Universidade de Lisboa

Faculdade de Medicina da Universidade de Lisboa

Excitatory and inhibitory effects of caffeine in the neocortex –

a transcranial magnetic stimulation study

Erica Rodrigues Marcelino

Master Degree on Neuroscience – VIII Edition

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Dissertação orientada pelo Professor Doutor Alexandre de Mendonça e pelo Professor Doutor Mamede de Carvalho

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CONTENTS

1. INTRODUCTION

1.1. Historical Aspects of Caffeine

The alerting proprieties of caffeine and its possible beneficial effects on motor and cognitive performance have long been touted. Legend has it, in fact, that an observant goatherd named Kaldi discovered coffee in Ethiopia somewhere between about 300 and 800 A.D.. He noticed that his goats did not sleep at night after eating coffee berries, so he took the berries to a local abbot, who brewed the first batch of coffee, noting its effects on arousal and cognition and motor performance (Smith *et al*., 2002). Coffee spread from Ethiopia into the Arabic world, was brought to Venice in the early 17th century and became known in Europe during that century, at first as a medicine, and then as a social drink in the Arab tradition (Fredholm *et al*., 1999).
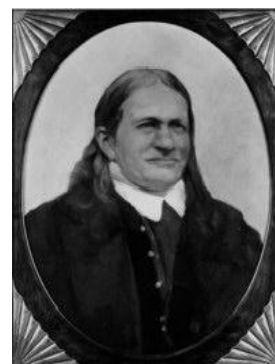
Figure1. Friedrich Runge

In 1819 the german chemist Friedrich Ferdinand Runge (figure 1 (http://www.shiga-med.ac.jp/~koyama/pain/Runge.jpg)) isolated caffeine at the behest of Johann Wolfgang von Goethe. As in the work "Faust" by Goethe the soul of Faust has been sold to the devil in exchange for "jeunesse", it appears that Goethe was anticipating in almost 200 years, the use of caffeine to treat diseases that predominate during ageing such as the case of the neurodegenerative diseases.

The structure of caffeine was elucidated near the end of 19th century by Herman Fischer (figures 2 (http://www.nobel-winners.com/Chemistry/emil_hermann_fischer.jpg) and 3 (http://www.regator.

com/blog/wp-content/uploads/2008/08/caffeine.jpg)(http://library.thinkquest.org/C0115926/drugs/Caffeine_2D_Structure_3541.gif)) (Ribeiro & Sebastião, 2009).

Figure 2. . Herman Fischer   Figure 3. Caffeine structure
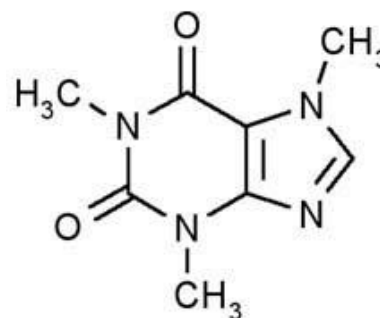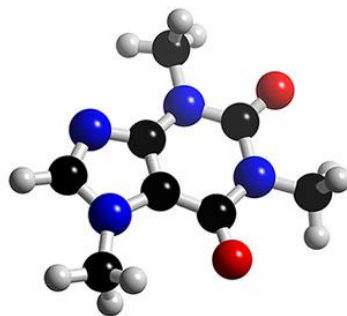
Coffee has ever since been valued as stimulating the mind and sharpening the senses. Caffeine, the psycho-active substance responsible for these effects, may be the most consumed psycho-active drug in the world (Fredholm *et al*., 1999). Caffeine is mainly present in coffee, which also contains trace amounts of theophylline, but no theobromine (Ribeiro & Sebastião, 2009).

Almost all caffeine originates from dietary sources, such as soft and energy drinks, hot chocolate, certain food products like cakes and candies, and over-the-counter medications, including analgesics and cold remedies, with coffee and tea being the most popular (Brice and Smith, 2002). The content of caffeine in these food items ranges from 71 to 220 mg/150 ml for coffee to 32 to 42 mg/150 ml for tea, 32 to 70 mg/330 ml for cola, and 4 mg/150 ml for cocoa (Debry, 1994). Consumption of coffee varies largely among different countries. The highest consumption (more than 10 kg/person/year) occurs in Scandinavia, Austria, and the Netherlands. In most western European countries, coffee consumption ranges from 6 to 9 kg/person/year. The lowest consumption (less than 5 kg/person/year) is found in the U.S. and Italy (D'Amicis and Viani,

1993; Debry, 1994). Furthermore, the content of caffeine per cup of coffee varies with the size of the serving, the mode of preparation of coffee (boiled, filtered, percolated, espresso or instant), and the type of coffee, Arabica or Robusta (D'Amicis and Viani, 1993; Debry, 1994). The size of the cup ranges from 50 to 190 ml and the content of caffeine in a 150-ml cup of coffee is a low as 19 mg/cup in instant coffee and as high as 177 mg/cup in boiled coffee. In Portugal, the volume of the cup ranges from 16 to 53 ml and the content of caffeine varies between 1,2 to 4,7 mg/ml depending the size of the cup (Casal *et al.*, 2009). Caffeine consumption from all sources can be estimated as 76 mg/person/d but reaches 210 to 238mg/d in the U.S. and Canada, and more than 400 mg/person/d in Sweden and Finland, where 80 to 100% of the caffeine intake comes only from coffee (Viani, 1993, 1996; Debry, 1994; Barone and Roberts, 1996).

1.2. Metabolism

Caffeine is metabolized in the liver by the cytochrome P450 oxidase enzyme system into three dimethylxanthines (figure 4 (http://www.psycheteria.org/iphone/CaffeineMetabolites.png)): paraxanthine (1,7-dimethylxanthine), which increases lipolysis, leading to elevated glycerol and free fatty acid levels in the blood plasma, theobromine, which dilates blood vessels and increases urine volume, and theophylline (1,3-dimethylxanthine), which relaxes smooth muscles of the bronchi (figure5 (http://journals.prous.com/journals/dot/20044001/html/dt400055/images/Hansel _f4.jpg)). The therapeutic dose of theophylline, however, is many times greater than the amount resulting from caffeine metabolism taking in non toxic amounts. Each of those xanthines is further metabolized and then excreted into the urine (Ribeiro and Sebastião, 2009).
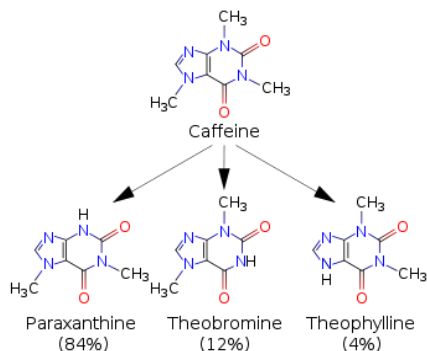
Figure 4. Caffeine metabolites
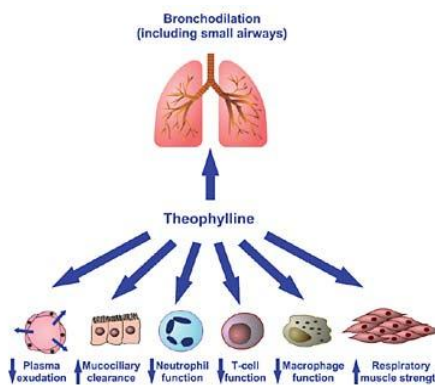
Figure 5. Theophylline actions

It has been shown that, after long-term caffeine ingestion, the levels of theophylline in the mouse brain may be higher than those of caffeine during a substantial part of the day and almost always higher then the level of paraxanthine (Johansson *et al*., 1996). This could mean that caffeine in the brain is metabolized partly via specific, local enzymatic pathways and that caffeine administration leads to high CNS concentrations of theophylline, whereas peripheral theophylline levels are kept low (Fredholm *et al*., 1999). The contention that most of the effects of caffeine in the CNS are direct or indirect consequences of adenosine receptors blockade increases in strength if local CNS concentrations of theophylline and/or paraxanthine are high after caffeine ingestion, since theophylline is some three to five times more potent than caffeine as an inhibitor of both adenosine A_1 and A_{2A} receptors, and paraxanthine is also at least as potent as caffeine (Benowitz *et al*., 1995).

Caffeine appears to be metabolized similarly in young and old individuals (Blanchard and Sawers, 1983). However, due to the lower lean body mass in older people, the bioavailability of caffeine in this group may be higher and may lead to higher blood and tissue concentrations.

The hydrophobic properties of caffeine allow its passage trough all biological membranes. Caffeine crosses the blood-brain barrier either in the adult or the young animal (Lachance *et al.*, 1983; Tanaka *et al.*, 1984), and the blood-to-plasma ratio is close to unity (McCall *et al.*, 1982), indicating limited plasma protein binding and free passage into blood cells. In newborn infants, caffeine concentration is similar in plasma and cerebrospinal fluid (Turmen *et al.*, 1979; Somani *et al.*, 1980). There is no placental barrier to caffeine (Ikeda *et al.*, 1982; Kimmel *et al.*, 1984) and unusually high levels of caffeine have been reported in premature infants born to women who are heavy caffeine consumers (Khanna and Somani, 1984).

There is ample evidence that smokers metabolize caffeine by approximately 50% more rapidly than nonsmokers (Benowitz *et al.*, 1989). Ex-smokers consume somewhat less caffeine than smokers (although more than nonsmokers), but they also metabolize the drug more slowly (Swanson *et al.*, 1994), thus, the level of caffeine may be at least as high. The effects of caffeine are probably due to a mixture of caffeine, theophylline, and paraxanthine, and the changes in the total amount of active drug are not known. Therefore, the speculation (Swanson *et al.*, 1994) that lowered metabolism of caffeine in ex-smokers may lead to increased toxicity remains unsubstantiated (Fredholm *et al.*, 1999).

