GENETIC HYPERTENSION: IN VITRO CHARACTERIZATION OF CELLULAR MEMBRANE PROPERTIES FROM NEONATAL SUPERIOR CERVICAL GANGLIA NEURONES FROM RAT

by

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GENETIC HYPERTENSION: IN VITRO STUDIES.

A mes parents, Lucien et Genevieve Jubelin,

sans qui je ne serais rien

et

A mes enfants, Jérémy et Katya Elder-Jubelin,

sans qui je serais beaucoup moins.

C'est aussi avec regret que je dédis cette thèse

à la mémoire de César (Dr C. Galeano)

mentor passionné devenu ami,

décédé bien trop prematurément

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ABSTRACT

This work intended to study cell membrane properties of cells from genetically hypertensive animals in culture. The spontaneously hypertensive rat from the Wistar-Kyoto strain (SH rats) was used as a model. Genetically related Wistar-Kyoto (WKY) and unrelated Spague-Dawley (SD) rats were used as controls. Neurones from the Superior Cervical Ganglia (SCG) were cultured in vitro, with and without target cells from the vasculature (aorta, A VSMC, and mesenteric artery, MA VSMC). Passive and active membrane electrical properties from SCG neurones and A and MA VSMC were studied with microelectrodes via injection of long-duration (400 msec.) depolarizing and hyperpolarizing square pulses of current.

Most of WKY and SD SCG neurones fired one or two action potentials upon depolarization. The majority of SH SCG neurones was found to have lost their accommodative properties and fired three or more action potentials upon depolarisation. In all strains, firing abilities were inhibited in presence of A VSMC. The firing frequencies distributions remained unchanged in SD and WKY co-cultures, but was shifted to lower values in the presence of MA VSMC in SH co-cultures. Multiple firing was found to be initiated by the absence of or lack of activation of a calcium-dependent potassium channel and carried by a regenerative calcium current. This cellular dysfunction is thought to be present in all cell types in genetically hypertensive individuals, and a general framework is

provided which tentatively explains the development, establishment and complex phenotype of essential hypertension.

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LIST OF ABBREVIATIONS

A : aorta

ADP : afterpotential-depolarisation

AHP : afterpotential-hyperpolarisation

AP : action potential

BP : blood pressure

Ca^{++} : calcium (ions)

CNS : central nervous system

EJP : excitatory junction potential

K^+ : potassium

$K_{(Ca)}$: calcium-activated potassium (channel)

LHP : long-(duration)-hyperpolarisation

MA : mesenteric artery

Na^+ : sodium

NE : norepinephrine

NGF : nerve growth factor

RMP : resting membrane potential

SCG : superior cervical ganglia

SD : Sprague-Dawley (rat)

SH : spontaneously hypertensive (WKY rat substrain)

SNS : sympathetic nervous sytem

TEA : tetraethylammonium

TTX : tetrodotoxin

VLHP: very long (afterpotential) hyperpolarisation

VSMC: vascular smooth muscle cells

WKY : Wistar-Kyoto (rat strain)

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INTRODUCTION

I. Blood pressure and its regulation: overview.

Regulation of blood pressure (BP) is a complex phenomenon as shown in the diagram of his computer simulation (Guyton, 1972). This graph in itself explains why we do not yet fully comprehend the regulation of BP, let alone its various pathologies. However, four major components of this system can be identified: the circulatory system (heart and blood vessels), the peripheral nervous system (sympathetic and parasympathetic), the central nervous system , and the kidneys/adrenal glands.

I.a. The circulatory system.

The circulatory system has two major components intervening in BP regulation: the heart (heart rate and stroke volume) and the arterial tree whose main BP regulatory component consists of small muscular arteries and arterioles (flow regulation, resistance, contractility). One of the basic levels of BP regulation involves the arterial vessel wall: elasticity, contractility, resistance to stretch, and stretch-induced contraction. All of these factors act on the diameter of the blood vessel which, in combination with the blood volume,

determines the blood flow and resistance and, therefore, the BP. The passive properties are characteristic of large vessels (such as the aorta,) while the active properties are associated with smaller (resistance) vessels. A gradient between passive and active properties can be described from large to small vessels, as well as a gradient of innervation (smaller vessels being more innervated). The heart and large vessels insure a gross regulation of BP by generating a regular flow of blood throughout the organism. Smaller (resistance) vessels are responsible for the fine tuning of the blood flow and, ultimately, for the arterial BP (Mulvany, et al., 1978; Black, et al., 1984) as well as an important participation in local BP regulation within organs. The number, size and contractility of smooth muscle cells in the media of these vessels is, thus, a fundamental element of BP regulation. Being heavily innervated, their sensitivity to neurotransmitters and the type of neuro-transmitter(s) liberated by nerve endings of the peripheral nervous system are also important. This regulation of blood vessel wall contraction is mirrored on the mucosal side by the effect of circulating hormones as well as relaxing and contracting factors (Endothelium Derived Relaxing, i.e. nitric oxide, and Contracting , i.e. Endothelin, Factors respectively) released by endothelial cells (Bassenge, 1989; Dinh-Xuan, 1990; Karwatowska-Prokopczuk and Wennmalm, 1990; Luscher et al.,1990)

I.b. The sympathetic nervous system (SNS).

Overall, the sympathetic nervous system is responsible for vasoconstriction and includes a number of reflexes, integrating baro- and chemo- receptors signals (Brody, et al., 1989). Vasorelaxation is mainly insured by the vagal afferent, the para-sympathetic nervous system modulation of the sympathetic system, and the release of endothelial factors (under a various array of neural and humoral controls). The amount and type of neurotransmitter liberated at the sympathetic neuro-vascular junction depends primarily on the type of neurone and its neurotransmitter (which is defined partly by genetics and partly by interactions with trophic factors and target cells during development) (Furhspan, et al., 1986; Potter, et al., 1986; see also sections II.a.2. and IV., this chapter); but it also depends on the overall activity of the autonomic nervous system and the frequency and intensity of firing of the pre-synaptic neurones (Brock and Cunnane, 1987; Lacroix, et al., 1988). This activity can be spontaneous or reflex, and can be generated and/or modulated by the inputs of pressure or chemical sensors, by the activity of a single nucleus along the path of the reflex, or by the last neurones of the reflex arc (pre-synaptic to the vascular smooth muscle cell, VSMC). There are numerous neurotransmitters possibly acting at the different levels of the SNS involved in BP regulation besides adrenaline/ noradrenaline, including serotonin (Ozaki, et al., 1986; Kawasaki, et al., 1987; Schlicker, et al., 1988; Tsuda, et al., 1988 c; Brody, et al., 1989), glutamate (Brody, et al., 1989; Smith and Barron,

1990), gamma amino butyric acid (Sazaki, et al., 1989), vasopressin and angiotensin II (Morris, et al., 1986; Hayashi, et al., 1988), Neuropeptide Y (Westfall, et al., 1987; Pernow, 1988), substance P (Brattstrom, et al., 1986; Scott, et al., 1990), non-Adrenergic non-Cholinergic (Inoue and Kannan, 1988) and purinergic transmitters (see Burnstock, 1985), epinephrine (Tsuda, et al., 1990, b) and opioids (Wang, et al., 1986; Bhargava and Gulate, 1988; Kapusta, et al., 1989; Tsuda, et al., 1989).

I.c. The central nervous system (CNS).

The CNS role in BP modulation is complex because of the high level of integration between the purely cardio-vascular reflexes and psycho-social and emotional behaviours (Folkow, 1987; Reis and Ledoux, 1987; DeQuattro, 1989; Folkow, 1989; Kapusta, et al., 1989; Reis, et al., 1989; Casto and Printz, 1990; see also entire section 15.2, IUPS Meet., Helsinki, Finland, p. 110, 1989). However, some CNS nuclei have been shown to be of particular importance, especially in relation to BP regulation disorders, such as hypertension. Some authors described the importance of CNS nuclei in general (Calaresu, et al., 1975; Reis and Ledoux, 1987; Chalmers, et al., 1988), while others suggested an active role of central cholinergic (Folkow, 1987; Giuliano and Brezenoff, 1987; Kawashima, et al., 1987; Brezenoff, et al., 1988) and catecholaminergic (Howes, 1984) pathways. More specifically, BP regulation appears to involve the participation of the cortex (Wang, et al., 1986; Schlicker, et al., 1988), the thalamus and

hypothalamus (Takeda and Bunuac, 1978; Wang, et al., 1986; Bohr, 1987; Bhargava and Gulate, 1988; Qualy and Westfall, 1988), the substancia nigra (Van den Bunse, et al., 1986), the hippocampus (Wang, et al., 1986; Bhatnagar and Meaney, 1989), the brain stem (Wang, et al., 1986), the nucleus of the tractus solitarius (Petty, et al., 1979; Catelli, et al., 1987, Catelli and Sved, 1988; Sazaki, et al., 1989; Stephenson, et al., 1989), and the medulla oblongata (specifically the C1 area of the rostroventro-lateral medulla) (Reis, et al, 1989; Stephenson, et al., 1989; Arneric, et al., 1990; Smith and Barron, 1990). Finally the parathyroid, through the parathyroid hormone (vasorelaxant) and its relation to calcium and sodium metabolism (Nicolov, et al., 1988; Zachariah, et al., 1988; Kaneko, et al., 1989; Pang and Lewanczuk, 1989; Zemel, et al., 1989) was also shown to influence BP regulation. Any modification in the activity of one or more of these areas is, thus, likely to modify BP.

I.d. The kidney.

The influence of the kidney on BP regulation is exerted mainly through overall regulation of blood volume through filtration. In addition, the kidneys secrete an hormone (renin), which regulates the function of numerous elements of this complex system, such as the hypothalamus/ pituitary/ adrenal axis, the intestinal wall, the blood vessel endothelium (which releases relaxing or contracting factors on the VSMC) or directly the VSMC (Loly et al., 1984; Scuba et al., 1985; Simonson, 1988). Thus, any dysfunction of the kidney is

likely to have large repercussion on BP.

I.e. Interrelations between the four components of blood pressure regulation.

When not modulating cardiac function, most of the BP control is directed towards the wall of the blood vessels: on the mucosal side, volume, pressure, hormones and the endothelium; intrinsic to the wall, the number, size and contractility of the VSMC, as well as their sensitivity to various chemicals; on the medial side the neurones and their neurotransmitter(s), the type and amount of which depends on the overall activity of the autonomic nervous system and the CNS. The BP of a living organism constantly oscillates, and this continual adjustment is fundamental to the survival of the organism. This ability to adjust the BP is primarily performed by the SNS, since even the kidney/adrenal complex is under SNS control. Indeed, the major complication noted in sympathetically denervated or deafferented (interruption of baroreflex) experimental animals is not a long-term drastic drop in BP (the pressure rapidly returns to near normal after an initial drop), but a lack of ability to compensate for sudden variations of pressure, such as those generated by a change of posture, animal handling or a startling noise (Haeusler, 1969; Jonhson and Macia, 1979; Trindade Jr and Krieger, 1984; Lee, et al., 1986; Hayashi, et al., 1988; Osborn and England, 1989). Thus, experimental set-ups where the animal is handled (stress) might lead to the wrongful assumption of heightened BP, as possibly in the results of

Petty et al.(1979). In such experimental animals, a close-to-normal resting BP is observed with drastic fluctuations, suggesting that while the sympathetic nervous system does not generate the resting BP, it is the essential factor in BP regulation. The SNS activity is also thought to regulate the VSMC number in arterial walls (Bevan, 1975; Lee, et al., 1986; Lee, et al., 1988), regulating BP in yet another, albeit indirect, way (but see section II.c.1.1.).

Thus, the four major components of BP establishment and regulation described above are intimately related and in a constant state of "dialogue", generating a system of interaction, co- and reciprocal regulation between these components so intricate that it is difficult to separate them (Smith, et al., 1984; Chalmers, et al., 1988). Any pathology is, thus, very likely to include a multi-factorial compensatory response at each stage of its development, which will make it difficult to identify the original default.

The most common dysfunction of BP regulation is hypertension (high BP). General classes of causes for hypertension have been defined: hormonal, metabolic (sensitivity to salt intake, etc.), dysfunction of blood volume regulation, kidney malfunction, and genetic (essential) hypertension. Genetic hypertension is the dysfunction studied in this thesis. The basic cellular dysfunction is not induced by external factor(s) (although it could be unmasked by such factors), does not result from injury to any organ in the body and appears spontaneously over time: the older the subject, the higher the BP. Its genetic transmission characteristics make it

possible to raise spontaneously hypertensive animals, providing models to study human essential hypertension. Any reference to hypertension, hypertensive state and its mechanisms, etc., in this text will refer to genetically determined spontaneous hypertension, unless specifically mentioned otherwise.

II. Genetic hypertension.

II.a. Human essential hypertension.

II.a.1. Characterisation.

Genetic hypertension is a widely spread disease in the human population which is defined by heightened mean arterial BP. It can be described as "a specific inherited disorder of middle age... [with a] significant familial resemblance... apparent at an early age... [and with] approximately 40% of the variance in... blood pressure [due] to environmental influences." (Luft et al, 1987). Even though there is no exact threshold to define abnormally high BP, hypertension is usually defined by a systolic pressure higher or equal to 150-160 mm mercury, with a diastolic pressure higher or equal to 95 mm mercury. These terms define a group of approximately 15% of the human population (10% of the younger (18 y old to mid-thirties) and 20% of the older individuals) (Freis, 1979; Folkow, 1989). Although not directly life-threatening, this disease induces cardiac (Macia, et al., 1988) and vascular (Furuyama, 1962; Naeye, 1967; Cook and Yates, 1972) hypertrophy, and may lead to sudden death and death

by myocardial infarction (Freis, 1979). It also threatens other organs, such as the kidneys, the eyes and the brain through a generalised vasculopathy (Izzo Jr., 1989); other complications are the tendency to develop arteriosclerosis and atherosclerosis from excessive collagen deposits in the vessel wall (Weber, et al., 1987), altered breathing patterns (Begelora, et al., 1989), and numerous behavioural changes (Esler, et al., 1989). It is not clear yet if those are consequences of high BP or part of the symptomatology.

Both central and peripheral SNS (Westfall and Meldrum, 1985; Folkow, 1989) as well as VSMC function (Westfall and Meldrum, 1985) are affected. An augmented norepinephrine spillover (Esler, et al., 1989) and an impairment of the cardio-pulmonary reflexes, probably due to cardiac hypertrophy (Mancia, et al., 1988), have been identified. Clement et al., (1979) suggest that BP variability is proportional to BP but not to sympathetic activity because adrenaline and noradrenaline levels do not parallel BP variations. This does not consider the possibility of an increased uptake system (Mulvany, et al., 1979) and the overall lack of reliability in considering catecholamine overflow (Folkow, et al., 1983) as indicator of sympathetic activity. All these elements suggest a general dysfunction of the SNS, but the original cause of essential hypertension still eludes researchers. The problem is compounded by the difficulty to evaluate abnormal variations of BP in children, who show a very labile BP (Levin, 1983, Diament, et al., 1986, Trevisan, et al., 1989), making early detection (and thus early

treatement) difficult. Many would agree with Westfall and Meldrum that "hypertension is a multifactorial disease involving many alterations in the nervous and endocrine systems as well as alterations in vascular smooth muscle function" (Westfall and Meldrum, 1985). Egan states that "thus, hypertension is initiated predominantly by neurogenic mechanisms [and] could be later sustained without excessive sympathetic drive" (Egan, 1989).

Theoretically, the kidneys possess a nearly infinite capacity to compensate for BP changes (Guyton, 1989). Thus, it appears that in order to observe a hypertensive state this capacity would also have to be impaired by, for example, a defective Angiotensin Converting Enzyme system (Hollenberg and Williams, 1989). See discussion on the role of kidneys in hypertension in the SH rat model for further arguments (section II.c.4.).

For some time, salt intake sensitivity has been intimately connected with essential hypertension (Morgan, et al., 1979, but see Robertson, 1979; Simpson, 1979; Wilhelmsen, 1979; Kaplan, 1990) and an imbalance in sodium metabolism has been identified (Ashida, et al., 1989; Kjelsen, et al., 1989; Resnick, et al., 1989; Semplicini, et al., 1989; Weder, 1989). However, except for a sub-group of individuals sensitive to salt intake, which a low salt diet could help (Wilhelmsen, 1979; Falkner, et al., 1986; Luft, et al., 1987), hypertension and salt intake sensitivity have been shown to be different pathologies, albeit polygenetic and often co-selected (Watt, et al., 1985; Falkner, et al., 1986; Harrap, 1986; Horan and Lovenberg,

1986; Horie, et al., 1986; Schlager, et al., 1986; Schofield, et al., 1986; Luft, et al., 1987; Williams, et al., 1987; Youngue and Myers, 1988; Folkow, 1989). Furthermore, low salt intake regimen (at levels effective to reduce BP to a satisfactory level) might even be more harmful than beneficial (Wilczynski and Leenen, 1987; Folkow, 1989; Kaplan,1990).

At the cellular and molecular level, many different dysfunctions have been identified, such as 1) a general default in Na^+/Ca^{++} metabolism (Resnick, et al., 1989); 2) an augmented activity of the Na^+/H^+ and Li^+/Na^+ antiport (Semplicini, et al., 1989) and K^+/Na^+ transport (Kyeldsen, et al., 1989) systems, as well as raised magnesium (Kjeldsen, et al., 1990) and free calcium (Tsuda, et al., 1988 b; 1990 a, Bohr, 1989; Cirillo, et al., 1989) concentrations in red blood cells; 3) an augmented Na^+/H^+ transport (without pH modification) (Weder, 1989), an augmented protein phosphorylation and intra-cellular free calcium combined with a more active Ca^{++}-Calmodulin and Protein-kinase C pathways (Haller, et al., 1989), a diminished activity of plasma membrane calcium pump (Takaya, et al., 1990), and a generalised membrane alteration, especially in its calcium binding capacity, (Bohr, 1989; Le Quan Sang, et al., 1991), in the platelets.

