitting monoexponential (for AMPA-mediated events) or biexponential (for GABA-mediated events) functions.

Statistical analysis was performed by pCLAMP 10 and Origin 6 softwares and the employed statistical tests were the Chi Square, the Levene's test (to validate the variances homogeneity) the T-Student (for parametric data) and the Mann-Whitney (for non-parametric data); differences were considered statistically significant when P value was less than 0.05.
AIMS

The specific goals of the present study are:

1. to investigate whether artificial manipulation of intracellular Ca\textsuperscript{2+} buffering, by chronic and acute treatment with the intracellular Ca\textsuperscript{2+} buffer BAPTA-AM, affects Ca\textsuperscript{2+} oscillations in ventral interneurons of organotypic spinal slices;

2. to analyze whether BAPTA-AM modifies the biophysical properties of neurons and neuronal network activity;

3. to explore, by employing a different intracellular Ca\textsuperscript{2+} chelator (EGTA-AM), the specificity of BAPTA-AM effects.

All results were obtained from slice cultures of embryonic mouse spinal cord during the second and third week of \textit{in vitro} growth (14-20 DIV), a developmental stage at which the expression of spontaneous Ca\textsuperscript{2+} oscillations is maximal. These cultures allow to evaluate the long-term effects of chronic treatments, and they also permit the combination of different investigation techniques such as electrophysiology and Ca\textsuperscript{2+} imaging. Moreover, since the \textit{in vitro} development of this preparation has been extensively characterized (Streit \textit{et al.}, 1991; Streit., 1993; Ballerini and Galante, 1998; Ballerini \textit{et al.}, 1999; Galante \textit{et al.}, 2000; Galante \textit{et al.}, 2001; Rosato-Siri \textit{et al.}, 2002; Avossa \textit{et al.}, 2003; Fabbro \textit{et al.}, 2007; Furlan \textit{et al.}, 2007; Sibilla \textit{et al.}, 2009), they represent an useful model to evaluate the changes induced by chronic and acute treatments on neuronal network function and on Ca\textsuperscript{2+} oscillations behavior.

In this thesis, I focused particularly on Ca\textsuperscript{2+} oscillations because they are an uncommon and peculiar type of intracellular Ca\textsuperscript{2+} signals: completely independent from action potentials and synaptic activity, and generated in a small subset of ventral interneurons of organotypic spinal cultures by the contribution of various Ca\textsuperscript{2+} stores and sources (Fabbro \textit{et al.}, 2007; Sibilla \textit{et al.}, 2009).
RESULTS

The main aim of my thesis was to investigate the effect of chronic and acute intracellular Ca\textsuperscript{2+}'s manipulation on Ca\textsuperscript{2+} oscillations behavior, biophysical properties of neurons and neuronal network activity of organotypical spinal cultures. To this end, I chronically treated spinal slices with two different intracellular Ca\textsuperscript{2+} chelators, BAPTA-AM and EGTA-AM, and I investigated their effects using Ca\textsuperscript{2+} imaging and single cell patch clamp techniques.

Impact of chronic intracellular Ca\textsuperscript{2+} buffering on Ca\textsuperscript{2+} oscillations

To gain insight into the role of intracellular Ca\textsuperscript{2+} buffering on the generation of Ca\textsuperscript{2+} oscillations, I routinely recorded spontaneous Ca\textsuperscript{2+} oscillations from a ventral field (of about 125\textmu m x 95\textmu m size, Figures 1A and 1B) of chronically incubated (24h with BAPTA-AM; see methods) and non-incubated (control) organotypical spinal cultures. I performed these experiments during the second and third weeks of in vitro growth because, as previously reported (Fabbro et al., 2007; Sibilla et al., 2009), at this time the majority of slices display repeated Ca\textsuperscript{2+} oscillations.

In this study, I reported that, under standard experimental conditions, untreated cultures displayed spontaneous Ca\textsuperscript{2+} oscillations in 72% (13 out of 18) of the recorded organotypic slices with 4±3 oscillating neurons detected in each field. Conversely, 90% (17 out of 19) of the slices chronically treated with BAPTA-AM were oscillating, a feature which was accompanied with a significant increase number of oscillating neurons (8±4) in each field (P<0.05; Figure 1C).
Figure 1 A-B) Pseudo-color images of the optical signals at random frame interval obtained from ventral fields in control (A) and BAPTA-AM-treated (B) slices. Slices were loaded with the fluorescent indicator FURA-2-AM; the Ca²⁺ maps were obtained by computing a ratio of 340/380 nm excitation wavelength values (ΔR). The red arrows indicate the oscillating neurons. Note that the field recorded from BAPTA-AM treated slices shows an increased number of oscillating neurons compared with control one. C) Quantification of oscillating slices and neurons in control (in black) and BAPTA-AM treated (in red) cultures by Ca²⁺ imaging experiments. The histogram and plot summarize the results. The fraction of oscillating slices rose from 72% in control cultures to 90% in BAPTA-AM ones (left panel). Even the number of oscillating neurons detected in each field was significantly increased (* P<0.05) from 4±3 in the control to 8±4 in the BAPTA-AM oscillating neurons (right panel).
Figure 2A shows an example of Ca$^{2+}$ oscillations recorded from BAPTA-treated and untreated slices. In standard solution, the kinetic of oscillations was affected by BAPTA treatment, in fact, the control oscillating neurons (n=30) exhibited a mean duration of 9±2s and mean period of 24±11s, while BAPTA-AM ones (n=51) presented a significantly increased mean duration (11±3s; P<0.01) with similar period (26±9s) (Figure 2).

Figure 2. A) Left panel: representative spontaneous Ca$^{2+}$ oscillations generated from one control (black trace) and one BAPTA-AM (red trace) oscillating ventral spinal interneurons. Right panel: Ca$^{2+}$ oscillations in an expanded time scale. B) Plots show the oscillations duration (left panel) and period (right panel) in control (in black) and BAPTA-AM oscillating neurons (in red). Note that oscillations recorded from BAPTA-MA neurons have a significantly increase duration (** P<0.01) with no difference in the period.
The next step was to verify if Ca\textsuperscript{2+} oscillations, recorded after chronic incubation with BAPTA-AM, represent the same phenomenon as the spontaneous ones, by testing their dependence on extracellular Ca\textsuperscript{2+} (Fabbro et al., 2007; Sibilla et al., 2009). Slices were perfused with Ca\textsuperscript{2+}-free solution, and I monitored the behavior of oscillating neurons. Ca\textsuperscript{2+} oscillations were fully suppressed in 38\% of the analyzed control neurons (13 out of 34 neurons from 6 control slices) and in 32\% of the analyzed BAPTA-AM neurons (13 out of 41 neurons from 6 treated slices) (Figure 3A bottom traces and Figure 3B). In the remaining fraction of neurons, although regular oscillations disappeared, some isolated, sporadic Ca\textsuperscript{2+} oscillations were still detected (Figure 3A top traces and Figure 3B). The similar extracellular Ca\textsuperscript{2+} dependence of control and chronic BAPTA-AM oscillating slices validated the nature of this phenomenon.