Moreover, a relationship between adenosine A_{2A} receptors antagonist and genetic variability in caffeine metabolism associated with habitual caffeine consumption has been proposed (Cornelis *et al.*, 2007), which provides a biological basis for caffeine consumption. In this study, persons with *ADORA2A TT* genotype were significantly more likely to consume less caffeine than carriers of *C* allele.

1.3. Therapeutic Use

Caffeine and theophylline have been or are used as adjuncts or agents in medicinal formulations. Methylxanthines have been used to treat bronchial asthma (Serafin, 1996), apnea of infants (Bairam *et al*., 1987; Serafin, 1996), as cardiac stimulants (Ahmad and Watson, 1990), as diuretics (Eddy and Downes, 1928), as adjuncts with analgesics (Sawynok and Yaksh, 1993; Zhang, 2001), in electroconvulsive therapy (Coffey *et al*., 1990), and in combination with ergotamine for treatment of migraine (Diener *et al*., 2002). A herbal dietary supplement containing ephedrine and caffeine is used as an anorectic (Haller *et al*., 2002). Other potential therapeutic targets for caffeine include diabetes (Islam *et al*., 1998; Islam *et al*., 2002), parkinsonism (Schwarzschild *et al*., 2002), and even cancer (Lu *et al*., 2002). Caffeine has also been used as a diagnostic tool for malignant hyperthermia (Larach, 1989).

1.4. Constituents of Coffee Other than Caffeine

One of the major constituents of coffee, caffeic acid (figure 6 (http://www.agrool.gr/files/caffeic%20acid.jpg)) is an effective antioxidant agent (Reneva *et al*., 2001). It has been suggested that the antioxidant activity of caffeoyl derivates emanates from the ability of caffeic acid (figure 7 (http://www.chemdrug.com/databases/dataimg/1/20.png)) to form an iron complex, which prevents the production of hydroxyl radicals (Kono *et al*., 1998; Sestili *et al*., 2002). It was demonstrated that the roasting process has no diminishing effect on the antioxidant activity of remaining caffeoyl derivates (Charurin *et al*., 2002). Because coffee contains a higher percentage of caffeic acid derivatives, such as chlorogenic acid and 1,3-dicaffeoyl quinic acid, than other beverages (Clifford, 2000; Mattila and Kumpilainen, 2002) the antioxidant effects and hepatoprotective activities must have been taken into account (Rechner *et al*., 2001).
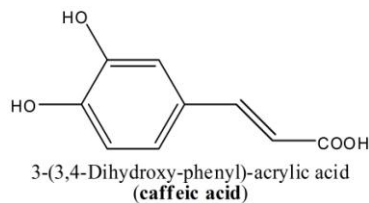
Figure 6 – caffeic acid   Figure 7 - dihydrocaffeic acid
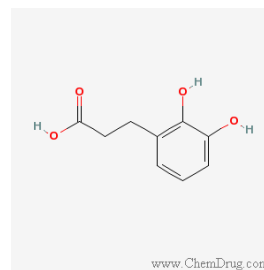
1.5. Mechanisms of Action

When we talk about caffeine actions on the peripheral and central nervous system, we must consider the major mechanism, blockade of adenosine receptors, in particular A_1 and A_{2A} adenosine receptors (see below). Other mechanisms should also be considered (figure 8 (http://www.biology-online.org/user_files/Image/caffeineF01.gif)), namely blockade of phosphodiesterases (PDEs) (e.g., PDE1, PDE4, PDE5), regulating levels of cyclic nucleotides. These effects (up to 40% inhibition of phosphodiesterases) according to Daly (Daly, 2007) may be observed in concentrations below those that cause toxic effects. In relation to PDE5 inhibition, it is interesting to note that caffeine acts like sildenafil, a PDE5 inhibitor, thus, the potential effects related to these actions need to be investigated, to see whether consequent vasodilatation might contribute to the net effect of caffeine.

Another mechanism involves actions on ion channels. Caffeine enhances the activity of a cyclic ADP- ribose-sensitive calcium release channel, the so called ryanodine-sensitive channel, thereby causing release of intracellular calcium from storage sites in the sarcoplasmic reticulum of muscle and the endoplasmic reticulum of other cells, including neuronal cells (MacPherson *et al*., 1991; Galione, 1994), Caffeine also acts on the receptors for the inhibitory neurotransmitters γ-

aminobutyric acid (GABA) and glycine (Fredholm, 1980; Daly, 1993; Nehlig and Debry, 1994; Fredholm *et al*., 1997, 1999; Daly and Fredholm, 1998).
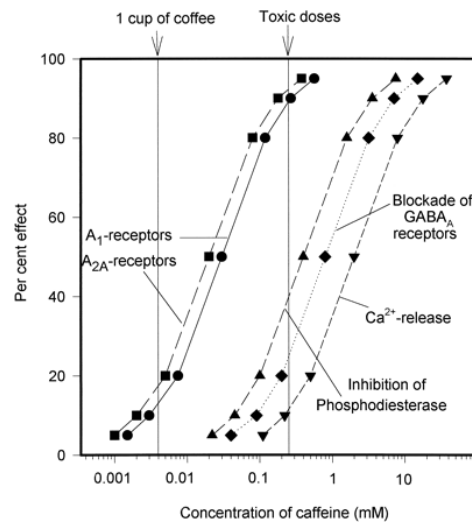
Figure 8 – Mechanisms of action

The effects of caffeine are biphasic. The stimulatory behavioral effects in humans (and rodents) become manifest with plasma levels of 5 to 20 μM, whereas higher doses are depressant. The only sites of action where caffeine would be expected to have major pharmacological effect at levels of 5 to 20 μM are the A_1- and the A_{2A}- adenosine receptors, where caffeine is a competitive antagonist (Daly and Fredholm, 1998). Major effects at other sites of action, such as phosphodiesterases (inhibition), GABA and glycine receptors (blockade), and intracellular calcium-release channels (sensitization to activation by calcium) would be expected to require at least tenfold higher levels of caffeine.

1.6. Peripheral Effects

Beyond activating the central nervous system (CNS), leading to behavioral, autonomic, and endocrine responses (Rall, 1980; Nehlig *et al*., 1992), caffeine also exerts effects peripherally, mediated by direct tissue effects along with hormonal and autonomic outputs. Caffeine increases circulating catecholamines and free fatty acids (Robertson *et al*., 1978; Pincomb *et al*., 1988). It also increases blood pressure, both at rest and during behavioral stress (Lane and Williams, 1985; Sung *et al*., 1990; James, 1993). Neuroprotective effects of coffee may be related to its estrogenic effects. In studies of dopaminergic neurons, estrogen appears to reduce the production of free radicals and protects against oxidative stress (Pita *et al*., 2002).

1.7. Toxicity and Abuse

Tolerance develops very quickly, after heavy doses e.g. tolerance to sleep disruption (400 mg of caffeine 3 times a day for 7 days), tolerance to subjective effects of caffeine (300 mg 3 times per day for 18 days). Withdrawal symptoms may appear (figure 9 (http://upload.wikimedia.org/ wikipedia/commons/thumb/c/c7/Main_side_effects_of_Caffeine.svg/526px-Main side_effects of_ Caffeine.svg.png)), including inability to concentrate, headache, irritability, drowsiness, insomnia and pain in the stomach, upper body, and joints (within 12 to 24 hours after discontinuation of caffeine intake, peak being at roughly 48 hours, and usually last from one to five days - the time required for the number of adenosine receptors in the brain to revert to "normal" levels) (Ribeiro and Sebastião, 2009).

In humans, caffeine may cause a wide range of unpleasant physical and mental conditions including nervousness, irritability, tremulousness, muscle twitching (hyperreflexia), insomnia, respiratory alkalosis, and heart palpitations. Caffeine increases production of stomach acid, and

high usage over time can lead to peptic ulcers, erosive esophagitis, gastroesophageal reflux disease (Ribeiro and Sebastião, 2009), feeling of weariness, weakness, and drowsiness; impaired concentration; fatigue and work difficulty; depression; anxiety; increased muscle tension; and occasionally tremor, nausea or vomiting (Griffiths *et al*., 1990; Silverman *et al*., 1992; Hughes *et al*., 1993; Nehling and Debry, 1994; Strain *et al*., 1994, 1995; Dews *et al*., 2002).
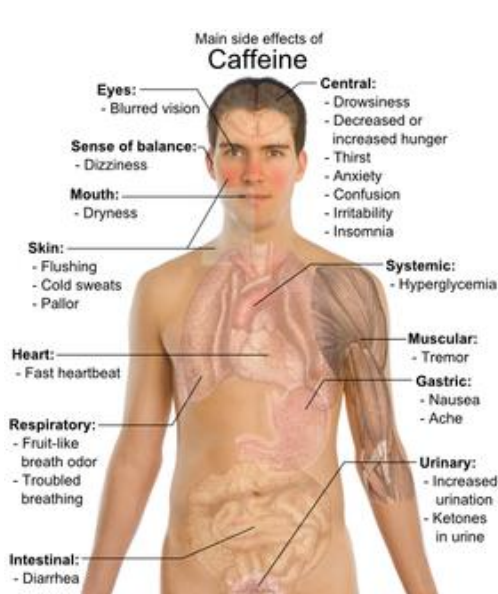
Figure 9 - withdrawal symptoms   Figure 10 - overuse

Overuse and dependency (figure 10 (http://upload.wikimedia.org/wikipedia/commons/thumb/6/ 66/Main_symptoms_of_Caffeine_overdose.svg/548px-Main_symptoms_of_Caffeine_overdose.s vg.png)) occurs after consumption of caffeine in large amounts, and in particular over extended periods of time, inducing caffeinism (toxic effects of caffeine often referred to at nonlethal levels). Convulsions and death can occur at levels above 300 µM. However, it cannot be excluded that subtle effects of 5 to 20 mM caffeine at sites of action other than adenosine receptor might have some relevance to both acute and chronic effects of caffeine. Extensive *in vitro* studies of the

actions of caffeine at such sites are usually performed at concentrations of caffeine of 1 μM or more, clearly levels that *in vivo* are lethal (Daly and Fredholm, 1998).