II.a.2. Treatments of essential hypertension.

The treatments available are numerous and, at best, limit the rise in BP to a more tolerable level. This will diminish the risk of

complications secondary to hypertension and could even reverse some (Mancia et al., 1988), except for coronary diseases (which represent the most threatening aspect of hypertension) (Freis, 1979). Furthermore, the patients compliance with the treatment is poor, partly because the side-effects are so unpleasant. Briefly, therapies usually combine two or more antihypertensive agents (including diuretics, calcium channel blockers, alpha- and beta-adrenoceptor antagonists, direct vasodilators, sympatholytics, ganglion-blockers and inhibitors of the Angiotensin Converting Enzyme) (Ibsen, et al., 1979; Matthews, et al., 1979; Scriabine, 1980; Opsahl, et al., 1989), and their side-effects vary from dryness of the mouth to cardiac problems, and include varied toxicities, cramps and fatigue, sedation, allergic reactions, rashes, headaches, impotence, gynecomastia, etc. (Scriabine, 1980). Although the extent and severity of the side-effects vary with the antihypertensive agent, none are free of them. This is no minor consideration when one is reminded that these treatments are life-long ones.

Indeed, recent efforts in developing new treatments focused on lessening the side-effects (Bhalla and Sharma, 1986; Shionoiri, et al., 1987; Nievelstein, et al., 1988; Opsahl, et al., 1989; Schoenberger, 1990). Among those, potassium channel activators (like cromakalim) are actively investigated (Lebel, et al., 1989) and supplements (such as magnesium, potassium, and antifibrotics) to the treatment have been suggested to lessen long-term side-effects (Saito, et al., 1988; Weber, et al., 1989; Tobian, et al., 1990;).

II.a.3. Summary.

Available treatments address the symptoms, but not the cause of the dysfunction. This is so because, even though numerous studies have taken place, the fundamental cause of hypertension has still not been identified. This is in part due to the complexity of BP regulation, but also to the fact that different dysfunctions might still be grouped under the same label. Most human studies are done on subjects in whom the hypertensive state is already present, which makes it difficult to separate the cause from the effect, especially when there appears to be a dissociation between the initiating factors of the hypertensive state and those maintaining it (Egan, 1989; Esler, et al., 1989). Some consequences of hypertension are reversible, but some only for a limited period of time after the establishment of high BP (Lundgren and Weiss, 1979; study in the rat). Also, the longer the organs are exposed to hypertension, the more serious is the insult to the tissues. Ultimately, life-long treatments are bound to induce complications on their own. For all these reasons, it is important to identify the cause of essential hypertension; it is even more important to find a marker to detect hypertension, so that individuals genetically inclined to develop the illness can be identified as early as possible to prevent the induction of secondary dysfunctions (cardiac hypertrophy, vasculopathy, etc). As for most human diseases, an animal model was needed to study the fundamental cause(s) of the dysfunction.

II.b. Animal models.

There are various strains of genetically hypertensive rats available, such as the GH, SH, Milan and Lyon rat strains. Although all of these strains can develop hypertension, they apparently do so through different pathogenic mechanisms (Horie, et al., 1986). Among the genetically hypertensive strains, rats from the Wistar-Kyoto sub-strain (the Spontaneously Hypertensive -or SH- rats) are the most commonly used (Frolich, 1986; Horie, et al., 1986; Lovenberg, 1986). Hypertension in these animals can be described in the same terms as for human hypertension: a "complex, multifactorial, polygenic disturbance of BP" (Horan and Lovenberg, 1986) (see section II.c.).

When compared to hypertensive humans, SH rats develop similar changes in the vasculature, central and peripheral nervous system, and kidneys. Many of these changes can now be described as induced by and contributing to the hypertensive state. Part of the confusion resides in the complexity of the dysfunction, as in human BP regulation, and the difficulty to isolate single facts in the whole animal or to relate the multitude of cellular and molecular abnormalities to the whole body function in addition to technical difficulties such as the availability of sensitive techniques, experimental conditions, animal handling (emotional modulation of BP), and age-related evolution of the variables.

A word of caution should be noted with respect to the controls used in these studies, since the genetically selected controls (WKY)

and SH strains define highly inbred populations (Kurtz, et al., 1989, Henry, et al., 1990). Indeed, WKY rats have been shown, in some instances, to be the strain with the genetically selected abnormality when a third, normotensive group of genetically unrelated animals is included in the study (Blennerhassett, et al., 1989). For these reasons, among others, care should be exercised when correlating an observed modification to the hypertensive state

The possible polygenic characteristic of hypertension should not be mistaken with cosegregation of genes located close to the potential hypertension-gene and selected at the same time by the inbreeding process necessary to generate hypertensive strains (the gene(s) involved appears to be recessive (Bruner, et al., 1986)). Such was the case for salt appetite and sensitivity in SH rats (Harrap, 1986; Youngue and Myers, 1988), confirmed by the existence of sub-strains of salt -sensitive and -insensitive Dahl rats and the effect of DOCA pellets which induce hypertension in normotensive rats when subjected to high salt diet (Motoyama, et al., 1988).

II.c. The SH rat.

In the SH model, the development of hypertension is usually divided into three stages: pre-hypertensive (0 to 40-50 days old), early hypertensive (up to 4-5 months old) and established hypertensive (older than 5 months) (Aoki, et al. and Okamoto, in Gray 1984 a; Warshaw, et al., 1979). This classification is disputed and there seems to be a more progressive rise in BP, starting at the

least at birth, that techniques such as the tail-cuff method could not detect (Gray, et al., 1984 a, b; Smith, et al., 1984).

II.c.1. Cardiovascular modifications.

The main target of hypertension, the cardio-vascular system, shows modified morphology and different cellular properties in hypertensive animals.

II.c.1.1. Vascular media hypertrophy.

Cardiac and resistance vessel wall media hypertrophy and hyperplasia (Folkow, et al., 1970; Warshaw, et al., 1979; Lee, et al., 1983; Clegg, et al., 1986; Sen and Tarazi, 1986; Cameron and Antonok, 1988; Schmid-Schonbein, et al., 1990) are observed. It is noted in pre-hypertensive (young) rats (Lee, 1985) and in other hypertensive strains (Lee and Triggle, 1986). When denervated, locally or completely, the vessels show a reduction of their cross-section (Lee, et al., 1986; Lee and Triggle, 1986; Nyborg, et al., 1986; Mulvany, 1986). However, the BP may already be elevated in "pre-hypertensive" animals (4 weeks old and younger) (Gray, 1984 a, b; Kaskel, et al., 1988; but see Campbell, et al., 1981; Cheung, 1984; Lee, et al., 1985; Rapp, et al., 1986; Tsuji, et al., 1989), and it is possible that these changes are a consequence of an already augmented BP.

Some experimental results support that notion: 1) media hypertrophy can be induced by stenosis (i.e., induction of higher pressure and wall stretch) (Gabella, 1979, 1984); 2) VSMC from SH rats in culture divide faster than their WKY counterparts (Yamori, et al., 1984; Grunwald and Fisher, 1985; Clegg, et al., 1986; Ishimitsu, et

al., 1988 Paquet, et al., 1989; DiGlio, et al., 1989; but see Blennerhassett, et al., 1989), but VSMC from normotensive strains in which hypertension has been induced by ligating ("clipping") one of the renal arteries also show a faster division rate in culture (Mey, et al., 1980); independently of the initiating signal, the total number of cell divisions is identical in all strains (Mey, et al., 1980); 3) together, these results suggest that a "stretch-induced" division signal, transferrable in culture, is present and not related to genetic hypertension; and 4) fibroblasts from the blood vessel wall (Durant, et al., 1989) and the skin (Guicheney et al, 1991) show the same accelerated division rate. Thus, vessel wall hypertrophy and hyperplasia would participate in the maintenance of hypertension, but would not be the original dysfunction.

II.c.1.2. Vascular tone.

Experiments carried on resistance vessels from SH rats show an augmented tone (Schmid-Schonbein, et al., 1990) and a faster development of tension (Bhalla, et al., 1987; Fouda, et al., 1987; but see Bukoski et al., 1989). Other vessels, such as the portal vein, have shown similar modifications (Malmqvist and Arner, 1988; Shimamura, et al., 1989). Also, VSMC from the mesenteric artery of the SH rat (SH MA VSMC) show an augmented receptor affinity for (Nyborg and Bevan, 1988), a change in beta-adrenoceptors (Kwan and Lee, 1990), and responsiveness to neurotransmitters (Tsuda, et al., 1984, 1988 a; Phelan and Simpson, 1986; Nickols, et al., 1986; Bhalla and Sharma, 1987; Cline Jr and Yamamoto, 1987; Fouda, et al.,

1987; Yamamoto and Cline Jr, 1987). Interestingly, Mulvany, et al., (1979) could not detect an increased sensitivity to neurotransmitters in SH MA VSMC without preventing noradrenaline neuronal uptake first, demonstrating the complexity of the possible compensatory mechanisms. The mesenteric artery has been found to be hyper-innervated (Shimamura, et al., 1987, Cassis, et al., 1988; Shimamura, et al., 1989), but the average excitatory junction potential (EJP) to be identical in SH and WKY MA (Fujii, et al., 1989). A combination of normal innervation and hyperplasia, without modification of VSMC reactivity or excess of neurotransmitter, has also been suggested (Warshaw, et al., 1979; Lee et al., 1986; Mulvany, 1986), as well as a smaller fraction of alpha-receptors on the VSMC membrane, with no difference in affinity (Suzuki, et al., 1986; Kojima, et al., 1989) and the exact opposite (Nyborg and Bevan, 1988). Similar conflicting results were communicated for the atrial natriuretic factor receptors (Nakamura, et al., 1989; Resnik, et al., 1989).

Some authors also suggested that these differences in vessel wall contractility could be attributed to a lack of endothelium derived relaxing factor (Criscione and Powell, 1986; Auch-Schwelk and Vanhoutte, 1989; Hoeffner and Vanhoutte, 1989). The endothelium-mediated responses have been shown to be impaired proportionally to the rise in BP, suggesting pressure-induced damages (Su, et al., 1986; Sunano, et al., 1989; Luscher, et al., 1990). These results, and the suggestion that the augmented responsiveness is an adaptation to higher BP (Fouda, et al., 1987), make it unlikely

that a genetic modification of endothelial cells be at the origin of the hypertensive state.

Finally, circulating factors, similar to the parathyroid factor in humans (see section I.c.), which influence VSMC ion homeostasis (Campbell, et al., 1989) and augment calcium influx (Lewanczuk, et al., 1989), have been described. This would imply that some of the modifications observed in SH vasculature would be independent of BP and of genetically modified VSMC properties. (see section relating to kidney for further comments).

II.c.1.3. Ions homeostasis and other intracellular VSMC changes.

Whether in situ (Campbell, et al., 1981; but see Fujii, et al., 1989) or in culture (Blennerhassett, et al., 1989), VSMC from SH rats appear to show a diminished resting membrane potential (but see Warshaw, et al., 1979). However, cells coming from rats with a clipped renal artery also show a depolarisation when compared to controls (Bryant, et al., 1986). The resting membrane potential, however, does not necessarily reflect the general ion handling of the cells.

Some modifications of ion handling by SH VSMC have been identified. Some involve calcium: basal intracellular calcium is considered normal (Phelan and Simpson, 1986; Nakamura, et al., 1989; but see Rapp, et al., 1986; Aviv and Livne, 1988), but calcium influx in response to transmitters is increased (Kannan, et al., 1986;

Rush and Hermsmeyer, 1986; Bhalla, et al., 1987; Hermsmeyer, 1987; Khalil and van Breemen, 1988; Bukoski, et al., 1989). Although part of these modifications of internal calcium might be induced by hypertrophy (Cameron and Antonik, 1988), a general disturbance of calcium homeostasis is obvious in these cells (Bruner, et al., 1986; Grammas, et al., 1986; Tsuda, et al., 1988 a; Koutouzov, 1987; Richardson, 1988).

One potential explanation for these conflicting results about calcium homeostasis is the possible existence of membrane associated micro-domains of calcium concentration (Rasmussen, et al., 1987; Nakamura, et al., 1989) and the stabilising effect of calcium on membrane fluidity: with a diminished capacity of high affinity binding sites, SH cell membrane would be more unstable, modifying the permeability of other ions (such as sodium) in the process (David-Dufilho, et al., 1986; Hermsmeyer, 1987; Tsuda, et al., 1988, b; Bohr, 1989; Rinaldi and Bohr, 1989; Storm, et al., 1990). Diminished membrane-bound calcium is still compatible with high internal free calcium, and this could explain the differing estimates of calcium in SH VSMC cells according to the techniques involved in measuring it. Aviv and Livne (1988) suggest that high internal calcium in SH VSMC is associated with an augmented Na^+/H^+ antiport activity, which would add to sodium imbalance. Also identified is a reduced $Na^+/K^+/Cl^-$ co-transport (O'Donnell and Owen, 1988), whose function is unknown. Finally, primary Ca^{++}/Na^+ counter-transport or Na^+/K^+/ATPase pump malfunctions resulting in modified Na^+

homeostasis have been discarded as a cause for SH VSMC modifications (Casteels, et al., 1985; Hermsmeyer, 1987).

Other molecular modifications in SH VSMC could include the prostaglandins pathway (Richardson, 1988), the thromboxane A2 homeostasis (Ishimitsu, et al., 1988), the inositol phosphate cycle (Ollenrenshaw, et al., 1988; Ek, et al., 1989; Labelle and Cox, 1989), altered G protein (Narayanan and Aronstam, 1989) and phospholipase C (Millanvoye, et al., 1988; Ek, et al., 1989) functions, and a blunting of cyclic GMP mediated responses (Nakamura, et al., 1989; Resnik, et al., 1989) but no difference in cyclic-AMP mediated response (Nabika, et al., 1985).

II.c.2. The peripheral sympathetic nervous system.

Overall, a general SNS hyperactivity is present in the SH rat (Judy, et al., 1976; Ekas and Lockandwala, 1981; Tsuda, et al., 1984; Zhang and Westfall, 1986; but see Shlicker, et al., 1988) with a possible hyper-innervation of some vessels (Shimamura, et al., 1987, Cassis, et al., 1988; Shimamura, et al., 1989). The experiments using sympathetic denervation with 6-hydroxydopamine (Nyborg et al., 1986) showed little changes in SH BP. Part of this negative result might be due to reinnervation (Schomig, et al., 1979), although Nyborg, et al., (1986) only noticed a limited reinnervation. The importance of the peripheral nervous system in the development of hypertension in SH was clearly demonstrated when the sympathectomy was complete, using both guanethidine and

antibodies to nerve growth factor (anti-NGF) (Jonhson and Macia, 1979; Lee, et al., 1986; Mulvany, 1986; Bhalla and Sharma, 1986; Motoyama, et al., 1988). This implies that the presence of a circulating factor (see above sections and II.d.) is not sufficient to induce hypertension. The fact that complete sympathetic denervation could be achieved in the SH rats only with the addition of antibodies to NGF also suggests that SH sympathetic neurones benefit from a specific protection from guanethidine. Again, it is interesting to note that total sympathectomy (in rats or in humans) brings the BP level down closer to normotensive level, but does not induce hypotension.

As in human essential hypertension, a certain number of cardio-vascular reflexes are suggested to be impaired (Thoren and Ricksten, 1979; Brody, et al., 1989; but see Mancia, et al., 1988). The baroreflex activity is increased, but apparently only to compensate for the elevated BP (Judy, et al., 1976; Murphy, et al., 1989), ability which is lost in older animals (Judy, et al., 1976). The central resetting of the baroreflex activity appears, thus, to be a consequence and not an initiating factor of high BP (Hayashi, et al., 1988).

The sympathetic ganglia, however, have demonstrated a certain number of modifications which cannot be described as consequences of hypertension. Nerve traffic pre- and post-synaptic to the superior cervical ganglia (SCG) shows a diminished silent period (Schramm and Barton, 1979). This represents an augmented efficacy of ganglionic transmission, i.e. the fact that pre-ganglionic

neurone activity is more likely to generate activation of the post-synaptic neurons. This could be due to a combination of hyper-responsiveness to acetylcholine (in young animals only) (Debinsky and Kuchel, 1989), an augmented number of beta-2 adrenoceptors (Saavedra, et al., 1990), and/or a loss of accommodation to depolarising pulses (Yarowsky and Weinreich, 1985).

A number of studies also suggest neural alterations at the level of the neuro-vascular junction (mainly in young animals), such as deficiencies in pre-synaptic modulation, (Kamikawa, et al., 1986; Masuyama, et al., 1986; Ozaki, et al., 1986; Tsuda, et al., 1988 a, c; Tsuji, et al., 1989) or increased neurotransmitter release (Ekas and Lockandwala, 1981; Tsuda, et al., 1984, 1990; Yarowsky and Weinreich, 1985; Kawasaki, et al., 1986; Masuyama, et al., 1986; Zhang and Westfall, 1986; Kawasaki, et al., 1987; Westfall, et al., 1987). This could explain why Yamamoto and Cline Jr (1987) do not observe any pre-synaptic modification of the neuro-effector junction in older SH rat and attribute the changes solely to modified post-synaptic mechanisms. Some of the increased release of neuro-transmitter may possibly be due to alterations of protein kinase C or Ca^{++}-dependent exocytosis (Tsuda, et al., 1990 a). As in many other SH cells, a disturbance in calcium handling in pre-synaptic neurones is strongly suspected (Grammas, et al., 1986; Nara, et al., 1986; Rapp, et al., 1986; Tsuda, et al., 1984, 1988 a; Hermsmeyer, 1987; Koutouzov, 1987; Debinsky and Kuchel, 1989).

II.c.3. The central nervous system.