The available data showed that, even if after chronic incubation with BAPTA-AM Ca\textsuperscript{2+} oscillations represent the same phenomenon as the spontaneous ones, there was an increase of slices and neurons showing Ca\textsuperscript{2+} oscillations. Hence, I wanted to explore how long these changes would persist. I addressed this issue by analyzing Ca\textsuperscript{2+} oscillations after 24-34h of BAPTA washing out. As exemplified in Figure 4, after prolonged washout, Ca\textsuperscript{2+} oscillations from BAPTA treated slices recovered the kinetic of control neurons, while the trend of increasing number of oscillating neurons and slices persisted.
Figure 3. A) Behavior of Ca\textsuperscript{2+} oscillations during perfusion with a Ca\textsuperscript{2+}-free solution in control (black traces) and BAPTA-AM (red traces) oscillating slices. The response was bimodal, Ca\textsuperscript{2+} transients could be completely removed (bottom traces) or not (top traces). B) The histogram summarizes the percentage of control (in black) and BAPTA-AM (in red) neurons in which the oscillations were blocked or continuous in Ca\textsuperscript{2+}-free solution.
Figure 4. A) Left panel: examples of spontaneous Ca\textsuperscript{2+} oscillations recorded in control (black trace) and chronic BAPTA-AM (red trace) cultures after 24h washing out. Right panel: expanded record of two Ca\textsuperscript{2+} oscillations. B) These plots display the values of duration (left panel) and period (right panel) of Ca\textsuperscript{2+} oscillations in control (in black) and BAPTA-AM (in red) neurons after 24h BAPTA washing out. Note that there is no difference in the duration (control = 13±4s, BAPTA-AM = 14±4s) and period (control = 26±11s, BAPTA-AM = 25±7s) between the two conditions. C) These graphics summarize that, after a prolonged wash out, the increased number of oscillating neurons per slice (right panel, control = 9±2 and BAPTA-AM = 15±5) and oscillating slices (left panel, control = 44% and BAPTA-AM = 86%) observed in BAPTA-AM treated cultures persists.
To conclude these sets of experiments, I investigated the effect of acute treatment with BAPTA-AM on oscillating neurons (n = 21). Figure 5 shows two representative Ca\textsuperscript{2+} oscillation traces recorded before and during acute BAPTA-AM perfusion. Acute treatment with BAPTA-AM induced a decrease of intracellular Ca\textsuperscript{2+} level. During this decrease the oscillations became less frequent and finally disappeared. This result shows that a correct intracellular Ca\textsuperscript{2+} homeostasis is necessary for the genesis of oscillations.

![Figure 5](image)

**Figure 5.** A) Example of two oscillating neurons from a control slice, recorded in the same visual field, before and during acute application of BAPTA-AM (30 µM). Note that Ca\textsuperscript{2+} oscillations are fully blocked and that Ca\textsuperscript{2+} signal baseline decreases during perfusion with BAPTA-AM

These results show that long-lasting application with BAPTA-AM induces opposite changes in Ca\textsuperscript{2+} oscillations when compared to acute treatment. In particular, I found that slices chronically treated have a higher probability of spontaneously generating Ca\textsuperscript{2+} oscillations when compared to control. This increased oscillating ability persisted after a prolonged BAPTA wash out. On the other hand acute treatment with BAPTA-AM fully blocked Ca\textsuperscript{2+} oscillations.
Modulation of synaptic activity by chronic BAPTA-AM treatment

The effects of chronic treatment with BAPTA-AM on biophysical properties of neurons were evaluated by measuring the membrane capacitance and the input resistance. Such properties are commonly used as indicators of neuronal function and of development stage conditions: in detail, membrane capacitance is an indirect measure of cellular dimension, and input resistance is related to the number of leak channels expressed in neuronal membranes.

Patch clamped interneurons from controls and BAPTA-AM slices displayed similar passive properties (input resistance: 384±30MΩ in control, 369±21 MΩ in BAPTA-AM; cell capacitance: 67±5pF in control, 62±4pF in BAPTA-AM; n = 28 and n = 24, control and BAPTA-AM neurons, respectively).

In a second set of experiments, the changes induced by chronic treatment with BAPTA-AM on interneuronal activity were assessed by recording spontaneous and miniature postsynaptic currents (PSCs) from ventral interneurons. This combined assay measures two aspects of network synaptic function. In particular, spontaneous PSCs mainly reflect random firing of local neurons, and thus provide an index of how changes in network activity can shape the function of a single interneuron. On the other hand, miniature PSCs are independent from action potential, and help to localize changes in synaptic transmission to pre and/or post synaptic level.

Spontaneous PSCs and miniature PSCs were therefore recorded from ventral interneurons in control cultures and from cultures chronically incubated with BAPTA-AM, by means of the whole cell patch clamp technique in the voltage-clamp mode. Holding potential (Vh) was set at -56 mV, a condition allowing the recording of heterogeneous currents (as both excitatory -glutamate-mediated- and inhibitory –GABA and glycine-mediated- synaptic currents are detected as inward).

I first investigated the spontaneous activity recorded from control and BAPTA-AM treated cultures to check if chronic incubation with BAPTA-AM induces any detectable difference in the overall spinal network. In standard solution, PSCs were recorded from 7 control and 8 BAPTA-AM ventral interneurons (Figure 6A). In BAPTA-AM neurons, an extensive and significant increase of the frequency of spontaneous PSCs was detected (control = 23.3±2.7Hz, BAPTA-AM = 30.9±1.4Hz; P<0.01) (Figure 6B).
Figure 6. A) Two representative traces of spontaneous activity recorded from one control (black trace) and one BAPTA-AM (red trace) ventral interneurons. B) Plots show the frequencies of spontaneous PSCs. Note that BAPTA-AM treated cultures display a significantly higher rate of PSCs in respect to the control (** P<0.01).