It should be mentioned that alteration of astrocytogenesis via A_{2A} adenosine receptors blockade during brain development raises the possibility that postnatal caffeine treatment could have long-term negative consequences on brain function, and should perhaps be avoided in breast-feeding mothers (Desfrere *et al*., 2007).

1.8. Adenosine

When we talk about the role that caffeine plays in the brain, we must refer their interaction with adenosine (figure 11 (http://wpcontent.answers.com/wikipedia/commons/thumb/5/53/Caffeine and adenosine.svg/350px-Caffeine_and_adenosine.svg.png)). As a result, the next lines are dedicated to describe briefly adenosine and its relation with caffeine on the nervous system.

Figure 11 – Caffeine and adenosine molecules

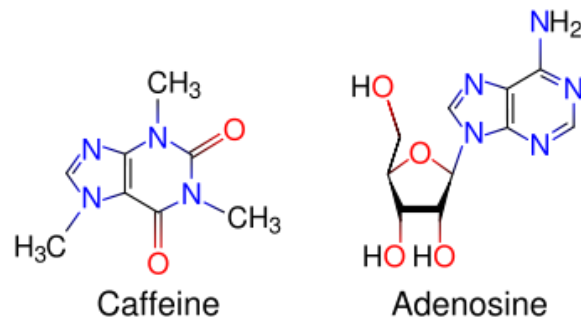Adenosine exists in all cells, and is released apparently from all cells, including neurons and glia. Adenosine is consensually recognized as a very important substance in the homeostasis of the cells in the nervous system, elegantly named by Newby (1981) a "retaliatory metabolite", or

according to others "a signal of life" (Engler, 1991). However, adenosine is involved in cell death namely in prevention or induction of apoptosis (e.g. Di Iorio *et al*., 2002).

Adenosine is able to regulate synaptic functioning through *tuning* and *fine-tuning*. *Tuning* synapses occurs when adenosine by activating its receptors is controlling e.g. the release of neurotransmitters by interfering with Ca^{2+} or other mechanisms directly related to neurotransmitter release (Ribeiro, 1994). In the case of *fine-tuning* adenosine is interfering with receptors for other neuromodulators (Sebastião and Ribeiro, 2000).

Adenosine is produced ubiquitously, and its neuroactive properties are determined by the presence of specific receptors in discrete regions of the brain that are involved in higher-order processes (e.g., the hippocampus, a brain structure that is critical for memory formation). At present, four heptahelical, G-protein-coupled receptors for adenosine have been identified and named A_1, A_{2A} and A_3 receptors (high affinity) and A_{2B} receptors (low affinity) (Fredholm *et al*., 1994). Of these subtypes, the rat A_3 receptor was originally shown to be but little affected by many methylxanthines, including caffeine. In humans, the A_3 receptor is blocked by caffeine with a K_D of close to 80 µM. Therefore, this receptor is not the best target for caffeine action in humans. The A_{2B} receptor has been shown to require higher concentrations of adenosine for activation than those found in resting animal tissues. Thus, inhibition of adenosine actions at this receptor is similarly unlikely to provide an explanation the actions of caffeine under physiological conditions. Under pathophysiological conditions, however, A_{2B} receptors are likely to be activated by endogenous adenosine and caffeine may then very well act also on these receptors.

Although A_3 and A_{2B} receptors are unlikely to be important, A_1 and A_{2A} receptors are activated at the low basal adenosine concentrations measured in resting rat brain. Thus, these receptors are

likely to be the major targets for caffeine and theophylline (Fredholm *et al*., 1999). The adenosine A_1 receptor is highly expressed in brain cortex, cerebellum, hippocampus and dorsal horn of spinal cord (Ribeiro *et al*., 2003). The A_{2A} adenosine receptor is expressed in the striato-pallidal GABAergic neurons, olfactory bulb and hippocampus (Cunha *et al*., 1994a, Sebastião and Ribeiro, 2009). A_{2B} receptors are expressed in low levels in the brain (Dixon *et al*., 1996) and the adenosine A_3 receptor has apparently intermediate levels of expression in the human cerebellum, hippocampus and low levels in the most of the brain (figure 12) (see Fredholm *et al*., 2001).
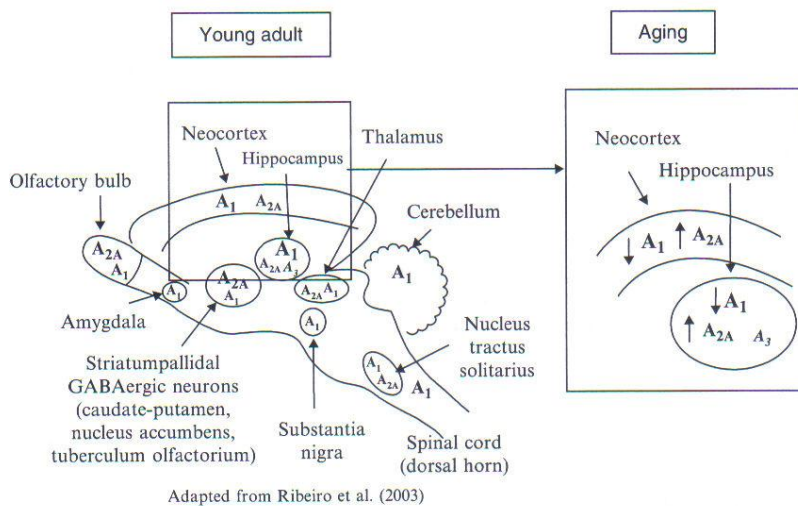
Figure 12 - Adenosine receptors distribution in the CNS (Sebastião and Ribeiro, 2009)

Since adenosine is ubiquitously present in all cells, with receptors distributed in all brain cells; any imbalance of such a widespread system is expected to lead to neurological dysfunctions/diseases. Due to its ability to antagonize adenosine receptors, to cross readily the blood brain barrier and to its safety, caffeine has therapeutic potential in central nervous system dysfunctions (Ribeiro and Sebastião, 2009).

1.9. Effect of Caffeine on the Nervous System

As a pharmacological tool, caffeine is not very useful since its affinity for adenosine receptors is low and its selectivity towards the different adenosine receptors is also poor. Caffeine is an antagonist of all subtypes of adenosine receptors and chronic or acute intake of caffeine may affect adenosine receptors in different and even opposite ways. The therapeutic or adverse effects of caffeine are considerably different depending on whether it is administered chronic or acutely. Chronic caffeine intake, with increases plasma concentrations of adenosine (Conlay et al., 1997), may be neuroprotective. This is in contrast with the consequences of acutely antagonizing A_1 adenosine receptors (de Mendonça et al., 2000).

The acute effects of caffeine are related to the adenosine A_1 and A_{2A} antagonism of caffeine in the brain, which in turn stimulates the release and turnover of several central neurotransmitter substances, including acetylcholine and noradrenaline (Nehlig et al., 1992; Fredholm et al., 1999). At this line, caffeine blocking of A_1 adenosine receptors may increase the levels of catecholamines and serotonin (Fredholm, 1995). When acting as an adenosine receptor antagonist, caffeine is doing the opposite of adenosine receptors activation whenever the levels of endogenous adenosine are tonically activating receptors. So, caffeine, like does adenosine, is exerting effects on all brain areas (Ribeiro and Sebastião, 2009), such as, facilitating neuronal activity by increasing excitatory neurotransmitter release and lowering the threshold for neuronal activation (Phillis et al., 1979). Caffeine causes an increase in spontaneous electrical activity in noradrenaline-containing neurons (Grant et al., 1982), as well as an increase of serotonin concentrations in the serotonergic neurons of the raphe nuclei (Berkowicz et al., 1971).

Chronic adenosine receptor antagonism with caffeine may also influence cognition and motor activity in a way that resembles the acute effects of adenosine receptor agonists (Jacobson et al.,

1996; Sebastião and Ribeiro, 2009). Such opposite actions of chronic versus acute treatment not only have important implications for the development of xanthine-based compounds as therapeutic agents but also constitute a frequent confounding parameter in research. Upregulation of A_1 adenosine receptors after chronic adenosine receptor antagonism with xanthines does occur, but A_{2A} adenosine receptors levels apparently do not change; in addition, there are changes in the levels of receptors for neurotransmitters with chronic administration of xanthines, namely a marked decrease in β-adrenergic receptors and an increase in 5-HT and $GABA_A$ receptors (Jacobson *et al*., 1996, Sebastião and Ribeiro, 2009). The increased expression of A_1 adenosine receptors in response to chronic antagonism of adenosine receptors by caffeine, as compared with A_{2A} adenosine receptors, may lead to a shift in the A_1/ A_{2A} adenosine receptors balance after prolonged caffeine intake (Ferré, 2008, Sebastião and Ribeiro, 2009). Moreover, chronic caffeine treatment leads to modifications in the function of the A_1R- $A_{2A}R$ heteromer and this may, in part, be the scientific basis for the strong tolerance to the psychomotor effects of chronic caffeine (Ciruela *et al*., 2006; Sebastião and Ribeiro, 2009).