As in the case of human hypertension, the activity of many nuclei of the CNS is modified in SH, but the relationship to hypertension is not firmly established. These include a central hyperactivity of the catecholaminergic (Howes, 1984), [along with an increase in alpha-1 adrenoceptors (Feldstein, et al., 1986)], cholinergic (Giuliano and Brezenoff, 1987; Kawashima, et al., 1987; Brezenoff, et al., 1988), and opioid (Wang, et al., 1986; Bucher, et al.,1987; Bhargava and Gulate, 1988) systems. Cultured neurones from hypothalamic nuclei from SH show an altered metabolism of vasopressin and oxytocin relative to cultured WKY neurones (Bennett, et al., 1989), and while basal gamma aminobutyric acid levels are normal in the SH hypothalamus, the turn-over rate is greatly diminished (Sasaki, et al., 1990).

The hypothalamus/pituitary/adrenal axis is also impaired (Bhatnagar and Meaney, 1989), but even if the stimulation of the hypothalamus generates a higher pressure response in SH rats (Takeda and Bunac, 1978; Qualy and Westfall, 1988), the basal BP does not rise following repetitive stimulation.

Another key area, the medulla oblongata, shows an increased norepinephrine overflow (Stephenson, et al., 1982) and an un-modified response to glutamate injection (Cline, et al., 1988; Smith and Barron, 1990) in the young SH rats, but shows a normal norepinephrine overflow (with a turn-over diminished by 80%) (Stephenson, et al., 1982) and an increased response to glutamate

(Cline, et al., 1988) in older animals. This area also shows, in SH rats, a diminished gamma aminobutyric acid turn-over rate, even if basal levels are normal (Sasaki, et al. 1990). Other nuclei with modified activities include the substantia nigra (Van den Buuse, et al., 1986) and the hippocampus (Bhatnagar and Meaney, 1989). Also, the parathyroid, when transplanted from SH to normotensive animals, induces hypertension, which suggests the production of a circulating hypertensive factor (PHF, Parathyroid Hypertensive Factor) (Kanako, et al., 1979; Pang and Lewanczuk, 1989).

Interestingly, a possible powerful modulator of the central nervous system activity, including its actions on the peripheral SNS, is seldom discussed in SH generation and maintenance: the cerebro-spinal fluid. Compounds present in the cerebro-spinal fluid could be responsible for a general modulation (tuning up or down) of the whole nervous activity. For example, WKY produces four times the amount of vasopressin in the cerebro-spinal fluid in response to hypertonic saline or angiotensin II, showing that vasopressin could act on BP centrally (via the cerebro-spinal fluid) or peripherally. Interestingly, SH rats showed the smaller increase in vasopressin and mean arterial pressure to the stimulus (Morris, et al., 1986). The same point could be made about central cholinergic receptors (Brezenoff, et al., 1988), gamma aminobutyric acid (Sasaki, et al., 1990), or the variations of pH (Kraig, 1989).

II.c..4. The kidney.

The role of the kidney in BP regulation is certain, but its

participation in the establishment of essential hypertension remains controversial. As noted in previous paragraphs, sympatholytic therapies do prevent the development of hypertension in SH rats; thus the kidney alone, or its circulating factor, is not sufficient to induce hypertension. This apparently contradicts results of transplants experiments carried out by Rettig et al. (1990), where normotensive rats implanted with kidney from SH rats develop hypertension, and with the results of similar transplants carried out in humans (for medical reasons) (Merino et al., 1976; Guidi et al., 1982; Curtis et al., 1983; Green et al., 1984; Guidi et al., 1985; Jespersen et al., 1986; Legendre et al., 1989). Also, the kidney function improves if BP is regulated (Mandal, et al., 1989).

Whether or not the kidney participates in the initiation of hypertension, some abnormalities in hormonal secretion have been identified in this organ and the adrenal glands (Howe, et al., 1986, but see Melby, et al.,1987; Marche, et al., 1986; Purkerson, et al.,1986; Adler, et al., 1988; Rosenthal, et al., 1988). Also, the key functional elements of the kidney, the proximal convoluted tubules, show many disturbances: 1) an augmented concentration of alpha-2 receptors (unusual subtype) (Smyth, et al., 1986; Stanko and Smyth, 1991), 2) a hyperactivity of the Na^+/ H^+ antiport (which induces high internal calcium and explains some sodium abnormalities) (Aviv and Livne, 1988), 3) a diminished activity of Na/K/ATPase (Melzi, et al., 1989), and 4) a diminished response to angiotensin II, while the response to vasopressin is normal (Uderman and Rodin, 1988).

II.c.5. SH defective cell types other than VSMC, nerve or kidney cells.

A number of cells throughout the SH organism show various abnormalities: 1) red blood cells show diminished sodium and potassium fluxes (internal sodium is normal), membrane phosphatidyl-inositol turn-over and calcium binding (David-Dufilho, et al., 1986), and membrane fluidity (Tsuda, et al., 1990 c). They also lack adducin, a membrane skeleton protein binding calmodulin and protein kinase C in a calcium-dependent manner (Salardi, et al., 1989). Their volume is smaller, their count is higher, and they have less calcium binding sites (Cirillo and Laurenzi, 1988; Cirillo,1990); 2) platelets show an increased Na/K/ATPase activity (David-Dufilho, et al., 1986), an augmented sensitivity of phospho-lipase C and phosphatidyl-inositol turn-over (Koutouzov, et al., 1987, 1989), and an abnormal aggregation (Huzoor-Akbar, et al., 1989);. 3) skeletal muscle cells (augmented sodium activity) (Pickar, et al., 1989); 4) pineal gland cells (modified phospho-inositol metabolism, up-regulation of alpha-1 receptors) (Laitinen, et al., 1990); 5) monocytes (Kerenyi et al., 1990); and 6) renal and intestinal epithelial cells (diminished $Cl^-/HCO3^-$ exchange and calcium flux rate, possibly related to sodium impairment) (Drueke, et al., 1989 a, b, 1990; Lucas, et al., 1988, 1989). Also, there is a general absence of kallikrein binding protein in the brain, heart, thymus, lung and liver of SH rats (Chao and Chao, 1988), whose relationship to hypertension is unknown at present.

II.d. Hypertension therapy in SH rats.

As noted in the previous paragraph, the SH rat is a good model for human hypertension: the two show similarities in the development and establishment of hypertension, defaults present in the nervous system, vasculature and kidney function. In addition, the SH rats respond to most therapeutic measures in a manner similar to humans: BP is diminished by oral Captopril (angiotensin converting enzyme inhibitor) (Antonaccio, et al.,1979; Norman, et al., 1987), sympatholytics (see previous paragraphs), ganglion blockers (Howe, et al., 1986), Nifedipine (Cattaneo, et al., 1986), and potassium efflux stimulators (Giardino, et al., 1988).

III. Summary.

Genetic hypertension is a progressive disease which affects numerous organs and cell types. It is not known which of these modifications is at the origin of the disease. An animal model, the SH rat, is available. Results of the studies in human and in SH rats suggest that essential (or genetic) hypertension is not induced by de-regulations of a single component of BP regulation, but rather appears to derive from the malfunction of an element common to many cell types in different organs. Although some different pathologies could be grouped under the same label, it is difficult to imagine that these many modifications in the physiology of different cells would be accidentally concomitant. Alternatively, it is difficult

to imagine that a single pathology would result from such an extensive polygenic character that it would lead to so many variations. There is a definite need to unify these dysfunctions and identify a common denominator. Most researchers would agree to a definite link to calcium metabolism, which intervenes in so many metabolic processes that it alone could generate a multitude of cellular dysfunctions. Studies in the SH rat detailed, among others, vascular and sympathetic nerve cells dysfunctions. More specifically, the diminution of the silent period in nerve activity described by Schramm and Barton (1979) could be explained by the lack of accommodation to depolarising pulses identified in SCG main neurones by Yarowsky and Weinreich (1985). In situ, the neuro-vascular junction has been described as hyperactive or hyper-reactive in SH rats (Smith, et al., 1982). The modifications could either be at the level of the VSMC (hypersensitive to neurotransmitters, augmented contractility, hyperplasia), or the nerve cell (discharge of more neurotransmitter per action potential, augmented firing frequency, or variation of co-neurotransmitter).

All these results were obtained in situ and in young adult SH rats when the effects of high BP were already present and no ionic mechanism was proposed for these results, except for the participation of calcium in multiple firing.

IV. Rationale for the in vitro study.

If the cellular behaviours summarised in Section II.d. were to be correlated with the initiation of hypertension, it would be important to first determine if similar modifications were present before any significant rise in BP and independent of any potential circulating factors or cardio-vascular influences. If the modification(s) of the cell properties is genetically defined, it should be expressed in vitro. Cultures of dissociated nerve cells from neo-natal SH SCGs were used, and the results compared to SD and WKY SCG neurones cell culture. This excluded hormonal influences and consequences due to the heightened pressure in the cardio-vascular system, while giving easy access to the cells for electrophysiological studies. If a change in nerve cell electrophysiology could be identified, its ionic basis could be determined.

Also, the degree of interaction between VSMC and nerve cells had to be evaluated because of the known influences of target cells on neurone functions. Trophic factors produced by target cells (VSMC in this case) have been shown to modify the nature of the neurotransmitter used by the nerve cell (Patterson and Chun, 1977; O'Lague, et al., 1978 a, b; Furshpan, et al., 1986; Potter, et al., 1986; see also review by Potter, et al., 1983). Indeed, as was noted earlier, the results obtained with sympathetic denervation in SH rats showed the necessity to add anti-NGF antibodies to obtain a sympathectomy as effective as guanethidine alone in normotensive animals; this

suggested a different dependency/protection for some peripheral nerve cells offered by an NGF-like growth factor likely produced by the VSMC from hypertensive rats. The extent of target cell-nerve cell interactions in vitro appears to be related to the density of sympathetic innervation in situ (Chamley, et al., 1973; Todd, 1986), probably because of a trophic effect exerted by the degenerating nerve terminals of the explant (Southwell, et al., 1985). Thus, first passage VSMC were preferred to explants. Overall, these results suggested the possibility that there would be a difference between trophic factors produced by different target cells from vessels with different in situ innervations or between cells from normotensive and hypertensive origin. This provided the rationale to include VSMC from aorta (A, poorly innervated in situ) and mesentery (MA, highly innervated in situ) in co-culture with SCG neurones from SH, WKY, Wistar and SD origin.

The rationale for this study was, thus, that the same, discrete, genetic modifications were present either in VSMC or neurones, or both, which could explain the hyper-reactivity of the neuro-vascular junction. The genetic modification(s) could be observed and characterised in vitro and related to the initiation of the hypertensive state.

V. Characterisation of SCG neurones and VSMC in culture.

V.a. Cell culture: the technique.

Cell culture is one of many techniques used in in vitro studies. Different in vitro techniques (organ bath, nerve tissue slices, cell culture, etc.) demonstrate different properties of the tissues studied and/ or of the cells present in the tissue. The more removed the technique is from whole body experiments, the less immediate the connection between the properties identified and their impact on a physiological function. The obvious draw-back of the cell-culture technique is that the cells are extracted from their normal environment, removing the influence of various physical (cell to cell interactions, three dimensional constraints, stretch), electrical (cell to cell contact) as well as chemical (neurotransmission, hormonal and growth factors, interstitial medium composition) factors. Thus, all conclusions from in vitro experiments are subject to caution until the validity of the conclusion can be assessed at least at the level of the organ.

However, cell culture has been a tool used since the turn of the century (Harrison, 1907) which has been applied to numerous tissues from most animal models used in modern-day science. This tool is particularly used to identify individual cell properties and their sensitivity to extrinsic factors. The drawbacks of the technique are also its main advantages, which include the absence of interference from the organ or the whole body (no hormonal action, stretch

response, etc.), the accessibility of the cells (membrane and intracellular markers, biochemical studies, use of microelectrodes, etc.), the opportunity to mix cell types, the absence of diffusion barriers to compounds introduced in the medium, the analysis of the medium to assess cell production or utilisation of compounds and, finally, the visual assessment of cell morphology, growth rate and growth pattern, etc.

In situations where the in situ data is too intricate to allow the identification of the original cause of the dysfunction, in vitro experiments appear as the necessary first step. Such is the case for essential hypertension.

V.b. VSMC in culture: morphology and membrane electrical properties.

Some objections have been raised about cultured muscle cells obtained from blood vessel explants: they apparently lose their contractile properties (loss of myosin) and the nature of the cells is somewhat uncertain because, in the initial stage of the culture, VSMC look more like macrophages than mature VSMC (Chamley, et al., 1977; Chamley-Campbell, et al., 1979). In essence, some critics of the method state that VSMC in culture regress to a stem-cell- like, un-differentiated stage.

First, the lack of contractility has been shown to result not from a lack of myosin but from a change in isomeric form of myosin which prevents its normal function as well as its detection by regular myosin markers (Taylor and Stull, 1988; Seidel, et al., 1989).

Second, cultured smooth muscle cells have been shown to retain in vitro properties observed in vivo (Sinback and Shain, 1979; Haimovich, et al., 1989) as well as preserving in vitro the changes induced in situ, such as their growth rate (Mey, et al., 1980; Grunwald and Wischer, 1985; Clegg, et al., 1986; Rosen, et al., 1986; Paquet, et al., 1989), a lowered resting membrane potential (RMP) (Blennerhassett, et al., 1989), membrane receptor presence and affinity (Gunther, et al., 1982; Bulbring and Tomita, 1985), changes in metabolism (Nakamura, et al., 1989), changes in protein synthesis associated with age (of the animal) (Eggena, et al., 1988) or pathology (Yoneda, et al., 1981; Nara, et al., 1986). This shows that the cultured A VSMC are, at the least, a good reflection of their in situ counterpart. **Third**, cultured smooth muscle cells seem to retain mature characteristics (RMP, contractility) from the beginning when the cells are prevented from dividing, by initially maintaining cell contacts or when they reach the plateau phase (contact inhibition of cell division) (McLean and Sperelakis, 1977; Chamley, et al., 1977; Lowenstein, 1979; McLean, et al., 1979; Chamley-Campbell and Campbell, 1981; Majack, 1987; Blennerhassett, et al., 1989). Cultured A VSMC divide to form a monolayer, followed by a characteristic "hills and valleys" configuration (Chamley-Campbell et al., 1979, Blennerhassett et al., 1987, Majak, 1987); at that stage A VSMC appear as pleiomorphic or ribbon shaped with a dark, somewhat granular, cytoplasm with a RMP of approximately -40 mV. If transferred to new culture dishes (passage) and replated at lower

density, the cells divide again; this can be repeated indefinitely.

The presence of other cell types (such as endothelial cells or fibroblasts) can be easily avoided or detected: the endothelium and the intima can be easily removed (and were removed in our experiment) from the tunica media, and the striking morphology of endothelial cells (polygonal, large cells) allows one to confirm that the endothelium has been totally removed, and to discard the dishes in which it was not. Fibroblasts in culture are possibly more difficult to detect because their morphology is similar to immature VSMC (Chamley et al., 1977), but they show a characteristic growth pattern, noticeably different from the VSMC, which allow their identification in older cultures; furthermore, the presence of fibroblasts in A VSMC is unlikely because studies show that VSMC in the tunica media of arteries represent the only cell type present (see Blennerhassett et al, 1990, discussion).

V.c. SCG neurones in culture.

Overall, culture of adult neurones is difficult (but possible), whereas neo-natal neurones are easier to culture. This method has been used extensively with SCG neurones and produced a large body of information. The neurones are dependent on NGF for their survival in culture (Main and Patterson, 1973); during the first two weeks in culture the neurones produce an extensive network of neurites, their cell body goes from small, rounded, and refractile to large, flattened, ovoid , with a darkened cytoplasm, and clearly

shows 1 to 3 centered nucleoli in the nucleus (O'Lague, et al, 1978 a). Over time the neurite network becomes very elaborate and the cell bodies regroup in ganglia-like structures embeded in accessory cells, which will also eventually cover the neurites. This poses a general problem of accessibility of the cells for electrophysiological studies; moreover, non-neuronal cells are thought to be responsible for neuronal death in some cultures (Burry, 1983). Various methods are available to prevent the multiplication of non-neuronal (dividing) cells in neurones cultures: specifically designed serum-free media (Ahmad, et al., 1983) exposure to the antimitotic arabinoside C (Furlong and Gresham, 1971 [in Seil, et al., 1980]; Aguayo, et al., 1975; Messer, 1977; Burry, 1983; Oorshoot and Jones, 1986; Patel, et al., 1988). However, neurones cultured in serum-free medium can apparently not develop synapses (they do not show synaptic activity) or spontaneous activity (Ahmed, et al., 1983), and arabinoside C has been shown to be toxic for neurones (Patel, et al., 1988; Martin, et al., 1990). Another method to prevent non-neuronal cell division in culture is irradiation: previous studies showed that irradiation (5 Krads) of cultures of neo-natal (post-mitotic) SCG neurones effectively prevented cell division and did not modify the membrane electrical properties and firing activity of the neurones (Patterson and Chun, 1977, O'Lague et al., 1978).

Like cultured VSMC, some minor modifications of neurone physiology were demonstrated in culture. Regenerated nerve cells (which is by definition the state of **cultured** SCG neurones) have

been shown to possess a different content of peptides (neuro - transmitter or -modulator) (Adler and Black, 1984; Bohn, et al., 1984; Nishi and Willard, 1985; Willard and Nishi, 1985 a, b; Roach, et al., 1986; Freidin and Kessler, 1989) and some modified electrical properties (Kelly, et al., 1988). These modifications are thought to be brought about partly by factors intrinsic to the neurones, partly by the cessation of pre-synaptic activity: some of these changes (such as the increase in substance P) can be reversed by neuronal depolarisation (Black, et al., 1984; Kessler, et al., 1984; Freidin and Kessler, 1989).

V.d. Neurones-VSMC co-cultures.