Under standard experimental conditions two PSC types can be clearly identified on the basis of their decay time course: PSCs with a fast decay time (< 5 ms), which are AMPA-mediated, and PSCs with slow decay time(> 20 ms) which are GABA and glycine-mediated (Galante et al., 2000). These two types of events were separately analyzed for their kinetic features and amplitude. Table 1 shows the absence of statistically significant variations in mean values of rise time, decay time constant (τ) and peak amplitude between control and BAPTA-AM treated samples (fast events: n = 7 control and n =8 BAPTA-AM neurons; slow events: n = 5 control and n = 7 BAPTA-AM neurons).
Acute application of 1 μM tetrodotoxin (TTX) dramatically reduced the recorded activity by eliminating its action potential-driven component and allowed the recording of the miniature PSCs, which were recorded from both control (n = 8) and BAPTA-AM (n = 8) interneurons (Figure 7A). The frequencies of heterogeneous miniature PSCs are shown in Figure 7B while Table 2 shows mean values of their rise time, decay time constant and peak amplitude, for both control and BAPTA-AM interneurons. No differences emerged between control neurons and BAPTA-AM ones when comparing the frequency (control = 5.6±1.2Hz, BAPTA-AM = 7.7±1.3Hz) and the kinetic features of miniature PSCs (fast events: n = 8 control and n =8 BAPTA-AM neurons; slow events: n = 4 control and n = 5 BAPTA-AM neurons).

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<th>Fast PSCs</th>
<th>Slow PSCs</th>
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<tr>
<td></td>
<td>Rise time (ms)</td>
<td>Decay time (ms)</td>
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<tr>
<td>CONTROL</td>
<td>0.88 ± 0.06</td>
<td>2.4 ± 0.18</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>0.87 ± 0.04</td>
<td>2.7 ± 0.2</td>
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Table 1
Figure 7. A) Two representative recordings of miniature PSCs (in the presence of 1 μM tetrodotoxin, TTX) from ventral interneurons from control (black trace) and BAPTA-AM treated (red trace) cultures. B) Plot shows the frequencies of miniature PSCs in control and BAPTA-AM neurons.

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<th>Fast PSCs</th>
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<tr>
<td></td>
<td>Rise time (ms)</td>
<td>Decay time (ms)</td>
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<tr>
<td>CONTROL</td>
<td>0.81 ± 0.04</td>
<td>3.08 ± 0.19</td>
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<tr>
<td>BAPTA-AM</td>
<td>0.76 ± 0.02</td>
<td>3.03 ± 0.21</td>
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Table 2
Effects of BAPTA-AM on voltage-dependent $K^+$ currents of ventral spinal interneurons

The results reported in the previous sections indicate that chronically treated BAPTA-AM slices show an increased excitability at the single cell level. I then decided to investigate whether $K^+$ currents were involved in this change. My choice was justified by the fact that $K^+$ currents are the main currents which contribute to the repolarization of neurons and therefore play a critical role in neuronal excitability.

Voltage gated $K^+$ currents were recorded, using whole-cell patch-clamp configuration in the voltage clamp mode, from ventral spinal interneurons after chronic incubation or during acute treatment with BAPTA-AM. To isolate voltage gated $K^+$ currents, in all the experiments described below, the inward $Na^+$ currents were completely blocked by perfusion with 1 µM TTX.

At the beginning I investigated if chronic incubation with BAPTA-AM induced changes in the $K^+$ conductance. For this reason, I recorded the amplitude of outward $K^+$ currents, from 7 control and 6 BAPTA-AM ventral spinal interneurons, by applying a series of voltage steps from -100 mV to +40 mV with a holding potential of -60 mV (Costantin and Charles, 2001). I repeated this protocol three times in each neuron, then I measured the mean amplitude of $K^+$ currents at steady-state for each step and, finally, I normalized all values to the capacity to obtain current density values. In Figure 8A an example of $K^+$ current recorded from untreated and treated slices is reported. The mean current density was significantly affected, starting from the -10mV potential, by chronic incubation with BAPTA-AM (at +30mV: control = 8.3±2.2pA/pF, BAPTA-AM = 23.2±4.2pA/pF, P<0,05; Figure 8B left panel); conversely, no difference was detected in the leak currents (at +30mV: control = 13.3±1.6pA/pF, BAPTA-AM = 14.5±2.9pA/pF; Figure 8B right panel).
Figure 8. A) Voltage-activated K⁺ currents in control (black) and BAPTA-AM (red) ventral spinal interneurons. Currents were evoked by a series of voltage steps from -100mV to + 40mV of 500 ms duration from a holding potential of -60 mV. The pulse protocol used to elicit the currents is shown in the inset. Tetrodotoxin (TTX, 1 µM) was present in the bath solution. B) Left panel: the plot shows the mean I/V relationships of K⁺ currents recorded from 7 control and 6 BAPTA-AM neurons. All values were obtained by subtracting the leak current and normalizing them to the capacity. Right panel: the plot shows the mean leak currents normalized by cell capacitance. Note the significantly increase amplitude of outward K⁺ currents (* P<0.05) in slices chronically treated with BAPTA-AM compared with control ones.