1.10. Overview of the Motor System Physiology

In order to understand the effects of caffeine on the motor system, a brief review of this system is presented.

The frontal lobe of each hemisphere is responsible for planning and initiating sequences of behavior. The frontal lobe is divided into a number of different regions, including prefrontal cortex, premotor cortex and primary motor cortex (figure 13). The function of the prefrontal cortex is to plan complex behaviors, but does not specify the precise movement that should be

made. It simply specifies the goal toward which movements should be directed. The prefrontal cortex sends instructions to the premotor cortex, which produces complex sequences of movement appropriate to the task. If the premotor cortex is damaged, such sequences cannot be coordinated and the goal cannot be accomplished.

Although the premotor cortex organizes movements, it does not specify the details of how each movement is to be carried out. Specifying the details is the responsibility of the primary motor cortex, which is responsible for executing skilled movements (Kolb and Whishaw, 2001).
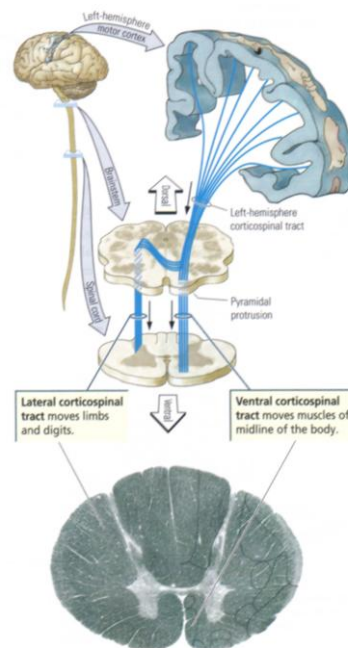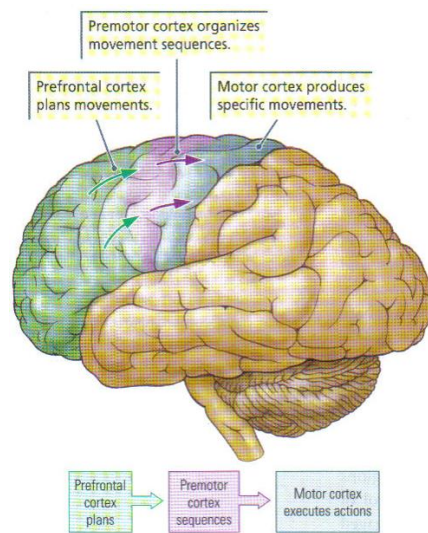
Figure 13 – The frontal lobe (Kolb et al., 2001)   Figure 14 – The corticospinal tracts (Kolb et al., 2001)

The posterior motor areas in the frontal cortex receive their main cortical input from the parietal lobe, and the anterior motor areas receive their main cortical connections from the prefrontal lobe. Many transcranial magnetic stimulation studies have focused on the intrinsic organization, output pathways, and function of a frontal motor region bordering the central sulcus. This region, called

the *primary motor cortex*, is essentially equivalent to area F1 (nomenclature according to Matelli et al., 1985) in monkey cortex or to cytoarchitectonically defined Brodmann area 4 in humans. It contains the largest output neurons of the cortex, the Betz cells, and it is probably the single most important source of crossed direct corticospinal projections controlling hand movements. It is noteworthy that the other frontal motor areas – the dorsal premotor cortex (superior part of Brodmann area 6, monkey areas F2 and F7 (Matelli et al., 1985), the ventral premotor cortex (inferior part of Brodmann area 6 and Brodmann area 44, monkey areas F3 and F6 (Matelli et al., 1985)) – also are important sources of corticospinal projections. A part of the dorsal premotor (F7) and a part of the mesial cortical area (F6) do not contain corticospinal neurons, but send their efferent projections exclusively to the brainstem.

The large pyramidal cells in the motor cortex send their axons to the contralateral side of the spinal cord and are able to activate their target motoneurons directly, but a number of interneurons in the brainstem or spinal cord are also influenced. These long projection neurons are called corticospinal neurons.

The corticospinal tract (figure 14) originates in primary and secondary motor cortices and are located on the precentral gyrus (figure 15) (Jones and Wise, 1977).
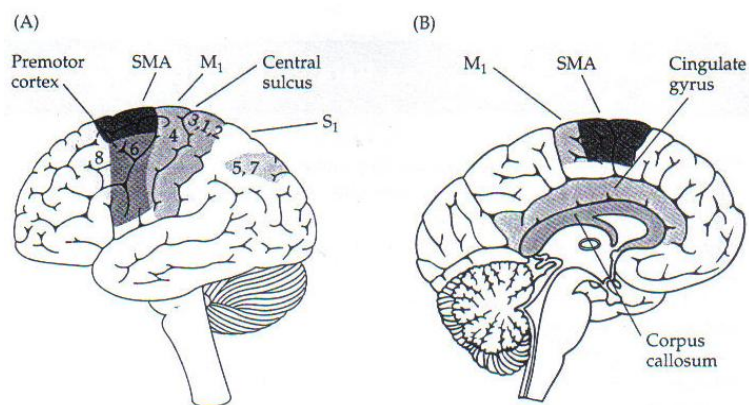
Figure 15 – The association motor cortices (Nicholls et al., 2001)

Cells from which the pathway originates are arranged in an orderly manner to form a somatotopic pattern in primary motor cortex – M_1 (figure 16) (Porter and Sakamoto, 1998; Kaneko *et al.*, 1994).
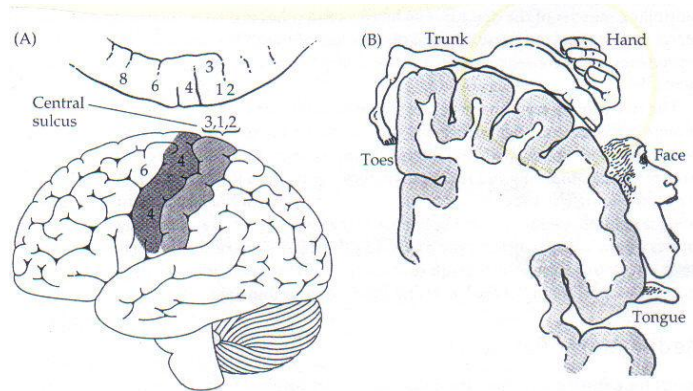
Figure 16 - Motor representation of the cerebral cortex (Nicholls et al., 2001)

Corticospinal neurons of the arm region in M_1 thus project to arm motoneurons in the spinal cord, as well as interneurons involved in the control of arm, hand and fingers. In addition to M_1, other areas in the frontal lobe, such as supplementary motor area and prefrontal areas are involved in other aspects of motor coordination (Grillner, 2001).

Sherrington (1910) called the spinal motoneurons the "final common path" because all the neural influences that have to do with movement or posture converge upon it. The major motoneurons of the spinal cord are called α motoneurons. When a motoneuron discharges, all the muscle fibers to which it is connected contract. The motor unit constitutes the elementary component of normal movement.

When α motoneurons are activated to produce shortening of extrafusal muscle fibers, other motoneurons (γ motoneurons) are also activated to cause contraction of the intrafusal fibres as well. These muscle receptors have fast conducting afferent axons that provide rapid feedback to

the spinal cord and take part in the autoregulation of the motor output to a given muscle. Thus, fast muscle spindle afferents provide direct monosynaptic excitation to the α-motoneurons that control the muscle in which the muscle spindle is located (Greer and Stein, 1990). In this way, the fusimotor system maintains the tension on the muscle spindle receptors over the full range of limb position, so that their sensitivity is undiminished. The fusimotor innervation of muscle spindles can be thought of as a "gain control" system, continually adjusting sensitivity to maintain dynamic range (Sears, 1964; Critchlow and von Euler, 1963; Greer and Stein, 1990).

Motor units are classified as either slow (S) or fast (F) according to their twitch contraction time for a motor unit to develop peak force following a single spike. The speed of the twitch and the rate of relaxation depend on the muscle fiber types of the motor unit and their geometry within the muscle (Floeter, 2001).

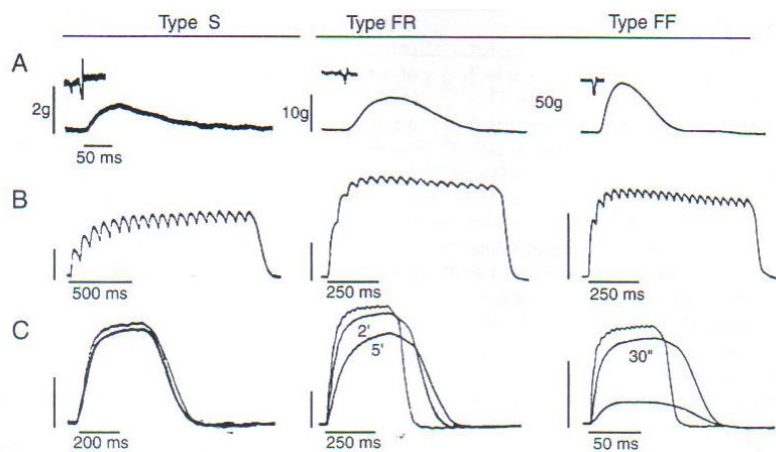Figure 17 – Contractile properties and fatigability of different motor unit types (S, FR and FF) (Burke et al., 1973)

Many of the motor areas contain complete representations of body movements. By virtue of their distinctive connections, each motor area may be involved in different aspects of sensorimotor transformations, in motor planning, and in motor execution (Rizzolatti et al., 1998). Studies have

revealed that the motor system creates internal representations of actions in addition to these classically accepted functions. The identification of parietofrontal circuits suggests that the motor system contains many functional units that act in parallel. A view of the motor system as being exclusively hierarchically and serially organized is no longer tenable based on a multitude of anatomical and physiological data (Classen, 2005).