Some of the most successful applications of cell culture include the study of nerve-target cell interactions. The use of cell culture allowed scientists to study the development of the synapse and the effects of the presence of the target cells on the neurone morphology and neurotransmitter status (see Wollenberg, 1985). When in co-culture, the nerve cells demonstrate a plasticity which was unsuspected before culture techniques were available. The neurotransmitter phenotype of embryonic neo-natal catechol-aminergic neurones can be regulated by their target cells (or medium conditioned by these cells) (Potter, et al., 1983, 1986; Patterson and Chun, 1977; Adler and Black, 1984; Konig, et al., 1987); the neurotransmitter phenotype and field arborisation are dependent on the pre-synaptic activity as well as the specific firing activity of the

neurone (Bunge, et al., 1974; Kessler, et al., 1983 a, b, 1984; Black, et al., 1984; Sretavan, et al., 1988) and possibly by nerve cell density (Adler and Black, 1985; but see Mains and Patterson, 1973 b, c). Membrane receptors are also regulated by target cells (Rotter and Frostholm, 1988; Insel, 1989) and NGF (Reed and England, 1986; Cooper, 1987). Neurones have been shown to develop functional synapses in vitro with target cells (Crain, 1970; Fischbach, 1972; Mark, et al., 1973; Bunge, et al., 1974; O'Lague, et al, 1974; Ko, et al., 1976; Furshpan, et al., 1986; Konig, et al., 1987; Vita, et al., 1988). Some target cells, though not all, provide cultured nerve cells with NGF (Chamley, et al., 1973, Jonhson, et al., 1972, Creedon and Tuttle, 1988 a, b) and innervation is suggested to be proportional to NGF content of the target tissue when the neurone is NGF dependent (Donohue, et al., 1989).

The technique of cell culture and co-culture of different cell types has been widely used over the years and proved to be extremely valuable in other contexts such as the studies of interactions between cardiac muscle cells/ nerve cells (see review by Wollenberger, 1985), spinal cord/ SCG neurones (Bunge, et al., 1974; Ko, et al., 1976), striated muscle cells/ neurones (Crain, 1970; Nurse, 1981 a, b; Haimovich, et al., 1986) and red nucleus/ substance nigra neurones (Konig, et al., 1987). The plasticity of the neurones is now accepted as a general feature in the development and, also, of the adult organism (Potter, et al., 1983; Adler and Black, 1984; Purves, et al., 1986).

SPECIFIC OBJECTIVES

The specific objectives of this study were the following:

*First, to develop a co-culture system allowing the study of vascular smooth muscle and nerve cells separately and in combination;

*Second, to characterise the cell types and their properties in such a system; to achieve that goal, the gross growth pattern and morphology of the cells were assessed, their membrane properties (active and passive) and their pharmalogical sensitivity were studied;

*Third, to identify differences between cells from hypertensive animals and from normotensive ones;

*Fourth, to identify the ionic basis underlying the difference in cell behaviour identified in the previous point;

*Fifth, to assess the effect of the presence of the other cell type on the different properties isolated in point three. For that part of the study VSMC from aorta (poorly innervated in situ) as well as Mesenteric Artery (highly innervated in situ) were included in neurones-VSMC co-cultures;

*Lastly, to suggest a possible cause of genetically determined hypertensive state.

MATERIAL AND METHODS

I. Animals and tissues.

I.a. Animals.

The animals used in these experiments were genetically hypertensive rats from the Wistar-Kyoto strain [the spontaneously hypertensive (SH) rats] and their genetic normotensive controls [the Wistar-Kyoto normotensive (WKY) rats] (both strains from SASCO Inc., Omaha, Nebraska, USA). To assess the validity of the observations made in regard to the hypertensive state, two more strains of rats, the Sprague-Dawley (SD) (BIO-LAB Corp., St. Paul, Minnesota, USA) and the Wistar (Animal Care Facility, McMaster University, Ontario, Canada) were used. The rats were bred in our own animal facility, and the progeny used as neo-natal pups (as a source of SCG neurones), or kept until they were young adults and used as a source of vascular tissue (aorta and mesenteric artery). (Note: all animals used in McMaster University, Ontario, Canada, came from the colony kept in our own animal husbandry).

I.b. Ganglia.

The pups were stunned by a blow on the head and decapitated

at the latest 48 hours after birth. The rest of the body was discarded and the SCG were isolated, removed immediately, cleaned of surrounding tissue and stored temporarily in working medium (see Section II.a). SCGs from all pups from one or more litter were pooled for cell extraction.

I.c. Blood vessels.

The young adult rats were stunned by a blow on the head and killed by cervical dislocation. A medial abdominal incision was immediately done to expose the abdominal cavity. The aorta and mesenteric artery were rapidly taken out, cleaned of surrounding tissues, split open longitudinally and the endothelium and intima removed by carefully rubbing the vessel between the two arms of a smooth end pair of dissecting forceps. The vessels from several animals were pooled and stored in working medium (see Section II.a) for each cell extraction.

II. Media.

The media used were of four kinds: working medium, dissociation medium, feeding medium and recording medium. They were prepared under sterile conditions and all reagents used were of analytical grade. The composition of each medium is described below.

II.a. Working medium.

The same medium was used for all cell type extraction procedures and consisted of Hank's balanced salt solution (HBSS) medium with 25 mM HEPES. HBSS (GIBCO, 310-4180AJ) had the following composition:

KCl	5.40	mM
KH2PO4	0.44	mM
NaCl	136.90	mM
Na2HPO4.7H2O	0.34	mM
D-Glucose	5.60	mM
Phenol red	0.01	g/l

II.b. Dissociation media.

Three different media were used: 1) a mix of collagenase (1 mg/ml) and elastase (0.025 mg/ml) in HBSS (same as above) complemented with $CaCl_2$ (250 uM) to dissociate cells from vascular tissue; 2) a solution of Trypsin (0.25%) in HBSS (same as above) to lift VSMC from the culture dish during the first (and only) passage; and 3) a mix of Trypsin (0.25%) and collagenase (1 mg/ml) in HBSS (same as above) to dissociate nerve cells from SCG.

II.c. Feeding medium.

Once plated, the cells were fed every three to four days with minimum essential medium (MEM, GIBCO 320-1095 AJ)

supplemented with 10% fetal calf serum (FCS, GIBCO), glucose (4.22 mM) and nerve growth factor (NGF, 7S, 100 ng/ml, SIGMA). MEM composition is shown below:

F15 (medium)	400	ml
NaHCO3 (7.5%)	12.5	ml
Antibiotic/anti-mycotic mix (100X)	5	ml
L-Glutamine (200 mM)	4	ml

II.d. Recording medium.

For the electrophysiological study, the culture medium was replaced with MEM (not supplemented with FCS or NGF) buffered with 25 mM HEPES, at pH 7.4 (GIBCO, 380-2370), with the concentration of calcium raised to 3 mM, to allow a more stable impalement of the cells (see Discussion).

III. Culture dish coatings.

Two types of coatings were used: collagen and poly-dl-ornithine. Rat tail collagen solution (Sigma, C 8897) was deposited in each dish (2 mls/dish) and then removed, leaving a thin film of collagen on the bottom. After drying overnight, the dishes were sterilised by exposure to U.V for an hour and stored in a sterile plastic sleeve until needed. The dishes were again exposed to U.V. for 20 minutes before plating the cells.

The poly-dl-ornithine (1 mg/ml, Sigma, P 8638) was dissolved in 0.15 M borate buffer (formula below), added in the dishes (2 mls/ dish) for 1 to 2 hours at room temperature (to allow polymerisation), then removed and the dishes rinsed with distilled water. After drying overnight, the dishes were exposed to U.V. for 30 minutes to be sterilised, and again for 20 minutes just before plating. Poly-dl-ornithine has been shown to be a better substrate for nerve cells than collagen (Saxod and Bizet, 1988).

Boric Acid	5.8	g/l
Sodium Borate	5.4	g/l

* dissolve in distilled water
and adjust pH to 8.6

IV. Cell extraction and culture.

IV.a. Vascular smooth muscle cells.

We used two methods to obtain smooth muscle cells from the vasculature: direct enzymatic dissociation (see Section II.b) or explant technique. The failure of the enzymatic technique to produce consistently viable cells for plating directed us to use the explant technique. Therefore, all our smooth muscle cells were obtained as follows: once cleaned and stripped from their endothelium, the vascular tissues were cut in small pieces (about 3 mm^3) and deposited directly on 60 mm sterile culture dishes (Falcon 3002). A

drop of feeding medium was then carefully deposited on each explant and 3 ml of the same medium added to each dish. The explants were fed every three to four days, after an initial period of one week during which the explants were left undisturbed. The explants were cultured (as all other cell preparations) in an incubator (Fisher Scientific, CO_2 Incubator 605) at 37°C under a humid 5% CO_2 atmosphere. After 21 to 28 days in culture, the smooth muscle cells had migrated out of the explants, divided and reached confluency. When the endothelium was not completely removed from the explant, epithelial cells would rapidly take over the dishes and could easily be recognised by their polygonal shape and their granular dark cytoplasm; such dishes were discarded.

Once the smooth muscle cells had reached confluency, they were lightly trypsinised for 15-30 minutes, the reaction stopped by the addition of 0.1 ml of FCS and the bottom of the culture dish gently scraped with a rubber policeman. The cell suspension was then gently triturated with a large bore pipette to break the clumps of cells. The explants were discarded and the suspension of cells was centrifuged at 1200 rpm using a table-top clinical centrifuge (International Equipment Co). The cell pellet was then resuspended gently in feeding medium and plated at the desired density on collagen-coated 35 mm culture dishes (Falcon, 3001). Only muscle cells obtained after the first passage were used in our experiments.

Part of the study was to find a way to obtain a stable culture that could be studied over time without changes related to time in

culture. Ultimately, this was obtained by irradiating the cells (see details in Results). Thus, all cells were irradiated three to five days after plating, using a Cobalt source (5 Krads), to prevent further cell division.

IV.b. Nerve cells from SCG.

Pooled SCGs were enzymatically digested for 25 minutes, then, after stopping the reaction with 0.1 ml FCS, were gently teased apart and triturated through a large bore pipette. The remaining large pieces were discarded and the dissociated nerve cells were collected by centrifugation at 1200 rpm using a table-top clinical centrifuge (International Equipment Co.). The cell pellet was gently resuspended in the feeding medium and plated on poly-dl-ornithine (SIGMA) coated 35 mm culture dishes (Falcon, 3001), at a plating density of 10^3 to 10^4 cells/dish, (single culture) or on top of a VSMC culture (co-culture). In all cases, the cells were irradiated three to five days after plating (Cobalt source, 5 Krads) to prevent further division of accessory cells. The feeding medium was replaced every three to four days. The cells were incubated in the same manner as previously described and studied two to five weeks after plating.

V. Electrophysiology.

On the day of the cellular recording, the feeding medium was replaced by the recording medium (described Section II.d). The culture was then placed at room temperature on the stage of an

inverted microscope (Nikon Diaphot-TMD, Japan) set on a vibration free table (TMC Micro-g, MA, USA).

Electrical recordings were obtained using glass micro-electrodes prepared from fiber-filled capillaries (1.2 mm o.d., borosilicate glass, Hilgenberg, FRG) pulled (needle/pipette puller, model 730, Kopf, California, USA) to produce microelectrodes with a 70 to 150 MΩ tip resistance when filled with 3 M KCl. The electrode was connected to the probe of a high input impedance electrometer (Model 707 microprobe system, WPI, Colorado, USA).

The passage of the current pulse through the electrode generates a voltage deflection due to the resistance of the tip of the electrode (tip potential); a Wheaston-bridge incorporated in the electrometer allows (through a variable resistance) to offset that voltage step (i.e. to compensate for the tip potential) so that the voltage trace remains flat during the passing of a current pulse: the electrode is then bridge-balanced; this was done in the medium and currents up to 1.5 nA could be injected without any notable deflection. The currents (0.1 to 1.5 nA) were generated through a stimulator (S-95 Tri-level stimulator, Medical System Corp., New York, USA). The culture dish was grounded via an Ag:AgCl half cell.

Through the use of micro-manipulators, the tip of the electrode is brought onto the surface of the cell membrane and tapped into the cell, and the membrane allowed to seal back onto the electrode tip. The voltage trace deflection noted then during the passage of current represents the voltage drop across the combined resistance of the

intra-cellular medium, the membrane, and the recording medium. The resistance of medium (cellular or recording) is negligible and the membrane resistance is considered solely responsible for the voltage deflection. One possible complication is the increase in tip resistance resulting from part of the membrane being torn and plugging the electrode tip; these fragments of membrane can be cleaned by a succession of hyperpolarising pulses; also, the trace was checked for tip potential upon withdrawal from the cell (voltage trace return to zero level, Figure 1-Out). Current and membrane potential traces were both displayed on a digital oscilloscope (Model 310, Nicolet, Wisconsin, USA) and stored on a video cassette (super-beta hi-fi, Sony) through a PCM2 A/D VCR adaptor (Medical Systems Corp., New York, USA). Pertinent traces were transferred on paper via computer interface (Henry programme, Nicolet, Wisconsin, USA) and a plotter (HP, Colour-pro).

Criteria for successful impalement included an initial sharp deflection of at least 30 mV for VSMC and 45 mV for nerve cells, that reached a steady state value within 30 seconds and showed no further shift over time. Successful impalements could be maintained for several minutes. Short duration hyperpolarising pulses (0.5 nA, 50-100 milliseconds) were used to measure membrane input resistance. For the nerve cells, long duration positive pulses (0.1 to 1 nA, 400 milliseconds) were used to elicit active membrane responses. A current-voltage plot (I-V plot) was also generated by injecting 100 milliseconds hyperpolarising and depolarising square current pulses

of varying intensities and recording the resulting changes in membrane electrotonic potentials. For post-threshold levels of current, the value of the plateau phase (steady state depolarisation) following the generation of action potential(s) was used to estimate the membrane resistance.

The variables measured included resting membrane potential (RMP) and membrane input resistance (MIR) for all cell types, plus maximum rates of depolarisation and repolarisation of the action potential (AP), as well as AP threshold (current and potential), maximum potential value and total amplitude. When present, multiple firing was evaluated by measuring the firing frequency during the first 100 msec and the last 300 msec of the stimulus. The presence of after-hyperpolarisation or -depolarisation was also noted (AHP or ADP respectively) and its amplitude and duration measured. The variables measured are illustrated in Figure 1, next page.

VI. Pharmacology.

The effects of different drugs on membrane potential, membrane input resistance, AP shape, and neurone firing frequency were studied by depositing drugs directly in the registration medium in the dish. In each dish, between 5 and 10 cells were impaled as controls prior to any drug addition. The compounds used were Apamin, Bay-K 8644, Norepinephrine, Tetraethylammonium chloride (TEA) and Tetrodotoxin (TTX), all from Sigma. All reagents used were of analytical grade.

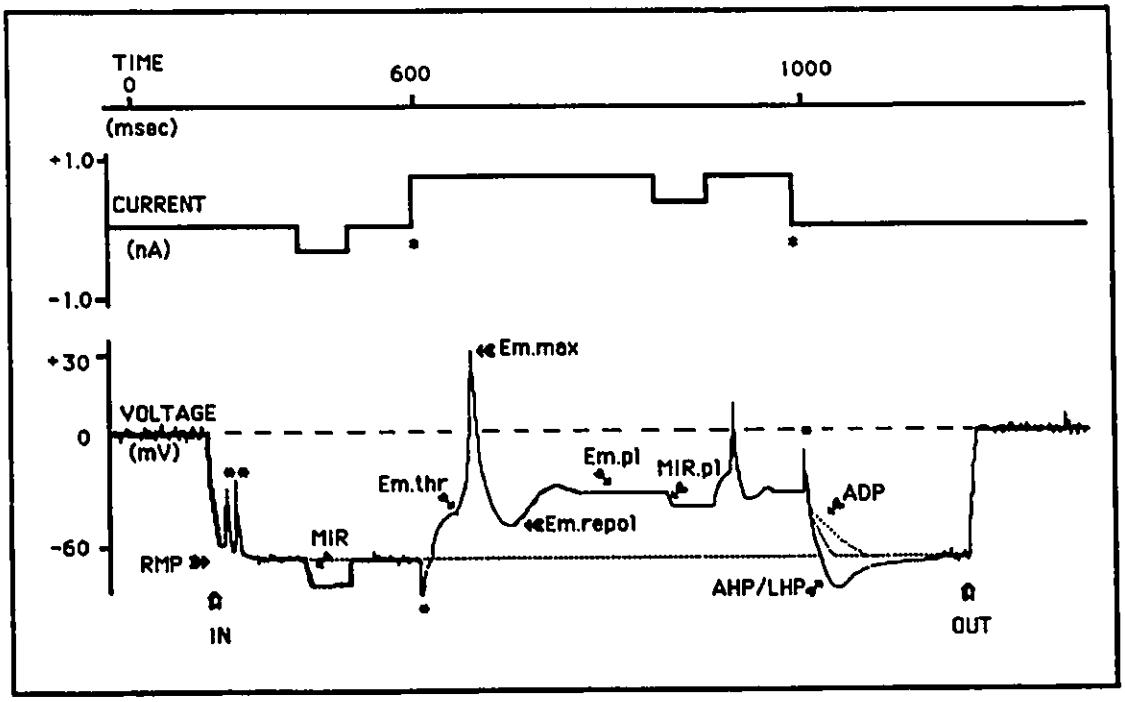


Figure 1: Variable measured during electrophysiological studies of neuronal membrane properties. RMP, resting membrane potential; MIR, membrane input resistance; $Em._{thr}$, membrane potential at the threshold of the spike; $Em._{max}$., membrane potential at the tip of the spike; $Em._{repol}$., lowest membrane potential level before the plateau phase; $Em._{pl}$, membrane potential during plateau phase; $MIR._{pl}$, membrane input resistance during plateau phase; ADP, after-depolarisation; AHP, after-hyperpolarisation; LHP, long after-hyperpolarisation. See text for calculation of these variables

VII. Electronmicroscopy.

A representative sample from each culture and co-culture set-up was selected to be processed for electronmicroscopy.