In order to analyse which type of K⁺ channel was involved in increasing the amplitude of outward K⁺ currents, I separately recorded each type of K⁺ currents. Since BAPTA-AM is an intracellular Ca²⁺ chelator, I started to investigate the presence of any changes in Ca²⁺ activated K⁺ current (I_{K(Ca)}). The amplitude of I_{K(Ca)} was obtained by subtracting from the value of K⁺ current recorded in standard Krebs solution (Figure 9A) the value
recorded during extracellular Ca\(^{2+}\)-free perfusion (Figure 9B) (Costantin and Charles, 2001). It can be seen from Figure 9C that there is no significant difference in the peak \(I_{K(Ca)}\) between control and BAPTA-AM neurons (at +30mV: control \(I_{K(Ca)} = 4.3\pm0.5\) pA/pF, BAPTA-AM \(I_{K(Ca)} = 4.5\pm0.5\) pA/pF). Similar results were obtained from 5 different culture series (n = 5 control and n = 5 BAPTA-AM neurons).
Figure 9. Separation of outward $K^+$ current into $Ca^{2+}$-dependent and independent components. A) Voltage-activated $K^+$ currents recorded from control (black) and BAPTA-AM (red) ventral spinal interneurons in the presence of normal extracellular $Ca^{2+}$ concentration (2mM). B) Reduces currents in the same cells after removal of extracellular $Ca^{2+}$. The pulse protocols used to elicit the currents are shown in the insets. Tetrodotoxin (TTX, 1 μM) was always present in the bath solution. All values were obtained by subtracting the leak current and normalizing them to the capacity. C) The plot shows the I/V relationships of $I_{K(Ca)}$ obtained by subtraction (A – B) of the traces on the top. Note that the $I_{K(Ca)}$ doesn’t seem to be involved in the increase amplitude of total outward $K^+$ currents.
The outward K\(^+\) current could be also separated, using two different voltage protocols, into a rapidly activating transient component (I\(_{K(A)}\)) and a more slowly activating delayed-rectifier component (I\(_{K(DR)}\)). In the first protocol the neurons, held at a membrane potential of -90mV, were hyperpolarized to -140mV for 250ms before applying a series of voltage steps from -100mV to +40mV (Figure 10A) (Bardoni and Belluzzi, 1993; Watkins and Mathie, 1994; Watkins and Mathie, 1996; Costantin and Charles, 2001). This voltage protocol allowed recording both I\(_{K(A)}\) and I\(_{K(DR)}\). The second protocol is quite similar to the first one except for the prepulse, which depolarized the neurons to -50mV. This depolarizing prepulse was used to inactivate I\(_{K(A)}\) so, I\(_{K(DR)}\) could be isolated and recorded (Figure 10B) (Bardoni and Belluzzi, 1993; Watkins and Mathie, 1994; Watkins and Mathie, 1996; Costantin and Charles, 2001). Figure 10C shows that I\(_{K(A)}\), recorded in both culturing conditions, was not significantly affected by chronic incubation with BAPTA-AM (at +30mV: control I\(_{K(A)}\) = 2.1±0.5 pA/pF, BAPTA-AM I\(_{K(A)}\) = 1.7±0.3 pA/pF). In contrast to its apparent lack of effect I found a significant increase (P<0.05) of I\(_{K(DR)}\) (at +30mV: control I\(_{K(DR)}\) = 21±3 pA/pF, BAPTA-AM I\(_{K(DR)}\) = 39±3.6 pA/pF) (Figure 10B). In four different series of cultures similar results were obtained (n = 5 control and n = 5 BAPTA-AM neurons).
Figure 10. Separation of outward K⁺ current into \( I_{K(DR)} \) and \( I_{K(A)} \) component. A) Voltage-activated K⁺ currents recorded from control (black) and BAPTA-AM (red) ventral spinal interneurons. Currents were evoked by a series of depolarizing steps from -100mV to +40mV, and 250ms prepulse to -140mV was delivered to remove any residual resting inactivation. B) The \( I_{K(DR)} \) recorded from the same neurons and under the same conditions as in A except that the prepulse amplitude was -50mV, which was used to inactivate \( I_{K(A)} \). The pulse protocols used to elicit the currents are shown in the insets. Tetrodotoxin (TTX, 1 µM) was always present in the bath solution. The plot shows the I/V relationships of \( I_{K(DR)} \) recorded from the traces on the top. All values were obtained by subtracting the leak current and normalizing them to the capacity. C) The \( I_{K(A)} \) that was inactivated by the depolarizing prepulse was obtained by subtraction (A - B). Note the increased amplitude of \( I_{K(DR)} \) in the neuron chronically treated with BAPTA-AM without any difference in the \( I_{K(A)} \).
In contrast with the results reported above, acute bath application of BAPTA-AM resulted in a decreased amplitude of outward K\(^+\) currents. This can be seen in the activity of a typical neuron in Figure 11 in which I found, during BAPTA-AM perfusion, a reduction of about 27\% in the amplitude of these currents at +30mV, without any difference in the leak currents. The results shown from this experiment are typical for spinal interneurons (at +30mV: mean reduction = 35\(\pm\)12\%; n = 5 neurons from 4 culture series).

Figure 11. A) Voltage-activated K\(^+\) currents recorded from the same neuron before (left panel) and during (right panel) BAPTA-AM (30 \(\mu\)M) perfusion. Currents were evoked by a series of voltage steps from -100mV to +40mV of 500ms duration from a holding potential of -60mV. The pulse protocol used to elicit the currents is shown in the inset Tetrodotoxin (TTX, 1 \(\mu\)M) was present in the bath solution. B) Left panel: the plot shows the I/V relationships of K\(^+\) currents recorded from the same neuron in the two different conditions. All values were obtained by subtracting the leak current and normalizing them to the capacity. Right panel: the plot shows the mean leak currents normalized by cell capacitance. Note that the acute treatment with BAPTA-AM inhibits about 27\% of outward K\(^+\) currents.
To summarize, acute treatment with BAPTA-AM reduces $K^+$ currents, in contrast to slices chronically incubated with BAPTA-AM showing an increase in amplitude of these currents. It is noteworthy that the $K^+$ channel mainly involved in these changes is $K_{DR}$, whereas BAPTA-AM has no effect on $K_A$ and $K_{Ca}$.

**Basic features of neuronal excitability in cultures chronically treated with BAPTA-AM**

The following experiments were aimed at investigating whether the increased amplitude of $I_{K(DR)}$, observed after chronic incubation with BAPTA-AM could induce changes in neuronal excitability. For this reason, I performed current clamp recordings of spontaneous activity from 14 control and 10 BAPTA-AM ventral interneurons. In all recorded neurons resting membrane potential (obtained with steady current injection) was set at $–60$ mV.

In Figure 12A sample tracings of spontaneous firing activity in control and BAPTA-AM neurons are shown. In standard Krebs solution no difference in action potential (AP) frequency was detected between control and BAPTA-treated cultures (AP frequency: $0.84±0.26$ Hz in control, $0.68±0.29$ Hz in BAPTA-AM) (Figure 12B).
Figure 12. A) Representative tracings of spontaneous activity recorded from control (black trace) and BAPTA-AM (red trace) ventral spinal interneurons. B) Mean action potential frequency is shown in the plot. No significant differences in the kinetic or in mean frequency values emerged between treated and control cultures.

I further analyzed in detail the resting membrane potential and the kinetic parameters related to spontaneous APs but, as shown in Table 3, they are not modified after chronic incubation with BAPTA-AM.
In the next series of experiments, I studied the evoked firing properties, from untreated and treated slices, to test whether the chronic incubation with BAPTA-AM affects the firing patterns of ventral interneurons.

Firing patterns were induced by depolarizing current steps of 500ms duration and 0.1 – 0.2 nA amplitude from a membrane potential of –60mV (Furlan et al., 2007). As shown in Figure 13, I could identify all different classes of interneurons present in organotypic spinal slices, on the basis of their firing pattern, as previously reported in the introduction (Furlan et al., 2007). However, treated cultures showed a slightly different distribution-profile of interneuron types. In fact, after chronic incubation with BAPTA-AM there was an increase in the fraction of tonic neurons (control = 44% and BAPTA-AM = 59%), the appearance of delay neurons (control = 0% and BAPTA-AM = 16%) and a decrease of adapting (control = 16% and BAPTA-AM = 10%), irregular (control = 20% and BAPTA-AM = 5%) and transient (control = 20% and BAPTA-AM = 10%) neurons (n = 13 control and n = 10 BAPTA-AM neurons) (Figure 13 B).