1.11. Transcranial Magnetic Stimulation (TMS) as a Tool to Investigate the Motor System

TMS has been used to improve our understanding of the human motor system. TMS studies have provided information about cortical connectivity and sensorimotor transformations of the primary motor cortex in surprising detail, as also have successfully been extended to study motor areas outside the primary motor cortex and to address more complex cortical functions.

TMS is based on electromagnetic induction (figure 18), which is described by Faraday's law. A time-varying current in a primary circuit (TMS coil) induces an electrical field and consequently a current flow (eddy current) in a secondary circuit (brain). The changing current in the coil generates a magnetic field $B(t)$, but the field itself has no effect on neuronal activation; the magnetic field merely mediates the interaction. The electrical field induced is proportional to the rate of change of B; no neural excitation occurs with slowly changing or stationary B. Because the tissue is transparent to the magnetic field in TMS, the scalp and the skull do not resist the field.
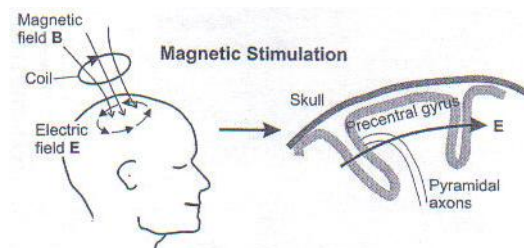
Figure 18 – Principles of transcranial magnetic stimulation (Ruohonen et al., 2005)

Transcranial stimulation is thus based on applying an electrical field in the brain tissue. The electrical field drives ionic currents in the tissue, charging the capacitances of neuronal membranes and thereby triggering the firing neurons.

The electromagnetic theory for TMS is well established and can be divided into three separated parts: the electrical operation of the stimulator circuit, the computation of the macroscopic electromagnetic fields being imposed on the brain due to current in the stimulator coil, and the detailed flow of current and buildup of charge on cellular membranes as result of imposing a macroscopic electric field in the brain.

Different orientations and intensities of TMS can recruit different proportions of D and I waves.

The D waves are caused by direct activation of pyramidal axons, whereas the I waves are thought to originate from the deep fiber system that is tangential to the cortex and that synaptically excites the pyramidal cells. Because D waves are the result of stimulation at the axon of pyramidal tract neurons, they are not greatly influenced by changes in the level of excitability within the gray matter of the cerebral cortex (Di Lazzaro *et al*., 1999). However, the situation is quite different for I waves, which are highly sensitive to the level of cortical excitability at the time the stimulus is given because they are synaptically induced. If we wish to use TMS to probe the excitability of motor cortex, we should try to employ it so that it induces the greatest proportion of I-waves volleys possible.

The fact that TMS evokes a complex series of repetitive discharges in the pyramidal tract of neuronal population has several implications for the electromyographic (EMG) responses that are evoked. These differ from the conventional compound muscle action potentials (CMAPs) produced by peripheral nerve stimulation in two important ways. First, the motor evoked

potentials (MEPs) are always smaller, longer than in duration, and more polyphasic than a CMAP. Second, the onset latency, amplitude, and threshold of an MEP change according to whether subjects are relaxed or active (Day *et al*., 1987; Thompson *et al*., 1987) (figure 19).
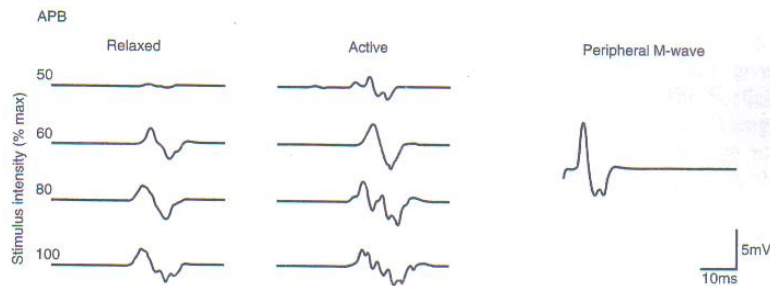
Figure 19 - EMG recordings (Rothwell, 2005)

The descending corticospinal volleys produced by TMS release excitatory postsynaptic potentials (EPSPs) at the motor neuron, and these develop over a period of several milliseconds, depending the number of I waves that has been evoked. Motor neurons are discharged asynchronously, and this leads to the longer duration MEP, especially at high intensities of stimulation. There can be cancellation between phases of motor units discharging at slightly different latencies.

The different latencies of MEPs evoked in active and relaxed muscles result from the time taken for EPSPs to depolarize a motor neuron to its firing threshold (figure 20). At rest, a single EPSP (e.g. that produced by the arrival of a D-wave volley) may fail to rise a motor neuron threshold and discharge will have to wait the arrival of the I1 volley some 1.5 ms later. However, if the same stimulus is given during voluntary contractions, there will always be some motor neurons that are near enough to their firing threshold to be discharged on receipt of the first EPSP that arrives at the motor neuron pool. MEPs evoked in actively contracting muscles always have the shortest possible latency, whereas those evoked in subjects at rest usually have a longer latency.
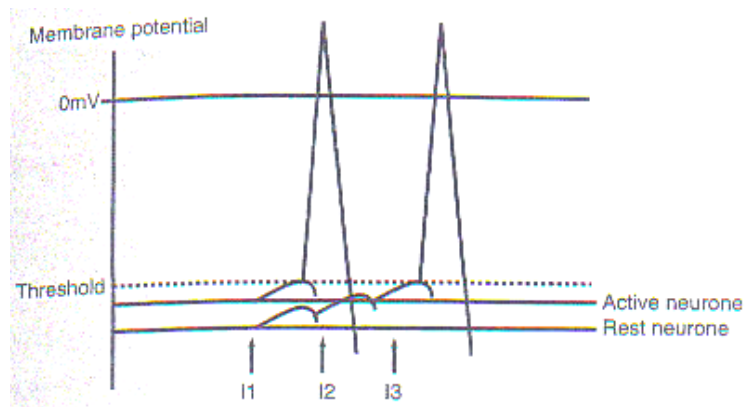
Figure 20 – Latency differences between responses evoked in relaxed and active muscle (Rothwell, 2005)

The difference in amplitude of MEPs at rest and during activation mainly results from increased excitability of spinal motor neurons during contraction. This means that a given amount of descending excitation will cause more spinal motor neurons to discharge during contraction, leading to a larger MEP. However, there is also some evidence that the response of pyramidal tract neurons to a TMS pulse increased during voluntary contraction (Di Lazzaro *et al.*, 1999). This may occur because the pyramidal tract neurons are more excitable and more readily discharge by synaptic input from I-waves generators during the course of voluntary contraction compared with rest.

The same factors contribute to the difference in threshold for evoking an MEP at rest and during contraction. The rest motor threshold (RMT) is always higher than the active motor threshold (AMT) because resting spinal (and cortical) motor neurons require more excitation to reach discharge threshold than during contraction. Some of the most important electrophysiological measurements obtained with TMS are briefly discussed below.

1.11.1 *Central Silence Period*

The cortical silent period (CSP) refers to an interruption of voluntary muscle contraction by electrical or magnetic stimulation of the motor cortex. Although the first shorter part of CSP can depend on peripheral mechanisms related to lower motor neuron inhibition, the larger last part depends on inhibitory cortical interneurons. It is currently thought is that it depends on GABA-B receptors, as derived from TMS-pharmacological studies (Inghillery et al., 1996).

1.11.2. *Corticomotor Threshold*

The corticomotor threshold (CMT) is the lowest stimulation intensity able to evoke a MEP of minimal size and is usually assessed in a small hand muscle. The CMT depends on the excitability of spinal motoneurons and motor cortex neurons (Mills, 1999). In theory, to define CMT, a minimal MEP should be elicited in 50% of the trials. In practice, the stimulus intensity is increased at 5% steps until reaching a level that induces approximately 100 μV responses in about 50% of 10 consecutive trials. There is a CMT at rest (i.e., resting threshold) and one during contraction (i.e., active threshold) that is lower. Distal muscles have a lower CMT than proximal ones.

The CMT augments with increasing age (Rossini et al., 1992) and is enhanced by sodium or calcium channel blocking anticonvulsants (Ziemann et al. 1996) but not by drugs acting on GABA or glutamate transmission. The CMT is based on mechanisms different from those of the CSP, which it ideally complements when investigating disease mechanisms. The CMT, for example, is raised in advanced cases of ALS (Eisen et al. 1990), multiple sclerosis (MS) (Ravnborg et al., 1991), in spinal injury above the lesion (Macdonell and Donnan 1995), and it is

reduced in early cases of ALS (Mills and Nithi, 1997), idiopathic generalized epilepsy (Reutens and Berkovic 1992), and progressive myoclonic epilepsy (Reutens et al., 1993).

Based on a large cohort of 89 normal subjects (ages range 12 to 49 years) using a circular coil and abductor digiti minimum (ADM) recordings, Reutens and coworkers (Reutens et al., 1993) found a mean CMT of 55.8 ± 12.9 (SD). Because of the large normal range, CMT is more useful for statistically comparing groups of patients and normal subjects rather than for determining abnormalities in individual cases.

1.11.3. *Short-interval Intracortical Inhibition*

Short-interval intracortical inhibition (SICI) was first reported by Kujirai and colleagues (Kujirai *et al*., 1993).