The cells were fixed in the culture dish and processed according to the technique used in Dr. Kannan's laboratory and described in Blennerhassett, et al. (1987).

VIII. Statistical analysis.

Values from all cells studied in identical conditions were pooled, averaged and compared by unpaired T-tests (significant at $p \leq 0.05$) using the computer programme Statistix. Results between co-cultures and single cultures were compared by Chi-square tests (significant at $p \leq 0.05$).

RESULTS

The first half of the thesis work was done at the Department of Neurosciences, McMaster University (Hamilton, Ontario, Canada), while the second half was done at the Department of Veterinary Biology, University of Minnesota (St. Paul, Minnesota, USA). Two completely different set-ups were used. Several factors contributed to some variations in RMP and MIR of the cells and the magnitude of some cellular activities: use of a vibration-free table available in St. Paul but not in Hamilton, better data storage in St. Paul allowing for analysis of more variables, and the experimentalist's increased ability over time. However, it was noted in all instances that the conclusions drawn in both places were similar. Thus the two sets of data were pooled when pertaining to the same part of the study.

It was known that neurones and cardiac cells could be irradiated in vitro without modifying their membrane properties or maturation process (Patterson and Chun, 1977, O'Lague et al., 1978), but nothing was known of the effects of irradiation on cultured VSMC. This part of the study, therefore, described mainly the effect of irradiation on VSMC cultures. In this section, the results obtained with non-irradiated cells are briefly described, followed by the description of the protocol developed to obtain stable cultures and its

effect on membrane morphology and membrane electrical properties.

Briefly, the results of this study can be summarised and will be presented as follow:

1) The morphology and membrane properties of SCG neurones and VSMC in irradiated cultures are similar to the morphology and membrane properties of these cells in non-irradiated cultures as described in the literature; and their membrane properties are similar to in situ. The model is valid to study the interaction of the two cell types in vitro.

2) When compared to neurones from normotensive strains, SCG neurones from genetically hypertensive (SH) rat show little difference in their passive or active membrane electrical properties. However, these neurones show a lack of accommodation to long-duration depolarising pulse (i.e. multiple firing of action potential) as opposed to SCG neurones from normotensive rats. Multiple firing can be inhibited by calcium antagonist (cobalt) and induced by calcium-activated potassium conductance blocker (apamin or tetraethylammonium).

3) The presence of vascular target cells in co-culture modifies some passive and active membrane electrical properties of the neurones: a) A VSMC specifically prevent SCG neurones from firing; b) MA VSMC from normotensive strains do not modify SCG neurones firing abilities; c) SH MA VSMC shift SH SCG neurones firing frequency distribution towards lower values; d) VSMC in culture appear to demonstrate a combination of general (VSMC), specific (A

vs MA), and strain related effects on neuronal membrane properties; and e) these results appear to define two sub-populations of multiple firing neurones in SCG: one (10-20% of all neurones) present in all strains and the other (40-50% of all neurones) specifically related to genetic hypertension.

I. Model: establishment and characterisation of VSMC and SCG neurones used in co-culture experiments.

I.a. Non-irradiated cell cultures.

I.a.1. Cultured vascular smooth muscle cells.

I.a.1.1. Identity and growth pattern.

When cells migrated out of an explant from rat aorta (A), they divided and grew to form a monolayer until they reached confluency (Fig. 2); they kept dividing further with the typical growth pattern known as "hills and valleys" (Fig. 3). At that time, most of A VSMC appeared as pleiomorphic or ribbon shaped cells with a dark, somewhat granular, cytoplasm with an average RMP of -40 mV (see Section I.a.1.2). In long-term experiments, overgrown cultures of A VSMC tended to generate characteristic arcs in the periphery of the dish (slow, low intensity contractile activity), then roll upon themselves and lift from the bottom of the dish. First passage A VSMC (only cells used in our study), showed characteristics (cell morphology, culture appearance and RMP) similar to explanted cells.

The cells explanted from mesenteric arteries (MA) showed a different growth pattern from A VSMC in that they never formed a

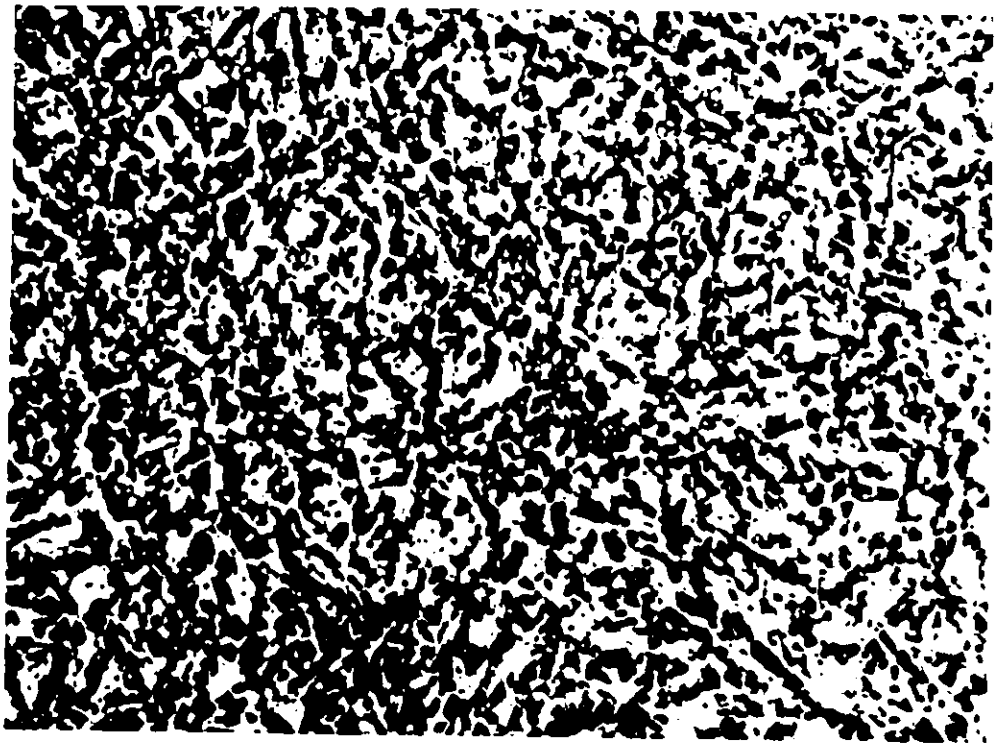


Figure 2: Aortic VSMC in culture (9 days), the monolayer. Magnification: X 340. See text for details.

Figure 3: Aortic VSMC in culture (3 wks), the "hills and valleys" configuration. Magnification: X 544 See text for details.

Figure 4: Mesenteric WKY VSMC in culture. Explanted cells (2 wks). Magnification: X 340. See text for details.

Figure 5. Mesenteric artery WKY VSMC in culture. First passage (5 days). Magnification: X 340. See text for details.

monolayer, but grouped themselves in hills separated by large spaces as soon as they exited the explant (Fig. 4). First passage MA VSMC settled themselves in the same manner (Fig. 5). Most cells in MA VSMC cultures showed a morphology identical to cultured A VSMC (compare Fig. 2, 3, 4, and 5).

As a general rule, cells cultured from explants and cells from the first passage showed identical characteristics (morphology, growth pattern). In both A and MA VSMC cultures, contamination by endothelial cells was easily identified and the contaminated dishes discarded. The presence of other cell types (such as fibroblasts) was not as easily detected because their morphology is similar to immature VSMC (Chamley et al., 1977). No effort was made to identify and/or eliminate these cells.

At least two culture plates from each set-up were treated for electronmicroscopy. The two sample electronmicrographs in Figure 6 and 7 show vascular smooth muscle cells, with their contractile filaments, healthy mitochondria and well developped endoplasmic reticulum (active synthesis).

I.a.1.2. VSMC passive membrane electrical properties.

Because VSMC were to be used only as target cells to evaluate possible interactions with nerve cells, the pilot studies did not include all strains in all cases. Table 1 summarises the membrane properties of A and MA VSMCs from WKY and SH rats in non-irradiated cultures. The cells showed RMP in the range of -33 to -52 mV. The average MIR was between 5 and 53 MΩ, and the cells very

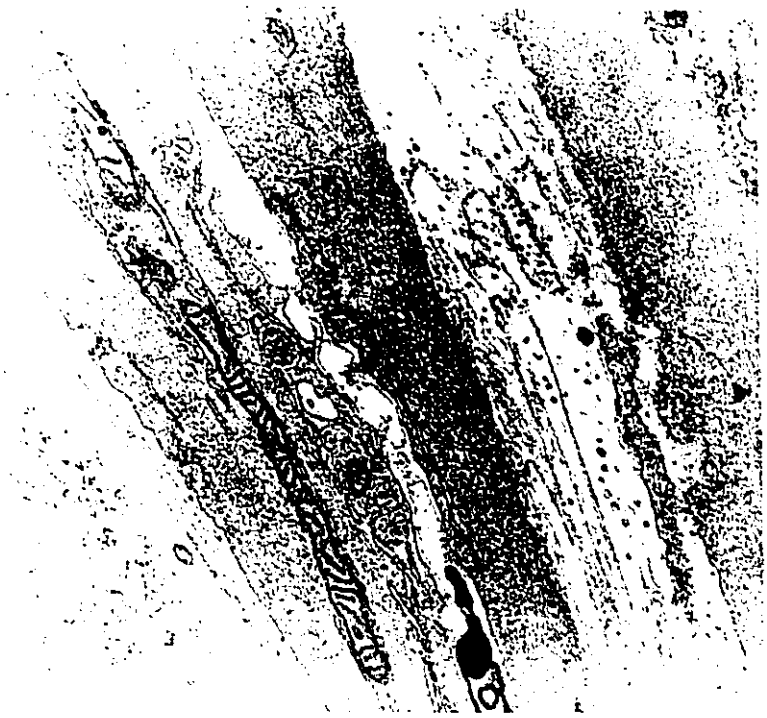


Figure 6. Irradiated WKY A VSMC.-SCG neurones co-culture. Micrograph shows profile of VSMC with mitochondria and contractile filaments in cytoplasm. Magnification: X 12180

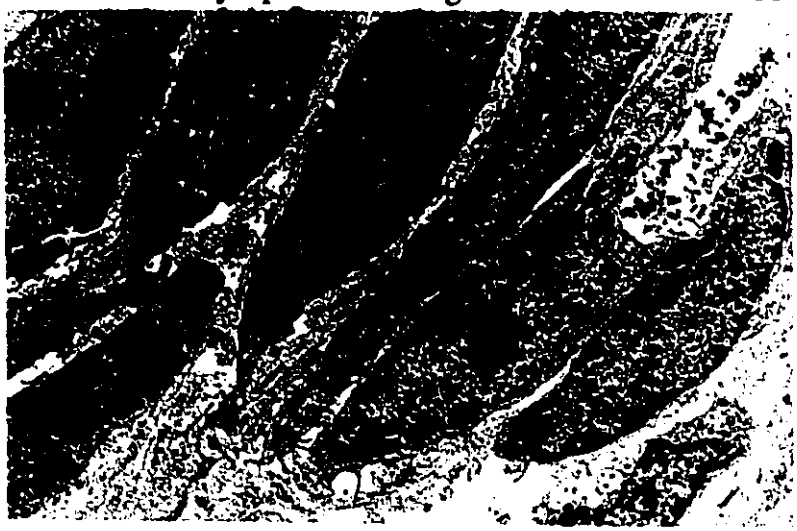


Figure 7. Irradiated WKY MA VSMC-SCG neurones co-culture. Micrograph shows profiles of VSMC with numerous mitochondria and contractile filaments and extensive endoplasmic reticulum. Magnification: X 12180.

TABLE 1 Membrane electrical passive properties of WKY, SD, and SH superior cervical ganglia neurones in co-cultures with their respective A and MA vascular smooth muscle cells.

VSMC	n	RMP,mV	MIR,MΩ
Explant A WKY	30	-34.6±1.3	22.6±3.3
First passage A WKY	12	-34.9 ±1.4	52.9±9.7
Explant MA WKY	20	-45.3 ±4.9	4.9±1.0
First passage MA WKY	12	-52.1 ±2.0	---
Co-culture A WKY-WKY	52	-28.5±2.5	13.3±3.0
First passage A SH	11	-33.7±1.7	15.3±4.5
Co-culture A SH-SH	11	-41.9±1.4	7.2±1.1

Values are given as mean±SE. VSMC: vascular smooth muscle cells; A WKY: aortic VSMC from WKY rats; MA WKY: mesenteric artery VSMC from WKY rats; A SH: aortic VSMC from SH rats; WKY-WKY or SH-SH: in co-cultures, the first term refers to nature of VSMC, the second to neurones; RMP: resting membrane potential; MIR: membrane input resistance. See text for further details.

seldom contracted upon injection of depolarising square pulses of current through the recording electrode. When contraction took place, it was only noticeable by a "tightening" of the cell (observed under high magnification (x540) in phase contrast) and the expulsion of the micro-electrode from the cell. A previous study in our laboratory (Blennerhassett et al, 1989) showed that RMP of cultured VSMC varies with time in culture; at the time, a direct relationship between RMP and cell density was inferred from the data, but not firmly established.

I.a.2. Cultured neurones from neo-natal rat SCG.

I.a.2.1. Cell morphology and growth pattern.

Neuronal membrane properties were studied two weeks (or more) after plating. During the first two weeks, the cell body grew and the cells produced an extensive network of neurites (Fig. 8). Under phase contrast microscopy, the cell body went from small, rounded, and very refractile to large, somewhat flattened, ovoid in shape, and with a darkened cytoplasm. A typical mature nerve cell body in culture also showed one to three nucleoli in the nucleus (Fig. 9). Later, the neuritic network became very elaborate and cell bodies grouped in ganglia-like structures (Fig. 10) encased in accessory cells, which would eventually cover the neurites. In some of the older cultures, accessory cells formed a monolayer which eventually lifted from the bottom of the dish, carrying the nerve cells with them.

I.a.2.2. Membrane electrical properties of cultured neurones. When square pulses of currents were injected above a certain threshold, the neurones were able to generate overshooting APs. The occurrence of spontaneous activity was relatively rare and consisted of random RMP oscillations giving rise to isolated APs when the threshold was reached. Other studies previously showed that the irradiation procedure did not modify the membrane electrical properties and firing activity of the neurones (O'Lague et al., 1978) so the details of these properties will be presented in section II.

I.a.3. Co-cultures of SCG neurones and A or MA VSMC.

I.a.3.1. Cell morphology and growth pattern.

When added to a culture of VSMC, the neurones settled down at random and developed an extensive network of neurites. The neuronal maturation process, neurite outgrowth and the regrouping in ganglia-like structures were not noticeably modified by the presence of VSMC, neither were the morphology and growth pattern of VSMC affected by the presence of neurones (Fig. 11).

I.a.3.2. Membrane electrical properties.

The membrane electrical passive properties of VSMC (RMP, MIR) were not modified by the presence of neurones (Table 1, p 56). However, neurones cultured with A or MA VSMC showed different responses to depolarising pulses: their RMP appeared to be lower (depolarised) and they seemed to be prevented from firing APs when cultured with A VSMC. This effect of vascular target cells was

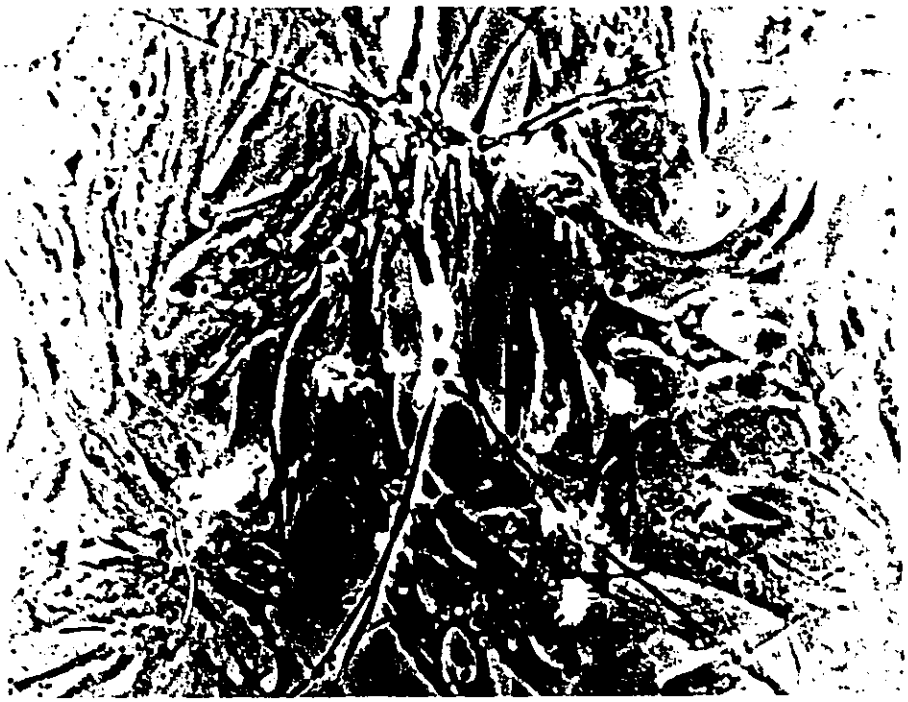


Figure 8. One week old culture of WKY SCG neurones. Magnification: X 340. See text for details.