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<thead>
<tr>
<th></th>
<th>Peak amp (mV)</th>
<th>Area (mV*ms)</th>
<th>Rise time (ms)</th>
<th>Decay time (ms)</th>
<th>Resting potential (mV)</th>
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<tr>
<td>CONTROL</td>
<td>73 ± 4.1</td>
<td>108 ± 7.34</td>
<td>0.63 ± 0.04</td>
<td>1.89 ± 0.23</td>
<td>- 48 ± 1</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>78.4 ± 5.3</td>
<td>113 ± 11.7</td>
<td>0.58 ± 0.04</td>
<td>1.71 ± 0.22</td>
<td>- 49 ± 1</td>
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Table 3
In summary, on neurons tested, chronic incubation with BAPTA-AM had no effect on resting membrane potential or network-driven firing activity, although it affected firing patterns.

**Exploring the specificity of BAPTA-AM effects: comparison with EGTA-AM**

Some of the BAPTA-effects reported in the previous sections, such as those observed in Ca\(^{2+}\) oscillations, are clearly correlated with the Ca\(^{2+}\)-buffering action of BAPTA; other ones, such as those observed on K\(^{+}\) conductance, are unexpected and did not show any
clear correlation with the changes in the Ca\textsuperscript{2+} transients. In literature there are only a few works which show this effect of BAPTA-AM on K\textsuperscript{+} currents (Watkins and Mathie, 1996; Constantin and Charles, 2001). Hence, it appeared interesting to investigate whether all the above results were reproducible by another intracellular Ca\textsuperscript{2+} chelator. To address this issue, I performed Ca\textsuperscript{2+} imaging and electrophysiological experiments from slices after chronic incubation (15 hours incubation; see methods) or during acute treatment with EGTA-AM.

**Impact of EGTA-AM on Ca\textsuperscript{2+} oscillations**

In a first set of experiments, where Ca\textsuperscript{2+} imaging experiments were performed after chronic incubation with EGTA-AM, I found a significant increase (P<0.05) in the number of oscillating slices in EGTA-AM treated cultures (67%, 6 out of 9) compared to control ones (24%, 4 out of 17), with a slight increase in the number of oscillating neurons in each field (6±1 in control and 10±2 in EGTA-AM) (**Figure 14**).
Figure 14. A-B) Pseudo-color images of the optical signals at random frame interval obtained from control (A) and EGTA-AM (B) ventral fields. Slices were previously loaded with the Ca²⁺-sensitive fluorescent indicator FURA-2-AM; the Ca²⁺ maps were obtained by computing a ratio of 340/380 nm excitation wavelength values (ΔR). The red arrows indicate the oscillating neurons. Note that the slices chronically treated with EGTA-AM show an increased number of oscillating neurons. C) The histogram and plot summarize the percentage of the slices in which the oscillations were found and the mean number of oscillating neurons observed in each field, respectively. Note the significantly increased probability of finding oscillating slices in EGTA-AM treated cultures as compared to control ones (* P<0.05). Even the number of oscillating neurons, detected in each field, was slight increased from 6±1 in the control to 10±2 in the EGTA-AM oscillating neurons.
Conversely, further analysis showed no significant differences in the kinetic of \(\text{Ca}^{2+}\) oscillations between treated and untreated cultures (mean duration: 15±1s in control and 18±2s in EGTA-AM, mean period: 29±2s in control and 33±3s in EGTA-AM; \(n = 9\) and \(n = 18\), control and EGTA-AM oscillating neurons, respectively) (Figure 15).

Figure 15. A) Left panel: spontaneous \(\text{Ca}^{2+}\) oscillations generated from control (black trace) and EGTA-AM (magenta trace) oscillating ventral spinal interneurons. Right panel: expanded record of two \(\text{Ca}^{2+}\) oscillations. B) Plots show the mean kinetic values in control and EGTA-AM \(\text{Ca}^{2+}\) oscillations. No significant difference in kinetic features was observed.
The next step was to verify if, after chronic treatment with EGTA-AM, Ca\textsuperscript{2+} oscillations maintained the dependence upon extracellular Ca\textsuperscript{2+}. I addressed this issue by perfusing slices showing oscillations with Ca\textsuperscript{2+}-free solution. After 10 minutes without extracellular Ca\textsuperscript{2+}, in 62% of control (18 out of 29 neurons from 5 control slices) and 53% of EGTA-AM neurons (10 out of 19 neurons from 4 control slices), sporadic oscillations were detected, while oscillations were fully suppressed in the remaining fraction of neurons (Figure 16).

Figure 16. A) Representative traces showing the typical behavior during Ca\textsuperscript{2+}-free solution in control (in black) and EGTA-AM (in magenta) oscillating neurons. Ca\textsuperscript{2+} oscillations could be completely abolished (bottom traces) or not (top traces) by perfusion with Ca\textsuperscript{2+}-free solution. B) The percentage of control (in black) and EGTA-AM (in magenta) neurons in which the oscillations were blocked or continued in Ca\textsuperscript{2+}-free solution are shown in the histogram.
Furthermore, in control cultures, I investigated the effect of acute treatment of EGTA-AM on Ca\textsuperscript{2+} oscillations behavior. As shown by the two representative traces of Figure 17, perfusion with EGTA-AM completely abolished Ca\textsuperscript{2+} oscillations (n = 10 oscillating neurons from two culture series).

Figure 17. A) Examples of two neighbouring oscillating neurons before and during acute treatment with EGTA-AM (20 \mu M). Note that Ca\textsuperscript{2+} oscillations are fully blocked by perfusion with EGTA-AM.

These observations thus confirmed those obtained with BAPTA-AM, supporting the needing of a correct intracellular Ca\textsuperscript{2+} homeostasis for the genesis of oscillations and validated the presence of homeostatic adaptation as a rebound to chronic manipulation of intracellular Ca\textsuperscript{2+}.

**Impact of EGTA-AM on Electrical Activity**

The available data suggested that chronic incubation with BAPTA-AM affects network electrical activity and that, in particular, it increases the amplitude of outward K\textsuperscript{+} current. It therefore seemed necessary to study whether chronic incubation with EGTA-AM could have a comparable effect. This issue was investigated by comparing the
amplitude of outward $K^+$ current in control and EGTA-AM ventral spinal interneurons. Figure 18A shows an example of outward $K^+$ current recorded from untreated and treated slices. The mean $K^+$ and leak current densities, normalized by cell capacitance, were not affected by EGTA treatment ($n = 11$ control and $n = 7$ EGTA-AM neurons) (Figure 18B).