They applied both stimuli through the same coil to the motor cortex hand area and found that a small subthreshold conditioning stimulus could suppress the response to a later suprathreshold test stimulus if the interval between stimuli was less than 5 ms (figure 21). Because the conditioning stimulus was below AMT, the investigators suggested that the interaction was occurring at a cortical level and the conditioning stimulus was suppressing the recruitment of descending volleys by the test stimulus. Direct recordings of descending volleys have confirmed this (Di Lazzaro *et al*., 2004). A small conditioning stimulus that itself evokes no descending activity suppresses late I waves if the interval between the stimuli is between 1 to 5 ms. The I1 wave is virtually unaffected, with the most sensitive wave being the I3 and later volleys (figure 22).
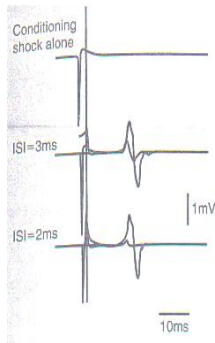
Figure 21 – Paired-pulse intracortical

inhibition (Rothwell, 2005)
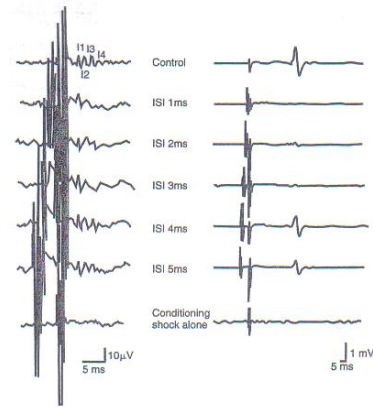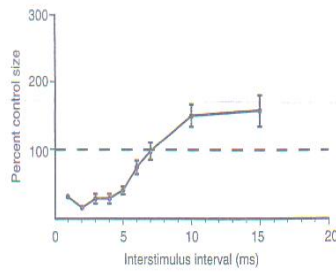
Figure 22 – Epidural Volleys (left) and EMG

responses (right) (Rothwell, 2005)

Because the I1-wave is unaffected, the results suggest that inhibition does not modify directly the excitability of pyramidal neurons. It seems more likely that the inhibition in this paradigm results from reduced excitability of inputs responsible for I waves.

The threshold for producing SICI (i.e., minimum intensity of S1 needed to reduce the response to S2) is remarkably low and averages about 70% of the active motor threshold in any individual (Orth *et al*., 2003). Increasing the intensity of S1 initially leads to increased inhibition, but this peaks at about 90% to 100%. Higher intensities give a smaller effect and eventually lead to facilitation. The later effect is thought to be caused by recruitment of descending corticospinal volleys at intensities above AMT that produce subthreshold excitation of spinal motor neurons. Although the threshold for inhibition is remarkably similar in different individuals (at least when expressed as a percent of that individual's AMT), the increase of inhibition at higher intensities is highly variable. This leads to a wide spread in normal values for SICI when measured with the usual S1 intensities of 80% to 100% AMT.

There is other piece of evidence that very small S1 stimuli that are below SICI threshold (at 2 to 5 ms) can nevertheless activate some cortical circuits. Pairs of S1 stimuli separated by intervals of 4 ms or less can show temporal summation and lead to clear inhibition (Bestmann *et al*., 2004). The time course of this interaction has been suggested to reflect convergence of synaptic inputs onto a common inhibitory interneuron that leads to SICI.

1.11.4. *Intracortical Facilitation*

Kujirai and coworkers (Kujirai *et al*., 1993) originally noticed that if the interval between the pulses was greater than 6 ms or so, the MEPs evoked by the test stimulus (S2) were weakly facilitated for at least 10 ms. This is now known as intracortical facilitation (ICF). The threshold for ICF is higher than that for SICI, and unlike SICI, it is sensitive to the direction of the current induced by S1, being maximal with a PA orientation (Ziemann *et al*., 1996). These features suggest that a different mechanism underlies SICI, but more work needs to be done to clarify its characteristics fully. There are no studies of how descending corticospinal tract volleys are affected by ICF.

1.11.5. *Long-interval Intracortical Inhibition*

Long-interval intracortical inhibition (LICI) was first described by Valls-Sole and associates (Valls-Sole *et al*., 1992). They found that a conditioning stimulus strong enough to evoke a MEP in the target muscle could suppress a response to a later stimulus of the same intensity if the interval was between 50 and 200 ms. Because the excitability of spinal H reflexes had recovered at this interval, the effect was though to be cortical, probably the result of activation of $GABA_B$ receptors (Werhahn *et al*., 1999). The cortical origin has been confirmed in recordings of

corticospinal volleys (Di Lazzaro *et al*., 2004). These recordings show that the S1 suppresses the size and number of I waves recruited by a S2 given 100 or 150 ms later. The I waves are facilitated if the interval between S1 and S2 is 50 ms even though the MEPs are suppressed. Presumably, the effect on the MEP results from the refractoriness of spinal motor neurons that discharge in response to S1 and other potential spinal mechanisms of inhibition.

1.12. Fatigue

Fatigue is generally measured as a failure to maintain a given level of physical performance, the causes of the failure of humans to perform has intrigued investigators for some time. Mosso (1904; cited by Sacco et al., 2005) is generally considered to be the first person to have systematically studied muscle fatigue in humans by recording movements of the middle finger using an ergograph. He found that the fatigue curves obtained when subjects performed repeated contractions could vary considerably. He attributed changes in performance to differences in the "nervous arousal" of subjects, who performed better at times when they had greater "mental energy". His conclusion was that muscle fatigue was primarily of nervous origin. Numerous studies using isolated muscle preparations have shown that considerable fatigue occurs in the absence of nervous input, and it is now generally agreed that under specific conditions all levels of the motor pathways can contribute to this phenomenon (Bigland-Ritchie et al., 1995). For convenience, these multifactorial processes have been designated *central* and *peripheral fatigue mechanisms*, with the former referring to changes proximal to the neuromuscular junction. Central fatigue can be defined as any loss of voluntary muscle activation associated with exercise.

Merton (1954) was the first to evaluate the role of central fatigue. In his classic study, the ability of subjects to achieve maximum force output of the thumb adductors during a sustained maximum

voluntary contraction (MVC) was evaluated using supramaximal tetanic electrical stimulation of the ulnar nerve. He found that no extra force was produced with addition of the stimulus, even when voluntary force had declined by more than 50%. He reached the conclusion that fatigue occurred only in the muscle, opposite to that of Mosso. Gandevia (2001) has provided a detailed view of the role of central mechanisms in muscle fatigue.

Brasil-Neto and colleagues (1993) were the first to describe the use TMS of the motor cortex to study responses to fatigue. They reported a transient decrease in resting motor evoked potential (MEP) amplitude of the flexor carpi radialis muscle after a bout of weighted wrist flexions repeated to exhaustion. They found a serial decrease in MEP size over the course of four stimuli at 5-second intervals. It is important to focus that sustained MVCs are extremely tiring and require a high level of motivation and compliance by the subject. For this reason, the duration of contractions tend to be short (usually 1 to 2 minutes). Many investigators have subsequently used motor nerve stimulation to evaluate voluntary muscle activation, with variations in measurement techniques, contraction types, and protocols and muscle groups used. It is now generally accepted that the inability to achieve full volitional activation of a muscle may contribute to fatigue in a variety of circumstances.

1.13. Effects of Caffeine on the Motor System

Caffeine has effects on the motor system. These effects may be exerted at distinct levels of the nervous system, namely the muscle, the lower motor neuron, the upper motor neuron, or involve modulatory effects from other cortical areas.

1.13.1. *Muscle*

It has been described that caffeine has a positive ergogenic effect. This is supported by 3 different mechanisms. First, alteration in fat metabolism, as caffeine promotes free fatty acid utilization, sparing muscle fiber glycogen reserve; second, a positive direct effect on calcium release from the sarcoplasmic reticulum by ryanodine receptors activation, a phenomenon readily observed in the muscle preparation but with a non-physiological level of caffeine; third, increasing excitatory neurotransmitter activity as a consequence of adenosine receptor antagonism (Graham and Spriet, 1995; Phillips *et al*., 1979).

Caffeine probably has no positive effect on the power of maximal muscle contraction, on the twitch contractile properties during exercise or on the M-wave amplitude obtained by electrical stimulation of a peripheral nerve (Williams *et al*., 1987; Meyers and Cafarelli, 2005; Bugyi, 1980; Wlliams *et al*., 1987; Hespel *et al*., 2002). However, the positive effect of caffeine in endurance and reducing fatigue is well demonstrated in a large number of studies (Meyers and Cafarelli, 2005; Kalmar and Cafarelli 1999; Plaskett and Cafarelli, 2001; Tarnopolsky and Cupido, 2000; Jackman *et al*., 1996; Van Soeren and Graham, 1998). It is not clear if this positive effect is related to a more effective muscle contraction. Detailed investigation of the muscle twitch evoked in contractions and relaxation times suggests an effect of the drug on sarcoplasmic calcium dynamics (Meyers and Cafarelli, 2005).

1.13.2. *Lower Motor Neuron*

As mentioned above, caffeine antagonizes adenosine at physiological doses, thus increasing excitatory neurotransmitter release which decreases neuron activation threshold (Phillips *et al*., 1979). The increased excitability of the serotoninergic neurons of the raphe nuclei may influence

spinal motor neurons as they receive input from descending raphe fibers (Berkowicz and Spector 1971). As a result, we could expect a greater lower motor neuron (LMN) excitability.

The monosynaptic Hoffman reflex (H reflex) is an indirect measure of LMN excitability. Two studies found no change in the ratio H-reflex/M-wave amplitude in a healthy population after caffeine (Kalmar and Cafarelli, 1999; Eke-Okoro 1982). The anticipated reduction of the H-reflex amplitude after exercise was not changed after caffeine ingestion (Motl and Dishman 2004). However, another study exploring the H-reflex stimulus-response curve to test LMN excitability detected significant increased excitability in 7 healthy controls after caffeine administration (6 mg/kg) (Walton *et al*., 2003). A different approach is to explore F-waves amplitude and peripheral silent period to assess LMN excitability, however, no changes in these measurements in a healthy control population after ingestion of 200 mg of caffeine were found (Cerqueira *et al*., 2006).