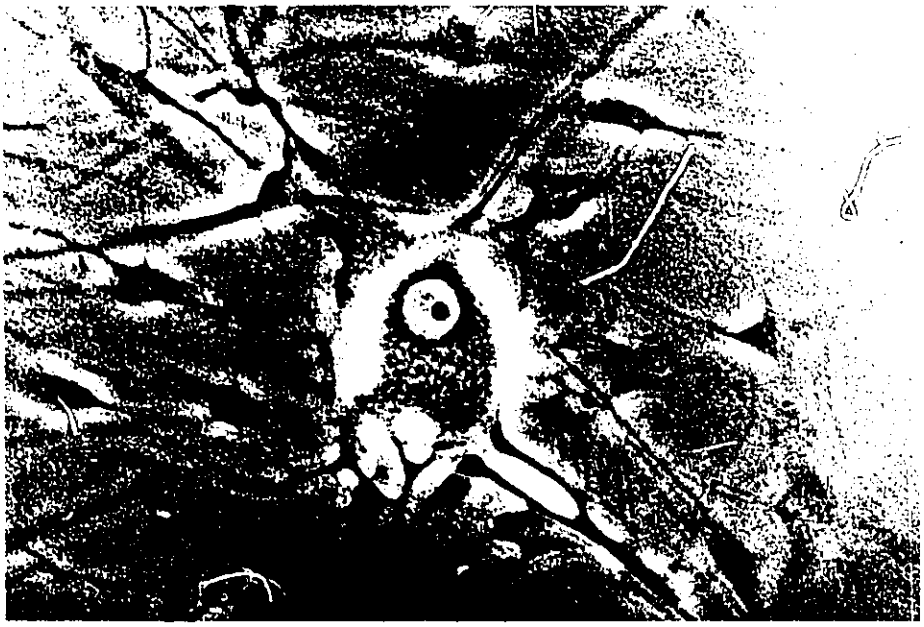


Figure 9. Four week old culture of WKY SCG neurones. Magnification: X 544. Note the difference in size (even with different mag.) and the clear nucleolus, when compared to Figure 8.

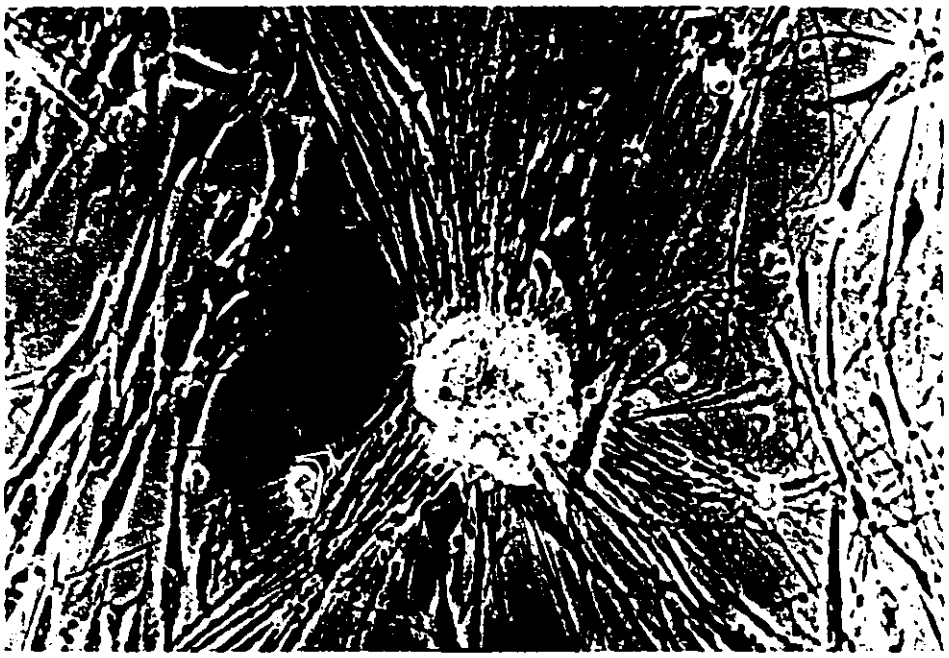


Figure 10. Rat SCG neurones in culture (3-4 wks); ganglion-like grouping. Magnification: X 340. See text for details.

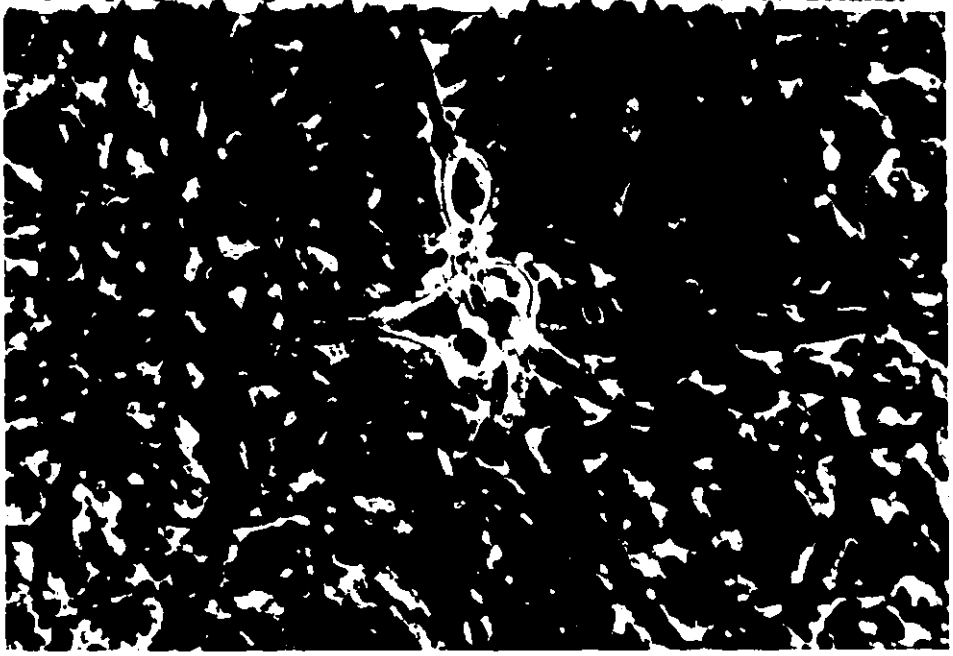


Figure 11. Rat SCG neurones and A VSMC co-culture (10 days). Magnification: X 340. See text for details.

noticed in non-irradiated cultures but was only studied in a systematic way in irradiated co-culture (see Section IV).

I.b. Establishment of stable cultures: irradiated cells.

The results in the previous section have shown that some vascular and nerve cells characteristics vary with time in culture (maturation) and that active division of vascular and ganglia accessory cells prevents reliable long-term studies. To study membrane electrical properties of VSMC and SCG neurones as well as the nerve-target cell interaction(s) in vitro, it was necessary to work with cells showing known stable characteristics over a long enough period of time; this would allow potential modification(s) of one cell type by the other to occur and be quantified. Since no previous study using non-dividing irradiated VSMC was available, the protocol had to be developed.

I.b.1. Irradiated single cultures of VSMC.

I.b.1.1. Morphology and growth pattern.

Figure 12 shows the effect of different doses of irradiation (Krads) on cell density (cell division and survival rate) of VSMC in first passage cultures. In the range tested (5.0, 5.2, 5.4, 5.7 and 6.0 Krads), the dose of radiation did not noticeably affect the cell morphology (compare Fig. 3 and 5 with Fig. 13 a and b).

Cell division was interrupted by all irradiation doses without altering short-term survival rates; cell density was nearly constant in each dish after irradiation. The constant density could have resulted

from a combination of cell division and cell death; if so, the two events would happen at the same (constant density) low rate, as typical cell phenotypes characteristic of cell division (pairs of rounded cells) were very seldom observed. In any event, VSMC stopped actively dividing and did not overtake the culture dishes. Irradiated VSMC survived for 6 to 7 weeks in culture and then died (Fig. 12 & 14 a(#)). There are a few possible explanations for that survival time and the suddenness of cell death (see discussion). Because 5 Krads was the dose previously used for neo-natal SCG neurones in culture (Patterson and Chun, 1977; O'Lague et al., 1978) and there was no added benefit with larger doses (if anything, it could raise the risk for cell damage), this dose was chosen as the single irradiation step for all cultures.

I.b.1.2. Membrane electrical properties.

Figure 14 shows the effect of time on A VSMC RMP and density after irradiation at day 3-5 in culture. In fig. 14 a, five different radiation doses were tested with no noticeable difference in their effects, and RMP stayed constant over time. Figure 14 b shows that the cell density of cultures irradiated stayed constant over time (as in fig.12, other experiement); two different densities (high and low) and different irradiation schedules (1, 2, 3, & 5 days after plating), were used to show a positive correlation with time in culture before irradiation and cell density: the longer we waited to irradiate the cells, the larger the density. To determine if time in culture (cell maturation in vitro) or cell density (cell division) was responsible for

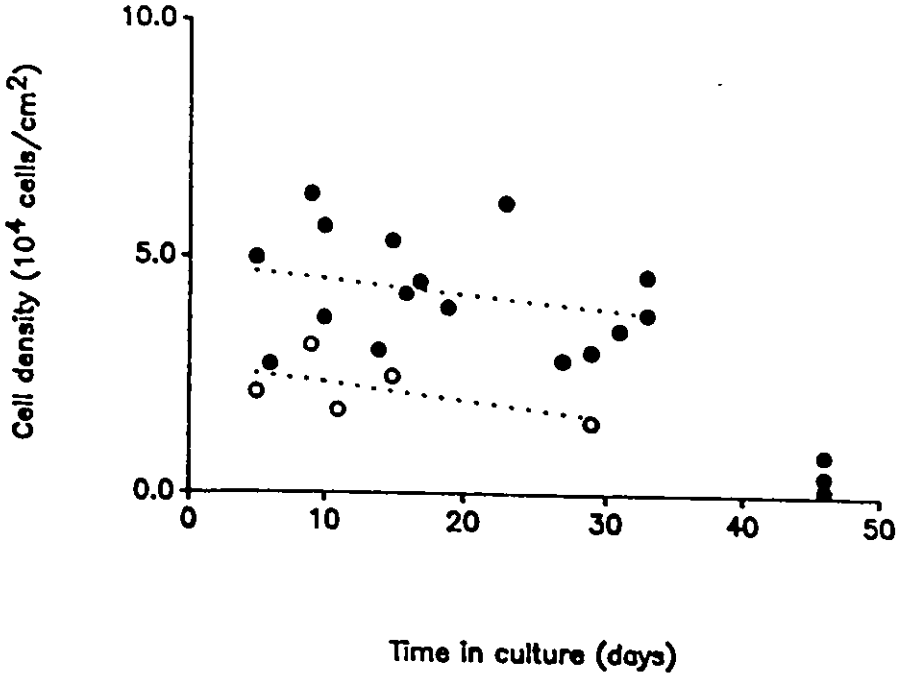
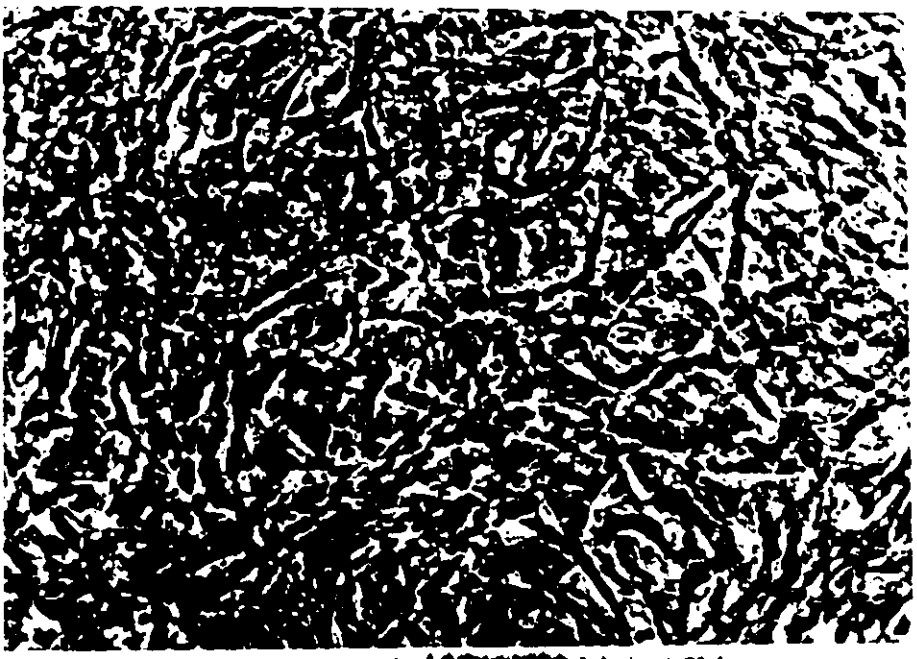


Figure 12. Cell density versus time in culture (A VSMC). Two different experiments with different initial plating densities ($25x10^4$ and $10x10^4$ /cm^2 for filled and clear circles respectively). Cell density stays relatively constant over the course of the first 5 weeks; massive cell loss occurs around day 47 (sudden drop in cell density).

A-



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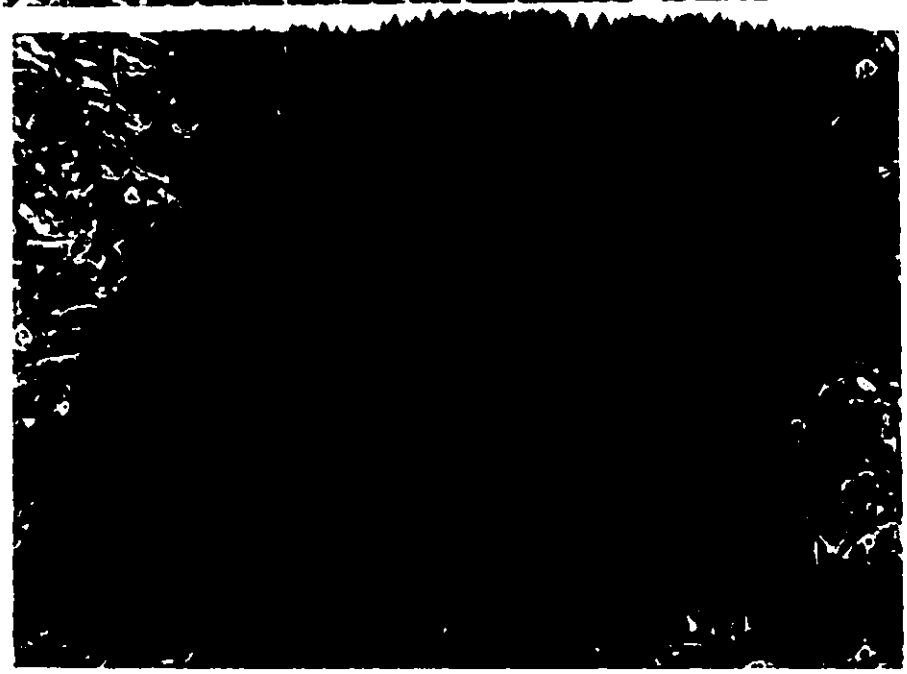


Figure 13. Irradiated 2-3 week old SH cultures (A-, A VSMC, B-, MA VSMC). Note that the gross morphology and tissue specific organisation of the cells within the culture is similar to non-irradiated cells. Magnification: X 340. See text for details.

the change in RMP, we studied sister cultures plated with different initial plating densities irradiated 3 days after plating. Figure 14-a shows that RMP is stable over time in culture for a given density. This experiment also shows a direct correlation between the density at the time of the experiment and the RMP: the higher the density, the larger the RMP (Fig. 14-c). Figure 4-d shows a transformation of the results using a correlation/regression test adjusted for the effect of time in culture (i.e. substracting the variations brought about by time- see fig. 14 a- from the general variations of RMP related to cell density); densities have been regrouped in 3 classes (0-2, 2-4, ≥ 4 $x10^4$ cells/cm^2); this test shows a good correlation between RMP and cell density in irradiated VSMC cultures. Similar results were obtained with cells obtained directly from explant, before the first passage. Even though it may appear that RMP of VSMC depended on time in culture, this study showed that density alone determines the RMP. To summarise, the RMP of irradiated VSMC was directly correlated to the cell density at the time of irradiation, irrespective of the initial plating density, and stayed constant over time afterwards. The RMP was also similar to that from non-irradiated cells with similar densities.