18. A) Voltage-activated $K^+$ currents in control (black) and EGTA-AM (magenta) ventral spinal interneurons. Currents were evoked by a series of voltage steps from -100mV to +40mV of 500ms duration from a holding potential of -60mV. The pulse protocol used to elicit the currents is shown in the inset. Tetrodotoxin (TTX, 1 $\mu$M) was present in the bath solution. B) Left panel: the plot shows the mean $I/V$ relationships of $K^+$ currents recorded from control and EGTA-AM neurons plotted. All values were obtained by subtracting the leak current and normalizing them to the capacity. Right panel: the plot shows the mean leak currents normalized by cell capacitance. Note that there is no significant difference in the amplitude of total outward $K^+$ currents (at +30mV: 44±7pA/pF in control and 41±6pA/pF in EGTA-AM) and leak currents (at +30mV: 24±10pA/pF in control and 21±3pA/pF in EGTA-AM) between control and EGTA-AM treated slices.
This data was further consolidated by the observation that also acute treatment with EGTA-AM did not affect the amplitude of outward $K^+$ and leak currents. Figure 19 shows outward $K^+$ currents and the corresponding I/V curves recorded from the same interneuron before and during perfusion with EGTA-AM. The same result was obtained on a sample of 5 ventral spinal interneurons.

**Figure 19.** A) Voltage-activated $K^+$ currents recorded from the same neuron before (left) and during (right) EGTA-AM (20 µM) perfusion. Currents were evoked by a series of depolarizing steps from -100mV to +40mV of 500ms duration from a holding potential of -60mV. The pulse protocol used to elicit the currents is shown in the inset. Tetrodotoxin, (TTX, 1 µM) was present in the bath solution. B) Left panel: the plot shows the I/V relationships of $K^+$ currents recorded from the same neuron in the two different conditions. All values were obtained by subtracting the leak current and normalizing them to the capacity. Right panel: the plot shows the mean leak currents normalized by cell capacitance. Note that the amplitude of total outward $K^+$ currents (at +30mV: 24pA/pF in control and 22pA/pF in EGTA-AM) and leak currents (at +30mV: 14,4pA/pF in control and 14,8pA/pF in EGTA-AM) recorded before and during acute treatment with EGTA-AM is comparable.
A possible explanation of the above results could be that BAPTA-AM exerts a specific effect on K\(^+\) channels that is not associated with the alteration in [Ca\(^{2+}\)], in contrast to EGTA-AM (Watkins and Mathie, 1996).

Spontaneous and miniature PSCs were also monitored in these experiments to inspect the effects of chronic treatment with EGTA-AM on synaptic activity. Heterogeneous spontaneous events recorded from ventral interneurons after chronic EGTA-AM treatment had a frequency of 22.5±1.74Hz (n = 7 neurons). This value is similar to the one observed in control cultures (20.38±2.35Hz, n =12 neurons) (Figure 20).

Figure 20. A) Spontaneous synaptic activity in control (black trace) and EGTA-AM (magenta trace) ventral interneurons. B) Frequencies of heterogeneous spontaneous PSCs are shown in the plot.

As shown in Table 4, also the separated analysis of the excitatory (fast-decaying) and inhibitory (slow-decaying) PSCs showed no significant difference between control and
EGTA-AM ventral spinal interneurons (fast events: n = 12 control and n = 7 BAPTA-AM neurons; slow events: n = 10 control and n = 5 BAPTA-AM neurons).

Moreover, the study of miniature PSCs by the application of 1µM TTX showed no effect of EGTA treatment either on their frequency nor on their amplitude or kinetic. As shown in Figure 21, mean frequencies of miniature PSCs were not significantly different between control and EGTA-AM interneurons (control = 2.96±0.64Hz; EGTA-AM = 2.54±0.97Hz; n = 9 and n = 5, control and EGTA-AM neurons, respectively).

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<tr>
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<th>Fast PSCs</th>
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<th>Slow PSCs</th>
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<tr>
<td></td>
<td>Rise time (ms)</td>
<td>Decay time (ms)</td>
<td>Peak amp (pA)</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.84 ± 0.03</td>
<td>2.91 ± 0.13</td>
<td>23.7 ± 3</td>
</tr>
<tr>
<td>EGTA-AM</td>
<td>0.93 ± 0.04</td>
<td>3.07 ± 0.23</td>
<td>21.7 ± 3.5</td>
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Table 4
Figure 21. A) Two representative recordings of miniature PSCs (in the presence of tetrodotoxin, TTX, 1 µM) from ventral interneurons from control (black trace) and EGTA-AM treated (magenta trace) cultures. B) Plot shows frequencies of heterogeneous miniature PSCs in the two conditions.

Also, the mean rise time, decay time and peak amplitude values of both fast and slow events were comparable in the two conditions (fast events: n = 9 control and n = 5 BAPTA-AM neurons; slow events: n = 7 control and n = 5 BAPTA-AM neurons; Table 5).

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<th>Fast PSCs</th>
<th>Slow PSCs</th>
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<tr>
<td></td>
<td>Rise time (ms)</td>
<td>Decay time (ms)</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.73 ± 0.04</td>
<td>2.61 ± 0.27</td>
</tr>
<tr>
<td>EGTA-AM</td>
<td>0.83 ± 0.02</td>
<td>3.07 ± 0.25</td>
</tr>
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Table 5

In summary, these results show that long-lasting application of EGTA-AM, in contrast to what has been observed after BAPTA-AM treatment, did not alter synaptic
transmission, and did not affect K$^+$ current. Hence, the comparison of BAPTA-AM with EGTA-AM confirms the view that BAPTA-AM exerts a specific effect on neuronal behaviour which is independent from the BAPTA Ca$^{2+}$-buffering ability.
DISCUSSION

The main finding of the present study is the novel observation that the chronic pharmacological manipulation of intracellular Ca$^{2+}$ homeostasis on spinal premotoneuronal networks \textit{in vitro} (by exogenous Ca$^{2+}$ chelators) induces profound changes in the Ca$^{2+}$ oscillations activity. Ca$^{2+}$ imaging recordings demonstrated that chronic increment of endogenous Ca$^{2+}$ buffering induces an increased recruitment of ventral interneurons into the oscillatory activity. Conversely, the acute treatment with Ca$^{2+}$ chelators fully suppresses Ca$^{2+}$ oscillations.

Another interesting result emerging from the present study is the unexpected specific effect of BAPTA-AM on K$^+$ conductance.

Organotypic spinal cultures

Organotypic cultures, prepared from embryonic rodent spinal cord, have been extensively used to study the intra-segmental neuronal maturation (Braschler \textit{et al.}, 1989; Streit \textit{et al.}, 1991; Ballerini and Galante, 1998; Avossa \textit{et al.}, 2003; Rosato Siri \textit{et al.}, 2004; Furlan \textit{et al.}, 2005; Furlan \textit{et al.}, 2007; Fabbro \textit{et al.}, 2007; Sibilla \textit{et al.}, 2009) and provide a unique method for the study of compensatory mechanisms in response to external perturbations (Galante \textit{et al.}, 2000; Galante \textit{et al.}, 2001). In fact, although they cannot exactly duplicate the \textit{in vivo} situation, organotypic cultures offer a useful compromise between dissociated cultures and \textit{in vivo} models. In fact, these cultures closely mimic the \textit{in vivo} environment, characterized by a variety of neurons and of glial cells that come together in a three-dimensional architecture (compare Avossa \textit{et al.}, 2003; Furlan \textit{et al.}, 2005 with Warf \textit{et al.}, 1991; Breckenridge \textit{et al.}, 1997; Wada \textit{et al.}, 2000; Gao \textit{et al.}, 2001; Woodruff \textit{et al.}, 2001; Liu \textit{et al.}, 2002; Tran \textit{et al.}, 2003).