A different behavior of the LMN could modify recruitment pattern of the motor units during muscle contraction, in particular in fatigue. There is no clear evidence that this occurs after caffeine consumption (Meyers and Cafarelli, 2005; Kalmar and Cafarelli, 2004). Nonetheless, caffeine augments the incidence of self-sustained firing (Walton *et al*., 2002), which is a consequence of increased LMN excitability and related to the presence of plateau potentials. Plateau potentials are facilitated by the tonic activity of descending serotoninergic and noradrenergic neurons (Crone *et al*., 1988). This can be a protective mechanism in conditions causing muscle fatigue, as an increase in plateau potentials spares the necessary enlargement of the excitatory drive to LMN pools (Walton *et al*., 2002).

1.13.3. *Upper Motor Neuron*

Transcranial magnetic stimulation (TMS) is the elective method to investigate non-invasively the cortical motor area function. The accumulated experience shows that TMS does not change motor evoked amplitude (MEP), central conduction time or cortico-motor threshold (Cerqueira *et al.*, 2006; Kalmar and Cafarelli, 2004; Orth *et al.*, 2005).

A recent study in 11 healthy subjects disclosed no change of cortical excitability after caffeine (3 mg/kg), as evaluated by motor threshold (rest and active), short interval intra-cortical inhibition (SICI), intra-cortical facilitation, cortical silent period (130, 150, 175% of active threshold) and size of the MEP (110%, 125% and 150% rest motor threshold) (Orth *et al.*, 2005).

As previously mentioned, the cortical silent period (CSP) refers to an interruption of voluntary muscle contraction by electrical or magnetic stimulation of the motor cortex. In a previous study, our group tested 200 and 400 mg of caffeine in a group of healthy controls and confirmed that stimulus intensity 50% above cortical threshold did no modify CSP. However, applying an intensity of 10% above threshold, a consistent and statistically significant increase in the CSP, between 12-16% in different upper limb muscles, was observed (Cerqueira *et al.*, 2006). It was hypothesized that caffeine, by antagonizing excitatory adenosine A2A receptors, could decrease the release of GABA and thus reduce GABAergic inhibitory transmission in the motor cortex. Alternatively, it could be that the decrease in the CSP after caffeine would not be caused by intrinsic changes in neocortical circuitry, but reflect instead modifications in the properties of extrinsic cortico-basal-thalamo-cortical pathways that control motor cortex activity (Cerqueira *et al.*, 2006).

1.13.4. *Central Fatigue*

As previously mentioned, the study of central fatigue with TMS is an exciting new area. The CSP lengthens and MEP increases during fatiguing contractions (Gandevia, 2001). Immediately after exercise, the MEP tested at rest shows an increase as compared to baseline response, a phenomenon termed postcontraction facilitation. After this period, the MEP response is markedly depressed for as much as half an hour – long-lasting depression. This postfatigue depression recovers rapidly during high-intensity muscle contraction. Some authors observed an increased post-activation potentiation after caffeine (Kalmar and Cafarelli 2004) but a similar fatigue-induced rest MEP depression. A more recent study shows that cortically driven twitch can be increased by caffeine ingestion, although maximal voluntary activation is not altered during fatigue or recovery, suggesting that voluntary activation is not limited by central excitability (Gandevia, 2001). Keeping a mild contraction of the target muscle after exercise, the expected MEP depression is minimized after caffeine, which reflects its role on the excitability of the motor cortex (Gandevia, 2001).

PART II – STUDY PRESENTATION:

2. AIM

Since caffeine intake consistently decreases CSP following TMS, and a fatiguing contraction increases CSP, we raised the hypothesis that the effect of caffeine on the CSP could be attenuated after muscle fatigue. The presence, or the absence, of interference with fatigue would be important to elucidate the mechanisms that underlie the central effects of caffeine on CSP.

In addition, we aimed to test in relaxed muscles the effect of caffeine in the short interval intra-cortical inhibition (SICI) and intra-cortical facilitation (ICF), as described elsewhere, as well as in long-interval intra-cortical inhibition (LICI) in which caffeine influence was not tested so far.

3. MATERIALS AND METHODS

3.1. Sample

Thirteen individuals (3 men and 10 women , mean age 27.5±3.3 years, ranging from 20 to 31) working at the Faculty of Medicine, University of Lisbon and at the Hospital de Santa Maria, were volunteers in this study. A structured questionnaire was applied by the same observer to all subjects, in order to appraise socio-demographic characteristics as well as caffeine consumption and smoking habits. Height and weight of the participants were registered to allow calculation of body mass index (BMI).

The inclusion criterion was age range between 20 and 40 and informed consent.

The exclusion criteria were the presence of cardiac pacemakers, preceding neurosurgical procedures, history of epilepsy, neuromuscular disorder, or drug intake that could affect cortical excitability, and pregnancy (Cerqueira *et al*., 2006).

It should be noted that participants were accepted independently of their caffeine intake habits.

3.2. Study Design

The study was performed at the Faculty of Medicine, University of Lisbon and the protocol approved by the local Ethics Committee.

All investigations started at the same hour (9 am). Subjects were asked to abstain from caffeine-containing drinks and foods for 24 hours before the evaluation. On arrival the participants were subjected to the experimental electrophysiological protocol. Following the first session they took a capsule containing caffeine (200 mg) *per os*. After one hour interval, the participants underwent the same set of electrophysiological tests. The capsules were prepared at the hospital pharmacy.

3.3. Neurophysiology

For magnetic stimulation a Mag2 magnetic stimulator (Medtronic, Skovlunde, Denmark) and a regular sized round coil with a biphasic pulse were used. This device was equipped with a twin-mode facility to permit double-stimulations paradigms. The electromyographic signal was registered and analyzed using a Counterpoint EMG machine (Dantec, Skovlunde, Denmark). Left ulnar nerve-ADM system was studied in each subject in the two sessions. Motor responses were recorded with surface electrodes (Ag-AgCl) using a belly-tendon montage, with a filter setting of 20 Hz – 10 KHz.

3.3.1. *Threshold and motor evoked potential determination*

The center of the coil was positioned flat over the vertex, but moved as necessary to obtain a maximal response at the lowest stimulus intensity. The subjects were asked to keep the hand relaxed during the investigation. Voluntary activation of motor units in the ADM was monitored through the surface electrodes by the audio system of the EMG device. We defined the resting MEP threshold in 5% increments of maximal stimulator output, from an initial stimulus intensity of 5%, as the minimum stimulus intensity that evoked at least 5 responses larger than 100 µV in 10 stimuli (Rossini *et al*.,. 1994). For recording the MEP latency and amplitude the stimulus intensity was set at 2% above threshold. In each subject, five to ten consistent responses were obtained, with an interstimulus interval of at least 30 seconds. MEP amplitude was estimated peak-to-peak from the largest response recorded.

3.3.2. *Paired Pulse Paradigm*

In each individual, the test stimulus intensity of 2% above threshold was chosen. For short-interval intracortical inhibition (SICI) (Chen, 2004). A conditioning stimuli was set at 0.8 motor threshold and the test stimuli was applied 4 ms later. For short-interval intracortical facilitation (SICF) (Chen, 2004) the conditioning stimuli was set at motor threshold and the test stimuli applied 15 ms later. For long-interval intracortical inhibition (LICI) (Chen, 2004) the conditioning stimuli was set at motor threshold and the test stimulus applied 100 ms later. For each paradigm the amplitude of the test response was averaged from 10 consecutive paired-stimuli.

3.3.3. *Muscle Fatigue*

The subjects were asked to perform full maximal isometric contraction of the left ADM muscle for 20 seconds to record EMG signal which was submitted online to power spectrum analysis to obtain the mean frequency of the signal. After 1 minute rest, the subjects were verbally stimulated to mantain a maximal isometric contraction of the left ADM muscle for 2 minutes. At the end of this period and during contraction a power spectrum analysis was again obtained and the median frequency registered.