The cells did not appear to be damaged, other than for their ability to divide, since they kept similar RMP and MIR for approximately 42 days (only healthy cells can maintain their RMP; damaged cells will "leak"). To further verify that VSMC were not damaged, the effect of a vascular neurotransmitter was tested

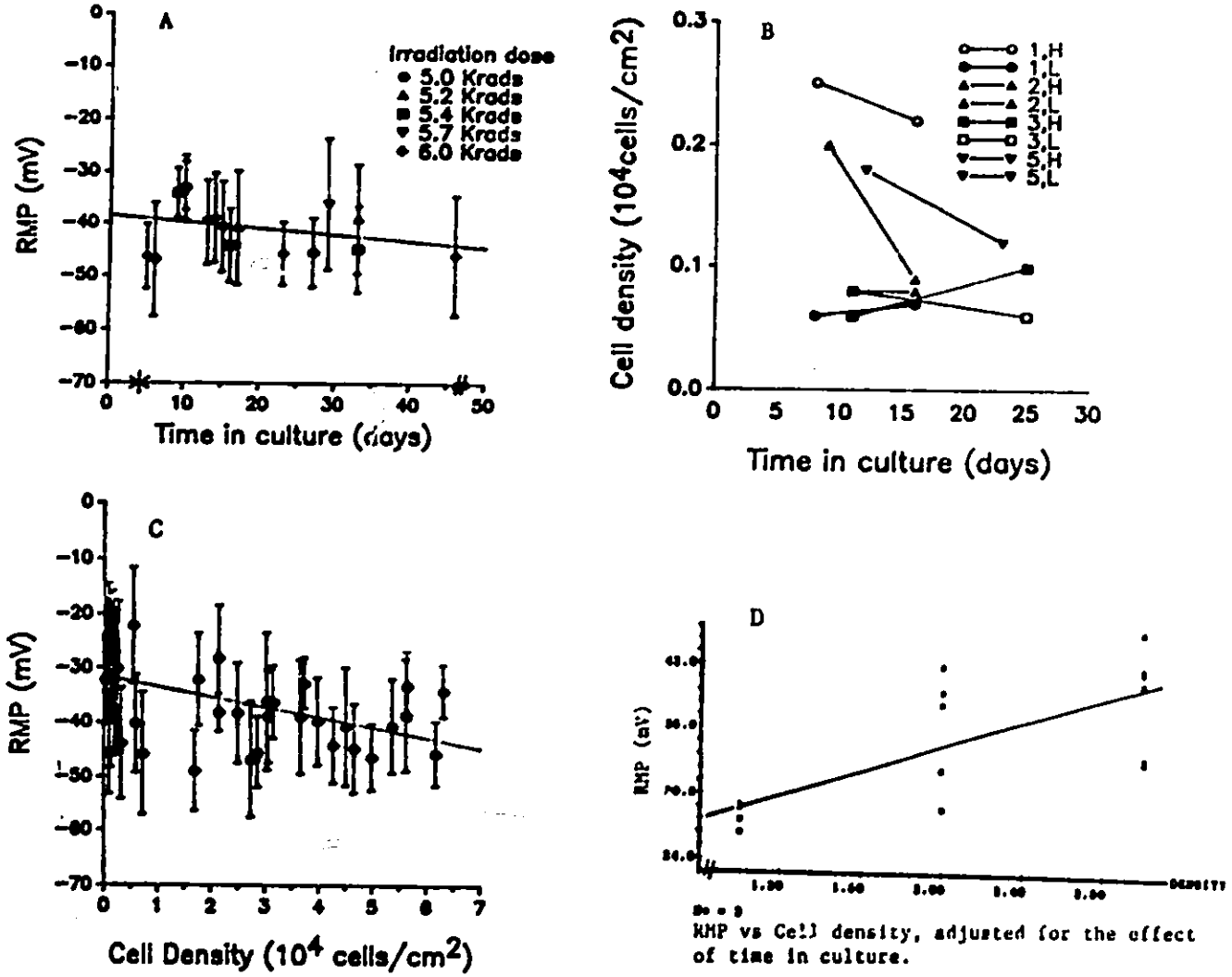


Fig. 14. Relationship between irradiation dose, RMP and time in culture (panel A), day of irradiation after plating, cell density and time in culture (panel B), and cell density and RMP (panel C and D). See text for abbreviations.

(norepinephrine, NE) (Figure 15). NE is known to induce VSMC depolarisation in arterioles (Hirst, 1977; Hirst and Edwards, 1989; see review by Bulbring and Tomita, 1987). NE was introduced directly in the medium and induced a progressive depolarisation (diminution of the membrane potential from RMP values towards 0 mV) at low concentrations (10^{-10} to 10^{-5} M) followed by a hyperpolarisation with higher concentrations (10^{-4} to 10^{-2} M). The depolarisation was reversible over time, with removal of NE (wash-out), and did not appear to show any desensitisation (same effect with a repeated dose of NE). NE also diminished the MIR of the A VSMC, which did not completely recover after wash-out. Similar doses of NE had approximately similar effects on A and MA VSMC, whether from WKY or SH, but the pilot study was not extensive enough to allow further conclusions. Figure 15 shows typical traces. As for the direct injection of depolarising current, neither an action potential nor the concomitant cell contraction were ever observed. Nevertheless, it showed that these cells were able to respond to a neurotransmitter in vitro.

A short study was conducted to see if these cells showed any sensitivity to traces of ascorbic acid, a necessary adjunct to protect NE from degradation. This was necessary not only as a control in itself, but also because ascorbate has been shown to induce membrane depolarisation and contraction of the smooth muscle cells from the toad stomach (Yamaguchi, et al., 1988). Results showed that this compound has no effect on the RMP of cultured VSMC (not

illustrated).

I.b.2. Irradiated single cultures of SCG neurones.

I.b.2.1. Cell morphology and growth pattern.

Irradiation (5 Krads) did not modify the neurone cell body shape, maturation or outgrowth of neurites. Division of accessory cells was effectively prevented by the irradiation procedures. The neurones showed the same propensity to regroup into ganglia-like structures over time. Figure 16 shows an electronmicrograph of an irradiated culture of SH neurones; healthy nerve cell bodies and neurites bundles with synaptic vesicles can be readily identified Ganglionic accessory cells did not noticeably divide.

A minor part of the study tested the survival rate of neurones when NGF was omitted from the medium to investigate a potential difference in NGF dependency between WKY or SD and SH neurones. None of the neurones survived more than a few days in culture.

I.b.2.2. Membrane electrical properties

Membrane properties of neurones are known not to be modified by irradiation. (See Section III for detailed comparative study of these variables.)

I.b.3. Irradiated VSMC and SCG neurones co-cultures.

Ib.3.1. Cell morphology and growth pattern.

In co-cultures, VSMC division was still prevented by the irradiation procedure and their gross morphology unchanged. The neurones showed the same propensity to grow, mature, extend neurites, and regroup into ganglia-like structures. The SCG accessory

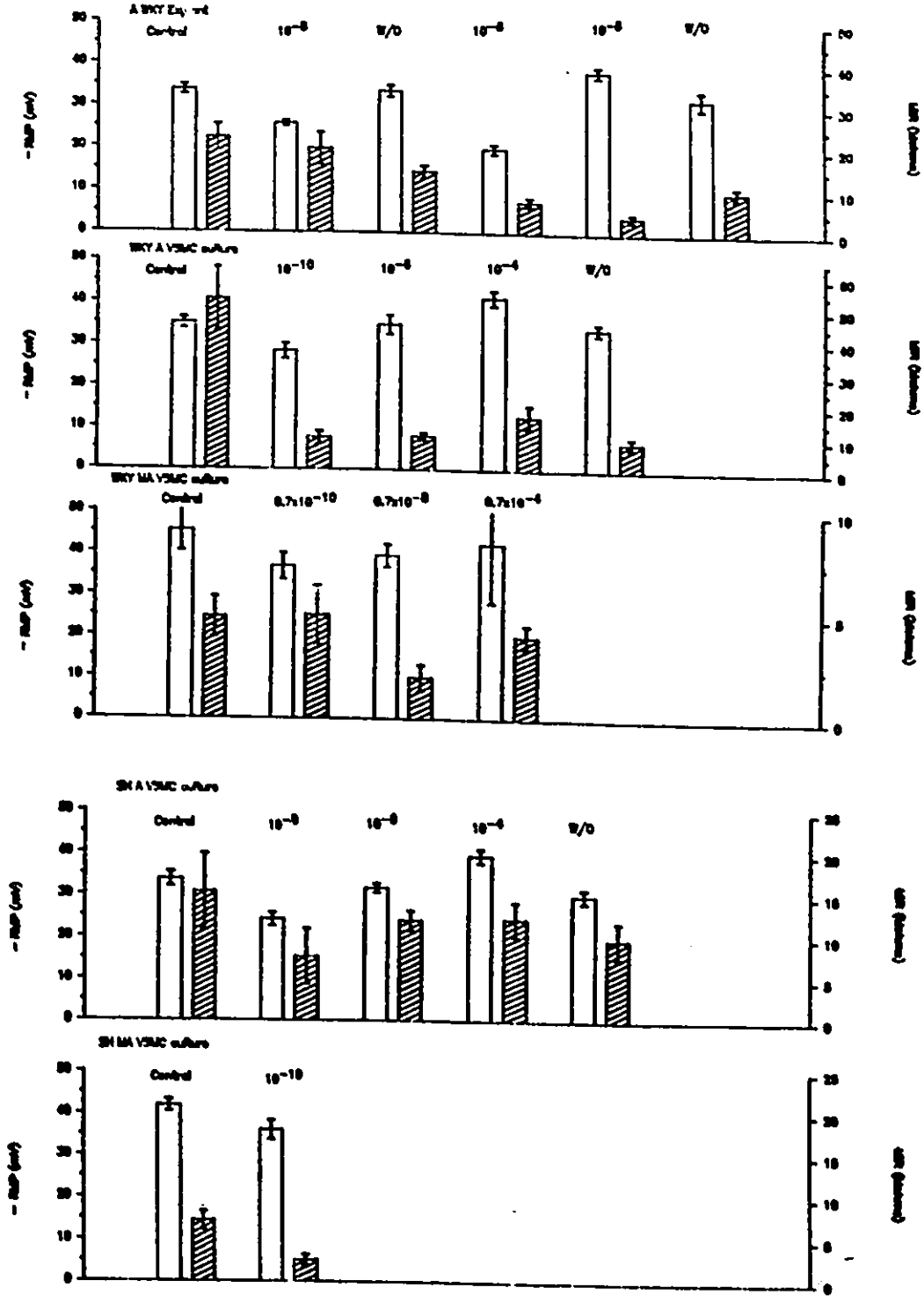


Fig. 15 Effect of norepinephrine on RMP (clear column, left scale) and MIR (hatched column, right scale). Number on top of columns represent NE doses (M). W/O: wash-out of NE. VSMC are from the source specified on top left corner of each panel.

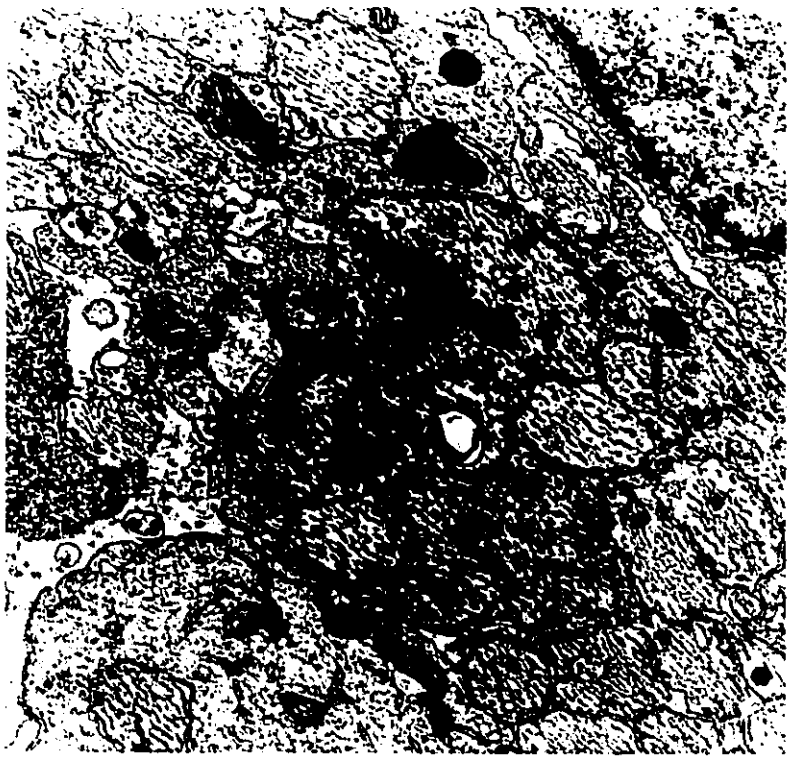


Figure 16: Electronmicrograph of irradiated SD MA VSMC-WKY SCG neurones co-culture. Note the numerous axon bundles, some of which show some synaptic vesicles. This area is adjacent to an area containing VSMC. Magnification: X 19500.

cell division was still effectively prevented. The electronmicrograph of a VSMC-neurones co-culture (Fig. 17) shows a typical neurites bundle, containing synaptic vesicles, in close contact to a VSMC (contractile filaments, mitochondria...).

I.b.3.2. Cell membrane electrical properties.

Irradiation of co-culture did not modify the VSMC membrane electrical passive properties (see section II); it did not change the RMP of the neurones nor did it prevent their ability to fire APs. Also, the inhibition of firing in co-cultures of neurones and A VSMCwas confirmed (see Section IV for a detailed comparative study of these variables).

I.c. Model summary.

In brief, the irradiation procedure on VSMC or SCG neurones efficiently prevented division of all normally dividing cells, without modifying VSMC or neurones respective morphology or membrane electrical properties. It also did not interfere with the outgrowth of neurite from SCG neurones and apparently preserved VSMC-SCG neurones relationship in co-cultures. The system was stable over time (at least 6 weeks), with VSMC membrane potential stable and proportional to cell density.

II Genetic hypertension: comparison of membrane electrical properties of A- and MA-VSMC from SD, WKY and SH rats in irradiated single or co-cultures.

The values of RMP and MIR from irradiated VSMC in co-

Figure 17. Electronmicrograph of an irradiated WKY MA VSMC-SCG neurones co-culture (same as fig.7). The nucleus of a neurone cell body (lower left corner) and bundles of axon processes can be noted. The tangential section through the axon bundles reveals synaptic vesicles, mainly of the large granular type. Magnification: X 19500.

cultures are similar (compare values in Figure 14, p. 64, and 15, p. 66 (control values) to the values in Table 1, p. 55). No significant differences were observed among the membrane properties of VSMC from WKY or SH, nor between VSMC in single culture and VSMC in co-culture. This was true overall of non-irradiated versus cells irradiated cells and of explanted cells, first passage cells, and A VSMC subjected to different doses of irradiation. The large variation of MIR values was also observed in another study from our laboratory (Blennerhassett, et al., 1989); low MIR was not related to cell damage (electrode or irradiation) since there was no correlation between RMP and MIR. Noted also was that the mean RMP of SH and WKY VSMC was very similar to the mean RMP of SD VSMC described in that same study. VSMC in co-culture were sensitive to NE as they were in single cultures (Fig. 15). In summary, the presence of nerve cells in the irradiated culture did not influence the membrane properties of A or MA VSMC

III. Genetic hypertension: comparison of membrane properties of neonatal SCG neurones from SD, Wistar, WKY and SH rats in single irradiated cultures.

III.a. Membrane passive electrical properties.

The criteria used to include a neurone in the study were a RMP between -50 and -90 mV, an MIR of 50 MΩ or greater, and the absence of an anode-break response to low intensity hyperpolarising

pulses. Wistar neurones membrane electrical properties were not calculated; only their RMP and firing frequencies were noted.

The presence of an anode-break response under these conditions was often associated with cell damage or incomplete seal in our preparation. In few cases where cell injury was accidental, one could observe a progressive cell depolarisation associated first with the appearance of an anode- break response, and then with the disappearance of all spike activity. Figure 18-a illustrates the reverse situation, where a cell sealed around the electrode. During the formation of the seal, while the RMP was low, an anode-break spike could be readily elicited by the injection of a low intensity hyperpolarising current pulse. However, after completion of the seal, shown by a lower (more negative) RMP, the same stimulus (same intensity) could not elicit the anode-break spike any more. The anode break response was also usually present in cells with a low (-55 mV or less) RMP and could be generated in some healthy cells only with larger (>0.8 nA) currents. When present, the anode-break response mirrored the cell response to depolarising current injections, i.e., consisted of a single spike in non-multiple firing cells and 2-3 spikes in multiple-firing ones. In contrast, an AP could always be generated at the end of a hyperpolarising current pulse during the plateau phase (steady-state) generated during a long-duration (400 msec,) depolarising pulse (Fig. 18-b).

The RMPs of cultured SCG neurones were in the range of -50 to -90 mV; their MIR, represented by the ratio of the voltage response

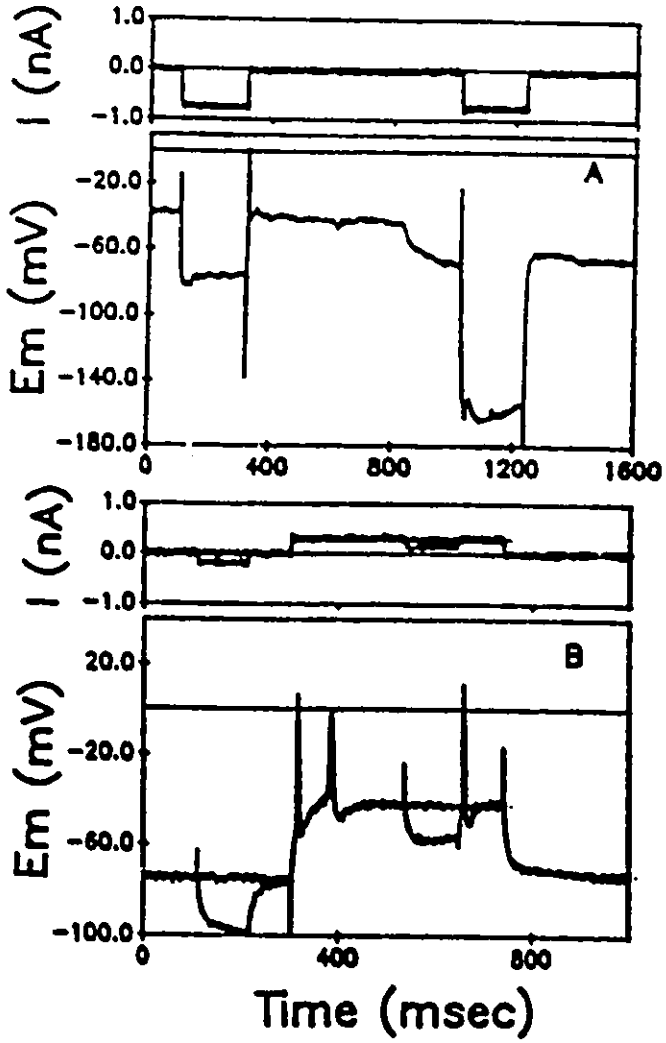


Fig. 18 Membrane response of superior cervical ganglia (SCG) neurons to various current injections in nonmultiple-firing neurons. In each panel, top trace represents injected current (I) and bottom trace represents neuron membrane potential (E_m) for this and all other figures. *A*: membrane responses to hyperpolarizing currents in a neuron in relationship to cell resting membrane potential. Note the disappearance of anode-break response at higher potential and the corresponding increase in membrane input resistance. *B*: injection of a hyperpolarizing current of small intensity at rest and during steady state of a depolarizing electrotonic potential (2 superimposed traces from the same cell). Note diminished membrane input resistance and anode-break spike during the plateau phase of depolarization.