From a functional point of view, several studies support the idea that organotypic slices mimic the development occurring \textit{in vivo}. First, the maturation of firing patterns of ventral interneurons is similar to those reported in acute spinal slices taken at different
postnatal ages (compare Furlan et al., 2007 with Szucs et al., 2003; Theiss and Heckman, 2005). Furthermore, the embryonic explants undergo a certain degree of maturation while growing in vitro replicating some of the basic synaptic properties (Rosato Siri et al., 2004; Furlan et al., 2007) and Ca\(^{2+}\) activity patterns (Sibilla et al. 2009) observed in the entire spinal cord (Branchereau et al., 2002; Hanson and Landmesser, 2003; Whelan, 2003; Momose-Sato et al., 2005; Momose-Sato et al., 2007; Momose-Sato et al., 2012).

On the basis of these results, I tried to clarify the mechanisms, in particular Ca\(^{2+}\) signaling, involved in the development and maturation of primitive motor networks into functional circuits. Due to the fact that it is quite difficult to manipulate endogenous cellular Ca\(^{2+}\) buffers, I started to use the cell-permeant form of BAPTA that has a high selectivity and affinity for Ca\(^{2+}\) over other ions and that exhibits fast Ca\(^{2+}\) binding (Tsien, 1980; Kao and Tsien, 1988; Pethig et al., 1989). The choice of BAPTA was justified by the fact that this synthetic Ca\(^{2+}\) buffer has features similar to those of endogenous Ca\(^{2+}\) binding proteins, including cytoplasmic mobility, Ca\(^{2+}\) affinity, Ca\(^{2+}\) binding rates, and measurable physiological effects (Scharfman and Schwartzkroin, 1989; Chard et al., 1993; Roberts, 1993; Roberts, 1994). An advantage of BAPTA compared to Ca\(^{2+}\) binding proteins is the reversibility of its physiological actions by it extrusion from the cell (Di Virgilio et al., 1990; Munsch and Deitmer, 1995; Ouanounou et al., 1996). These features make BAPTA-AM ideal for reversibly manipulating cytoplasmic Ca\(^{2+}\) buffering in neurons to directly examine the association between Ca\(^{2+}\) signaling and neuronal maturation.

**Ca\(^{2+}\) oscillations in BAPTA-AM treated cultures**

The shape of Ca\(^{2+}\) oscillations reflects the balance between the Ca\(^{2+}\)-influx/release and the underlying Ca\(^{2+}\)-extrusion/store. My first set of experiments was focuses to test the hypothesis that the Ca\(^{2+}\)-buffering capacity, and thus the intracellular level of Ca\(^{2+}\), affects the dynamics of Ca\(^{2+}\) oscillations. For this purpose, the intracellular Ca\(^{2+}\) concentration was manipulated by introducing the cell permeable acetoxy-methylester form of the Ca\(^{2+}\) chelator BAPTA into the cell.
In the experiments of chronic treatment with BAPTA-AM, I showed, for the first time, that pharmacological manipulation of healthy intracellular Ca\textsuperscript{2+} homeostasis induces a homeostatic compensation in spinal oscillating interneurons. I have determined that these compensatory changes created new, stable oscillatory patterns featuring broader duration of Ca\textsuperscript{2+} oscillations and an increment of neurons recruited to generate them, which persisted also after a prolonged wash out of the Ca\textsuperscript{2+} buffer. Furthermore, the observation that in almost half of the neuronal population oscillations could continue in the absence of external Ca\textsuperscript{2+} (although at an irregular pace), let to conclude that these new oscillatory pattern maintained the basic features of Ca\textsuperscript{2+} oscillations. Conversely, acute treatment with BAPTA-AM completely blocked Ca\textsuperscript{2+} oscillations and decreased the baseline fluorescence, strengthening the importance of Ca\textsuperscript{2+} buffering system in the control of the extent of Ca\textsuperscript{2+} rise.

I do not rule out the possibility that the changes observed could be due to the effect of BAPTA-AM metabolites, however there are a number of reasons why I believe that this interpretation is erroneous:

- the rate at which BAPTA-AM inhibits Ca\textsuperscript{2+} oscillations is similar to that seen for other drugs, such as thapsigargin, CGP and CCCP (Fabbro et al., 2007; Sibilla et al., 2009);
- acute exposure to BAPTA-AM induces a decline in basal level of intracellular Ca\textsuperscript{2+} concentration before a complete suppression of Ca\textsuperscript{2+} oscillations.

Excitability of ventral spinal neurons and networks in BAPTA-AM treated cultures

I then set out to investigate whether elevation of intracellular Ca\textsuperscript{2+} buffering, elicited by chronic BAPTA-AM treatment, induces modifications in synaptic activity. To check this, I recorded the spontaneous and miniature PSCs. After a prolonged BAPTA-AM treatment all neurons recorded in standard solution displayed a significant increase in the frequency of PSCs with both fast and slow decay (excitatory and inhibitory, respectively). These events had similar kinetic to those observed in untreated cultures. Despite the raise in frequency of spontaneous activity, the frequency of heterogeneous miniature PSCs was unchanged.
The increase in frequency of spontaneous synaptic activity, observed after chronic BAPTA-AM treatment, probably reflects an enhanced excitability of these neurons. A family of channels extremely important in the regulation of neuronal excitability are voltage-gated K⁺ channels. Surprisingly, a significantly increased amplitude of I_K were found in BAPTA-AM treated cultures. This finding is consistent with that of Watkins and Mathie (1996) who found a specific effect of BAPTA-AM on the magnitude of K⁺ conductance.

To investigate which type of K⁺ currents is involved in the up-regulation I individually recorded different types of K⁺ current. In BAPTA-AM treated cultures, the I_{K(Ca)} component, extracted by the removal of extracellular Ca²⁺, and I_{(KA)} component, obtained by using specific protocols, were similar to those reported in control cultures. Conversely, I found a functional potentiation of I_{K(DR)} leading to an overall increase in the maximal current amplitude.

Furthermore, in agreement with a previous work (Watkins and Mathie, 1996), I showed that bath application of BAPTA-AM partially reduces the magnitude of total I_K. The decrease of I_K by BAPTA-AM is time dependent, such that the current is inhibited to a greater extent at the end of the test step than at the beginning, and the inhibition is also voltage-dependent.