3.3.4. *Cortical Silent Period*

Immediately following the exercise protocol the subject was permitted to rest for 15 seconds, and then a new short period of left ADM maximal contraction was requested once again. This was monitored on screen and through a loudspeaker. The motor cortex was stimulated at 10% above threshold in ten consecutive tests, with at least 30 seconds between tests. This TMS stimulus was followed by a silent period in motor unit activity (CSP) in the ADM recording, defined as the

interval between the motor response and the return of motor unit activity of at least 100 μV. The shortest CSP in the ten tests was taken as definitive (Cerqueira *et al*., 2006). In a previous work our group has confirmed that the value measured for CSP latency as defined using this method has a high interater reliability and shows high correlation to the latency defined with the rectified signal (correlation coefficient > 0.9 for both) (figures 23 and 24) (Cerqueira *et al*., 2006).
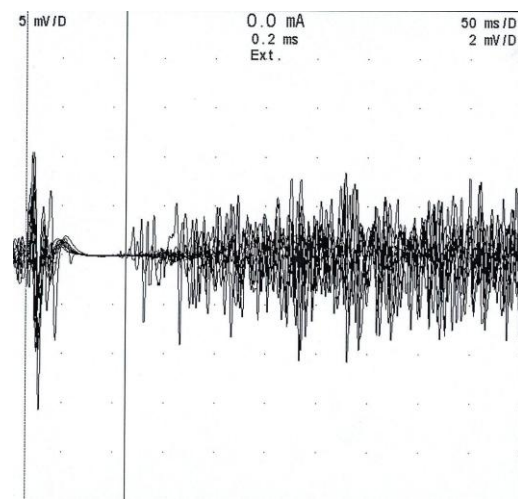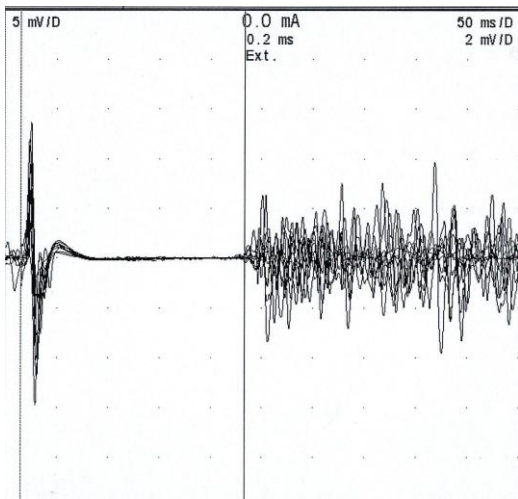
Figure 23. Cortical silent period recording before caffeine   Figure 24. Cortical silent period recording after caffeine

3.4. Statistical Analysis

Statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS) software (version 14.0 for Windows). Values are be given as mean ± standard deviation (SD). The paired Student's *t* test was used to analyse the effects of caffeine on electrophysiological parameters in the motor cortex. Repeated measures ANOVA was used to study the effects of both fatigue and caffeine on the median frequency of the responses spectrum. The absence or presence of fatigue was considered in the within-subjects analysis, and the effect of caffeine in the between-subjects analysis. *P* values <0.05 were considered statistically significant.

4. RESULTS

4.1. Demographic Characteristics

Ten female and three male volunteers were studied (see Table 1). Nine were regular and moderate coffee consumers and four were non-consumers, although one of these four non-consumers had coca cola. Just one of the females was a smoker.

	N	Minimum	Maximum	Mean	Std. Deviation
Age (years)	13	20	31	27,7	3,4
Weight (kg)	13	53	79	63,3	7,9
Height (cm)	13	153	186	171	8,6
Nº coffees (per day)	13	0	7	2,00	2,2

Table 1. Demographic characteristics

One of the subjects had felt tremor during all day after the experiment, it has been considered a side effect of the amount of caffeine given. Two other subjects felt a significantly deprivation of caffeine, but one of them said that the headache was gone after the intake of the pills in the experiment.

4.2. Electrophysiological Results

Electrophysiological results are summarised in Table 2.

The contraction protocol applied in this study was effective in inducing fatigue as evaluated by subject symptoms and the change of the median frequency of the EMG electrical signal. Fatigue caused a decrease on the median frequency of the motor response $85,6 \pm 17,1$ Hz to $60,0 \pm 17,5$

Hz before caffeine (*P*=0,001) and decrease from 78,8 ± 18,8 Hz to 58,8 ± 15,5 Hz after caffeine

(*P*=0,001). However, there was no effect of caffeine on the median frequency of the motor

response (*P*=0,127) and no interaction between fatigue and caffeine intake (*P*=0,274).

Groups	N	Before caffeine intake Mean ± SD (Min-Max)		One hour after caffeine intake Mean ± SD (Min-Max)		*P* value
		Rest	Fatigue	Rest	Fatigue	
Cortical threshold (%)	13	49,4 ± 5,5 (41-56)		48,6 ± 5,0 (41-59)		0,370*
Median Frequency	13	85,6 ± 17,1 (66 – 122)	60,0 ± 17,5 (40 – 98)	78,8 ± 18,8 (62 – 135)	58,8 ± 15,5 (31 – 90)	0.127** **0.001***
Amplitude (µV)	13	249,4 ± 183,0 (70 – 610)		304,5 ± 206,5 (82 – 646)		0,252*
Short-interval intracortical inhibition (SICI)(4 ms) (%)	13	87,5 ± 16,3 (46,4 – 100)		83,8 ± 24,5 (21,8 – 100)		0,657*
Intracortical facilitation (ICF) (15 ms) (%)	13	835,6 ± 1086,3 (-77,1 – 3542)		510,4 ± 421,5 (-82,5 – 1325,6)		0,3297*
Long-interval intracortical inhibition (LICI) (100 ms) (%)	13	77,7 ± 37,5 (-18,1 – 100)		49,0 ± 68,6 (-137,7 – 100)		0,201*
CSP (ms)	13		214,0 ± 43,7 (150 – 324)		177,5 ± 44.4 (110 – 251)	**0.004***

Table 2. Electrophysiological results

* Student's *t* test, comparison of the mean values before and after caffeine intake

** Repeated Measurements ANOVA test, effect of caffeine (between-subjects analysis)

*** Repeated Measurements ANOVA test, effect of fatigue (within-subjects analysis)

In spite of the persistence of the fatigue effect, caffeine could still decrease the CSP elicited under fatigue conditions as previously shown under resting conditions. Thus, when applying a stimulus intensity of 10% above threshold, the CSP obtained in fatigue conditions was decreased after caffeine intake (177,5 ± 44.4 ms), in comparison with the control value before caffeine (214,0 ± 43,7 ms, *P*=0,004).

As shown in Table 2, the threshold, SICI, ICF and LICI were not significantly changed by caffeine intake.

5. DISCUSSION

A remarkably consistent effect for caffeine on the CSP studied by TMS was previously described by our group (Cerqueira *et al*, 2006). This effect of caffeine was even proposed as a biomarker for the central effects of caffeine. CSP is thought to be a complex phenomenon, involving spinal mechanisms at the beginning and cortical mechanisms at the end of the process, and ultimately reflects the GABAergic inhibitory transmission at cortical interneurons. Experimental manipulation of $GABA_B$ receptors, which are inhibitory autoreceptors, is known to modulate the CSP (Inghilleri *et al*., 1996; Priori *et al*., 1994; Roick *et al*., 1993; Ziemann *et al*., 1996). In this context, caffeine might interfere with GABAergic neurotransmission in different ways (Cerqueira *et al*, 2006). It was proposed that caffeine, by antagonizing A_{2A} receptors, could inhibit the release of GABA, thus reduce GABAergic inhibitory transmission in the motor cortex, and hence decrease the CSP (Cerqueira *et al*, 2006). Experimental support for this possibility comes from the excitatory effect for adenosine A_{2A} receptors described on GABA release in the hippocampus (Cunha and Ribeiro, 2000). However, the alternative possibility that caffeine could decrease the CSP by modifying the properties of extrinsic cortico-basal-thalamo-cortical pathways that control motor cortex activity could not be ruled out. Adenosine A_{2A} receptors are found in brain areas rich in dopamine such as the basal ganglia, where they are associated with D_2 dopamine receptors (Ferre *et al*., 1997; Fisone *et al*. 2004) and any significant changes induced by caffeine within the basal ganglia would very likely exert a downstream effect on the motor cortex.

In the present work, taking into account that the fatiguing contraction physiologically increases CSP in the target muscle by a build-up of intracortical inhibition (Gandevia, 2001; Taylor *et al*.

1996) and that caffeine has known ergogenic properties and reduces the CSP, we tested whether the effect of caffeine on the CSP might be prevented in a fatigued muscle. The fatigue protocol applied in this study was effective, as confirmed by the significant decrease of the median frequency of the ADM electrical sign. This reflects the decrease in the muscle fiber conduction velocity (Lindström *et al*., 1970) as well as the synchronization of the motor units (Bigland-Ritchie *et al*., 1981). Caffeine did not prevent the decrease of the median frequency of the ADM electrical sign using this simple fatigue protocol. Remarkably, the magnitude of CSP reduction observed for caffeine in the ADM fatigued muscle (17%) was similar to the one previously observed in the ADM rested muscle (21%) (Cerqueira *et al*, 2006). Apparently, fatigue is potentiating intracortical inhibition (increasing CSP) and caffeine is attenuating intracortical inhibition (decreasing CSP) by mechanisms that are distinct and do not interact in the experimental conditions tested.

Our findings regarding the effects of caffeine on threshold, SICI and ICF essentially confirm previous results (Orth *et al*., 2005; Cerqueira *et al*, 2006). As mentioned above, the threshold reflects the excitability of the motor neurons or the associated interneurons, and SICI and ICF result from the activity of the cortical interneurons as mediated by $GABA_A$ and glutamate, respectively (Kujirai *et al*., 1993). The effect of caffeine on LICI was addressed here for the first time. Since both LICI and CSP result from a cortical inhibition, probably mediated by GABAergic neurotransmission and regulated by $GABA_B$ receptors (Werhahn *et al*., 1999), one might anticipate that caffeine could cause parallel changes on both LICI and CSP. However, caffeine, as discussed above, reduced the CSP, but did not modify the LICI significantly. This may signify that the mechanisms involved in both inhibitory phenomena are probably different

(Udupa *et al*., Epub ahead of print). Another possibility would be that the CSP would be more sensitive than LICI to reflect changes mediated by $GABA_B$ receptors. To this regard, it is interesting to consider again the effects of fatigue. Concerning LICI, as it happens with the CSP, there is also a dissociation between the effects of fatigue and the effects of caffeine. Fatiguing contraction is known to reduce LICI (Benwell *et al*., 2007) whereas caffeine does not modify LICI (as shown in the present study).

In conclusion, the present findings suggest that caffeine does not counteract centrally the effects of fatigue. Caffeine decreases and fatigue increases the CSP by mechanisms that do not interact and are thus are probably distinct, and caffeine does not affect, whereas the fatigue reduces, the LICI.

6. ACKNOWLEDGEMENTS

7. REFERENCES

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