When I analyzed the action potential parameters I found that they were never affected by elevation of I_{K(DR)}. This observation is probably explained both by the fact that I_{K(DR)} is the main current, but not the only one, which contributes to regulate spike properties, and by the fact that BAPTA-AM could affect other ion channels. Unfortunately, in this study I have not studied the effects of BAPTA-AM on other types of ion channels.

I next tested the effect of increasing I_{K(DR)} on five ventral spinal interneuron classes. The increased amplitude of I_{K(DR)} by BAPTA-AM evoked a different distribution profile of interneurons classes. In particular, the number of ventral spinal interneurons that displayed tonic firing pattern increased after chronic treatment, and the number of neurons that displayed adapting pattern decreased reciprocally. The acquisition of repetitive firing is a characteristic feature of maturation in ventral spinal interneurons in vivo (Szucs et al., 2003; Theiss and Hackman, 2005) and in vitro (Furlan et al., 2007), as such change occurs in coincidence with the maturation of K⁺ channels. These data confirm that I_{K(DR)}, in synergy with other membrane conductances, operates to mediate the modulation of discharge properties.
Effects of EGTA-AM on ventral interneuron activity

I have reported that treatment with BAPTA-AM in ventral spinal interneurons showed an unexpected effect on K⁺ conductance beside the more foreseeable change on Ca²⁺ oscillations. Hence, one question was raised whether these changes are really associated with the alteration in [Ca²⁺], or if they depend on a different Ca²⁺ buffering-independent action of BAPTA-AM (Watkins and Mathie, 1996). To examine this aspect chronic and acute treatments with another intracellular Ca²⁺ chelator (EGTA-AM) have been performed.

As a consequence of a prolonged EGTA-AM treatment, I reported an increased recruitment of neurons in which it was possible to detect Ca²⁺ oscillations without a global change in spontaneous event kinetic. The probable explanation for this behavior could be due to a greater buffering capacity and faster kinetic for Ca²⁺ ions of BAPTA buffer compared to EGTA one (Tsien, 1980). The Ca²⁺ oscillations also maintained the same pattern of behavior during Ca²⁺-free perfusion, concurring to support the idea that they represent the same phenomenon (Fabbro et al., 2007; Sibilla et al., 2009) amplified by the prolonged increased of intracellular Ca²⁺ buffering. Furthermore, similar to those detected during BAPTA-AM perfusion, we reported that acute exposure to EGTA-AM induced a clear decrease in Ca²⁺ baseline before the complete suppression of Ca²⁺ oscillations.

Taken together, these findings concur to support the idea that the Ca²⁺ buffering capacity can play an important role in setting pattern of Ca²⁺ oscillations. In this respect it is interesting to note that, in the same culture model in a corresponding time point of in vitro development, calbindin, a Ca²⁺-buffering protein, was detected only in a small cluster (≈10%) of ventral horn interneurons (Sibilla et al., 2009). If one hypothesizes that oscillating neurons are the ones positive for calbindin, this could suggest that these neurons need, more than other interneurons, to finely regulate intracellular Ca²⁺ homeostasis to be able to generate Ca²⁺ oscillations.

Afterwards I examined synaptic activity in control and EGTA-AM neurons, without revealing a compensatory increase in the frequency or changes in the shape of both fast and slow events in spontaneous and miniature PSCs. This lack of effect might be explained by a lack of effect of EGTA-AM on I_K; therefore, I focused my attention on K⁺ conductance. My results confirmed this hypothesis, as I did not detect any change in I_K both in experiments of chronic treatment and in those in which I directly applied
EGTA-AM in the bath solution. Therefore, these data exclude that the chelation of intracellular Ca\textsuperscript{2+} by BAPTA induced modification of I\textsubscript{K}, supporting the idea that BAPTA directly inhibits K\textsuperscript{+} channels.

The observation of chelation-independent effect of BAPTA-AM on I\textsubscript{K} and the related changes in spontaneous synaptic activity should be therefore taken into account when interpreting data obtained in experiments using this Ca\textsuperscript{2+} buffer. Future studies are needed to clarify the mechanisms underlying the reported homeostatic regulation of spinal neural activity and to establish whether this regulation can contribute in maintaining the appropriate level of activity during development.
CONCLUSIONS

Neurons normally generate spontaneous transient elevation of intracellular \( \text{Ca}^{2+} \) at early stages of development. These \( \text{Ca}^{2+} \) signals represent a widely used cellular code to implement developmental processes with different roles depending on the mechanisms by which they are generated (Gu and Spitzer, 1997). For instance, neurite growth or neuronal differentiation depend on different patterns of \( \text{Ca}^{2+} \) transients (Gu and Spitzer, 1995; Gu and Spitzer, 1997). Thus the origins and patterns of \( \text{Ca}^{2+} \) transients, as well as their functional consequences, are of interest.

In this thesis, I investigated the effects of pharmacological manipulation of intracellular \( \text{Ca}^{2+} \) homeostasis on the most peculiar type of \( \text{Ca}^{2+} \) signals of premotoneuronal networks in vitro, \( \text{Ca}^{2+} \) oscillations.

By means of \( \text{Ca}^{2+} \) imaging technique, I reported that the intracellular \( \text{Ca}^{2+} \) buffering capacity is important in setting pattern of \( \text{Ca}^{2+} \) oscillations in organotypic spinal cultures, with a mechanism likely involving endogenous \( \text{Ca}^{2+} \)-binding proteins. The complexity in the temporal pattern of expression of buffering proteins during development, previously characterized in this culture (Sibilla et al., 2009), suggests that these proteins have different roles (i.e. modulation of \( \text{Ca}^{2+} \) activity) beyond their “simple” protective function against excessive rises of [\( \text{Ca}^{2+} \)]. Taken together, my and previous findings suggest that a function of these proteins could be indeed the setting of \( \text{Ca}^{2+} \) oscillations patterns.

Another significant finding emerged from this study is that the commonly used intracellular \( \text{Ca}^{2+} \) chelator, BAPTA-AM, by itself influences the \( \text{K}^{+} \) currents. This result could not be mimicked by EGTA-AM, thus ruling out the possibility that the observed effect is caused both by chelation of intracellular \( \text{Ca}^{2+} \) and by AM de-esterification byproducts.

Notably, I reported for the first time that prolonged treatment with both BAPTA-AM and EGTA-AM produced an homeostatic compensation in neurons. This evidence further validates this in vitro model as a valuable instrument for studying the effects of chronic treatments on neuronal activity. However, future investigations need to be done to establish the mechanisms underlying the homeostatic regulation and its functional implication in developing spinal cord.
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