(shift of membrane potential away from the RMP) over the intracellular injection of 0.1 to 0.5 nA hyperpolarising current, ranged from 50 to 200 MΩ. As shown in Table 2, there was no statistical difference among the mean values of RMP or MIR of the neurones in the three strains studied. The decrease in MIR during the steady state (plateau phase) of a depolarising electrotonic potential was smaller for WKY (-27.4%) than SD (-50.6%) or SH (-36.9%) (n= 38, 27 and 55 respectively, $p \leq 0.05$).

Current-voltage plots were generated for most cells by injecting 50 to 100 milliseconds square current pulses and plotting the current against the resulting electrotonic potential. Cells from all strains showed a similar slope for small current intensities (no statistical differences among MIR of the three strains, Table 2) and some rectification at higher current intensities, both in the negative and positive domains of the curve. Although this rectification appeared to be more prominent for SH than WKY or SD neurones, the variability within each group and the difficulty of comparing three families of curves prevented a statistical comparison between the strains or multiple and non-multiple firing neurones (see next section). Figure 19 shows a representative curve for each group.

III.b. Membrane active electrical properties.

Active membrane responses of the cultured neurones consisted mainly of AP generation and the presence or absence of AHP or ADP following the termination of the depolarising stimulus. Figure 1 (in section: Material and Methods) shows the parameter measured for

TABLE 2. Membrane electrical passive properties of cultured WKY, SD, and SH superior cervical ganglia neurones.

Neurones	n	RMP ,mV	MIR ,MΩ	MR_{thr} ,MΩ	MR_{pl} ,MΩ	MIR_{pl} ,MΩ
WKY	38	-72.9±1.1*	90.9± 7.3	99.4±4.3+	69.3±3.7+	65.8±5.9+
SD	27	-67.7±1.4	92.8±10.7	110.1±6.6	60.0±5.8	45.8±4.3
SH	55	-70.0±1.5	91.7± 6.4	118.8±7.8	57.2±3.8	57.9±7.7

RMP: resting membrane potential; MIR: membrane input resistance; MR_{thr}.: membrane resistance at action potential threshold; MR_{pl} : membrane resistance during plateau period; MIR_{pl}.: membrane input resistance during plateau period; * : different from SD at $p \leq 0.05$; + : different from SH at $p \leq 0.05$.

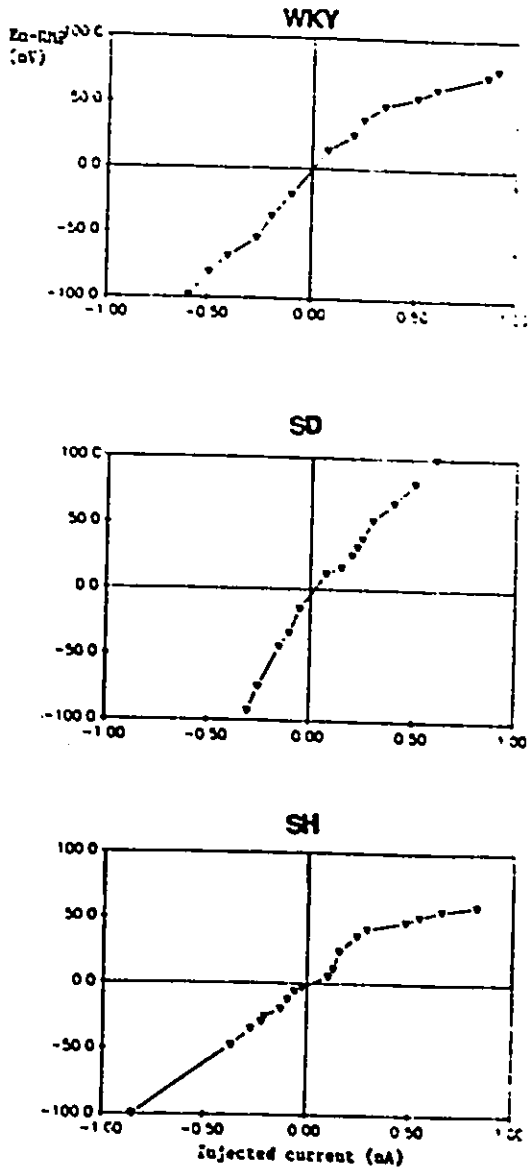


Figure 19. Representative I-V curves from SCG neurones in irradiated cultures. These curves are obtained by ploting the voltage deflection (Em - RMP) induced by intracellular current injection in the cell at rest against the intensity of that current. When AP is generated (post-threshold currents), the value of the potential during plateau (Em.pl) is taken as an approximation.

each neurone studied. Although the rate of depolarisation to reach threshold ($rate_{pp}$) and the AP threshold ($E_{m.thr}$) were similar for neurones from all strains (Table 3), the depolarising current necessary to reach that threshold (I_{thr}) was 28.6 % higher for WKY (0.36 nA) than for SD (0.28 nA, $p \leq 0.05$) or SH (0.28 nA, $p \leq 0.05$). Furthermore, the mean amplitude (AP_{ampl}) and mean peak value (AP_{max}) of the first spike were similar for all strains. Finally, the rates of depolarisation and repolarisation of the initial AP were slower for WKY neurones than for SD ($p \leq 0.05$) or SH ($p \leq 0.05$) neurones.

Moreover, striking differences were apparent during long duration (400 msec) depolarising pulses. Under these conditions, the membrane response of most SCG neurones from normotensive strains (79% for WKY, 81% for Wistar and 72% for SD nerve cells) consisted of 1-2 spikes followed by a plateau (steady-state) phase (Fig. 20 a, b). In contrast, most neurones from SH SCG (61%) showed a lack of accommodation to the depolarisation and generated multiple firing (Fig. 20 c). A cell was classified as multiple firing when it generated 3 APs or more during the depolarising pulse. In these cases, all APs were generated in a continuous train, with the frequencies described in Table 3. For all neurones, the frequency of firing was calculated for two periods (0-100 and 100-400 msec) of the stimulus and was systematically higher during the first part of the stimulus. For each of the two periods of the stimulus, the frequency of firing was systematically greater for SH than SD or WKY neurones. Most

TABLE 3. Membrane electrical active properties of cultured WKY, SD, and SH superior cervical ganglia neurones in response to injection of depolarising square pulses of current.

Neurones	n	Em thr , mV	I thr , nA	Rate pp., V/s	AP max., mV	AP ampl , mV	Rate, depol. repol.	Multiple firing	f 0/100 , Hz	f 100/400 Hz
WKY	38	39.8 ±1.7	0.36 + ±0.02**	3.2 ±0.3	12.3 ±2.4	85.2 ±2.6	20.1 ±2.2 *,+	a=21%	21.4	5.4
							-7.3 ±0.8 *,+	b=79%	11.7	0.5
SD	27	40.7 ±1.1	0.28 ±0.02	3.1 ±0.3	16.9 ±2.3	84.6 ±2.7	28.8 ±2.3	a=[illegible]8%	17.1	7.1
							-10.2 ±0.8	b=71%	11.1	0.5
SH	55	41.6 ±1.4	0.28 ±0.02	2.8 ±0.2	14.3 ±2.4	88.4 ±2.6	26.4 ±1.5	a=61%	24.2	5.9
							-9.3 ±0.5	b=39%	16.7	0.9

Em thr.: membrane potential at action potential(AP) threshold; I thr : current necessary to reach AP threshold; Rate pp.: rate of rise of pre-AP; AP max. :value of membrane potential at maximum of AP; AP ampl. :AP max. - RMP; Rate depol(arisation) and repol(arisation) of AP; f 0/100, f 100/400, frequency of firing during the first 100 and last 300 milliseconds of a 400 milliseconds stimulus respectively; a and b : proportion of multiple firing and non-multiple firing neurones respectively; * : different from SD values at p≤0.05; ** : different from SD value at p≤0.005; + : different from SH values at p≤0.05.

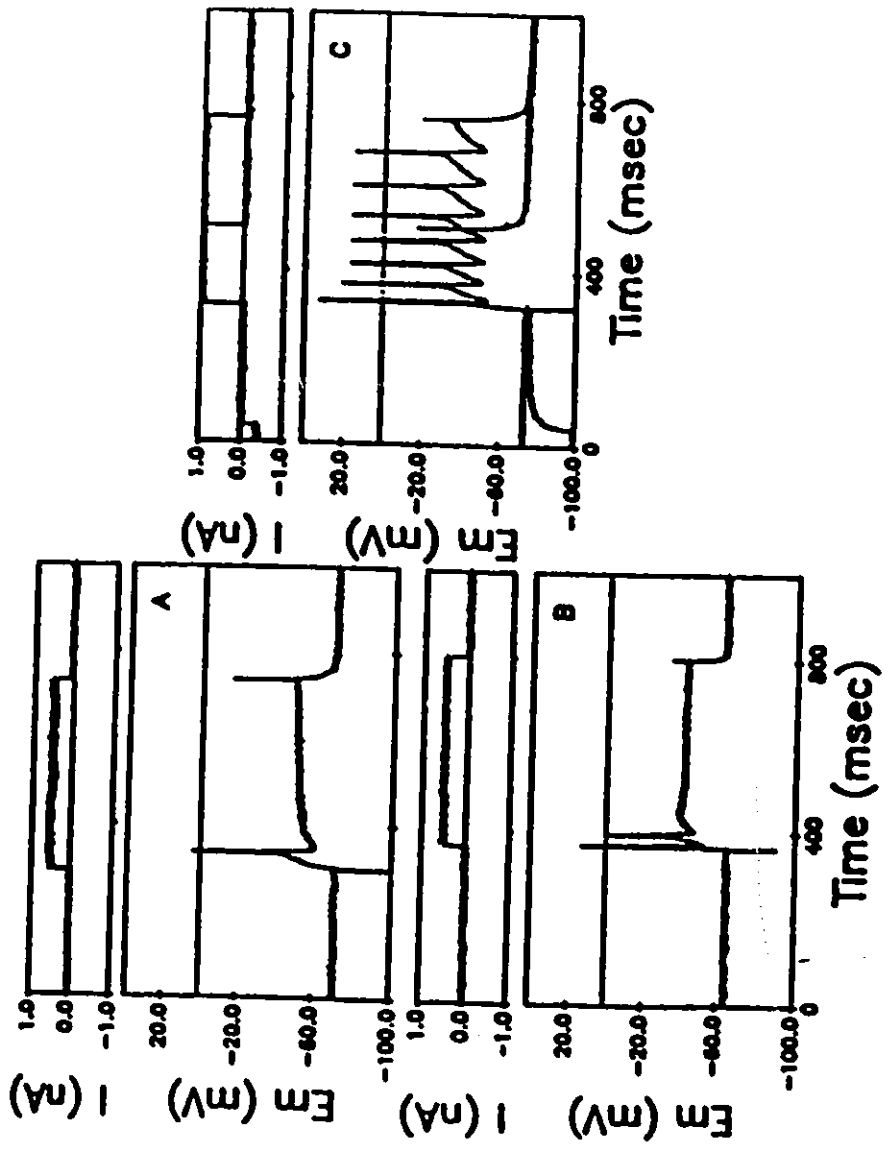


Fig. 20 Membrane responses to long-duration depolarizing currents showing typical nonmultiple-firing neurons from Wistar-Kyoto (WKY; *A*) and Sprague-Dawley rats (SD; *B*) and multiple firing in spontaneously hypertensive rat (SH) neurons (*C*). Note in *C* the 2 superimposed traces from the same cell showing multiple firing proportional to stimulus duration.

neurones eventually accommodated and generated a plateau phase, although on rare occasions neurones able to fire continuously for one second or more were found. In all cases, the cell firing lasted only as long as the stimulus. There was no difference in the very low occurrence of spontaneously firing neurones between SH and SD or WKY cultures.

The membrane resistance calculated during the plateau phase of depolarisation (MR_{pl}) was higher for WKY than for either SD or SH neurones (although significantly so only for SH neurones) (Table 2). The lack of, or termination of, multiple firing in the neurones could depend on time, voltage or inactivation of conductance(s) associated with the spike(s). To determine which it was, a depolarising current of small amplitude was superimposed on the last 200 msec of a 400 msec electrotonic potential generated by a long-duration sub-threshold depolarising pulse, as well as on the plateau phase of a long-duration supra-threshold stimulus. In both cases, these current pulses generated one or more spikes in a manner similar to the initial firing profile of the neurones: one or two for non-multiple firing cells, more for multiple-firing ones (Fig. 21 c, d). This suggested that the plateau and cell accommodation did not result from the inactivation of ionic conductances following the initial spike activity or due to time after the beginning of a depolarising step (delayed inactivation), but rather were voltage-dependent.

After the termination of the stimulus, the membrane potential returned to resting levels. Some neurones showed an after-

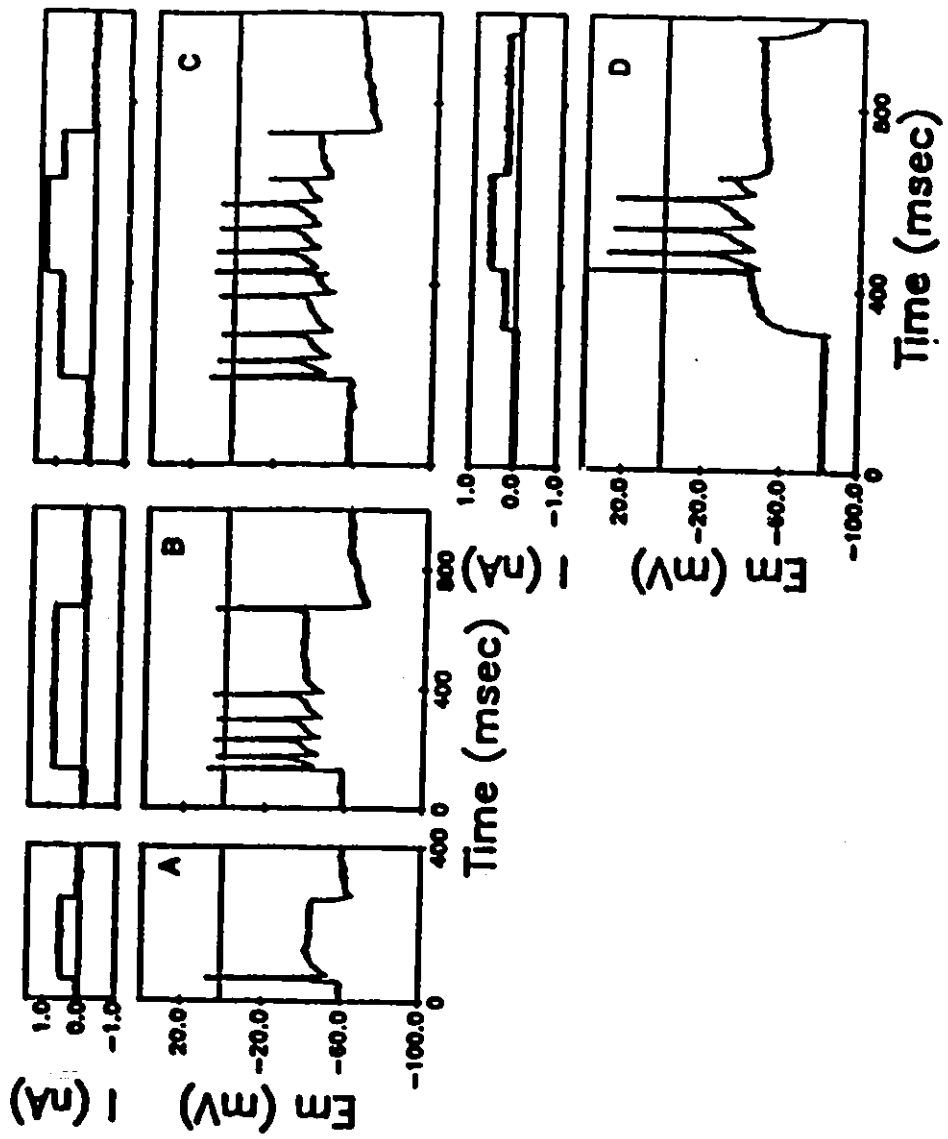


Fig. 21 Membrane responses to depolarizing currents in a multiple-firing neuron from SH. *A*: single spike in response to a low-intensity short-duration current. *B*: multiple firing in response to a higher intensity current in the same cell. *C*: membrane response of the same cell to a low-intensity current (same as in *A*) during the plateau phase of depolarization (same as in *B*); note superimposition of multiple-firing activity similar to the initial response to the stimulus. *D*: membrane response in another cell to a depolarizing current superimposed on the response to a subthreshold stimulus; multiple-firing activity is not prevented by previous depolarization, following an action potential (*C*) or not (*D*).

hyperpolarisation (AHP): the membrane potential rapidly returned towards RMP values, became more negative than RMP, and slowly returned to RMP value over 100 to 400 msec (Fig. 21 a, b, c). Some showed an after-depolarisation (ADP): the membrane potential slowly declined from plateau phase value to RMP, without ever becoming more negative than RMP (Figure 18). Finally, some retrieved rapidly (<10 msec) their pre-stimulus RMP value with no further variations (Fig. 20, a, b, or c). The three neurones illustrated in Figure 20 (WKY, SD, and SH) were chosen because they all lack ADP or AHP/LHP, have similar RMP, and apparently differ only in their multiple firing activity. Although the proportion of neurones showing an ADP was higher in SH neurone culture than in SD and WKY neurone cultures (36%, 12%, and 16%, respectively), there was no obvious correlation between the occurrence and amplitude of ADP and AHP and multiple firing. Indeed, this parameter appeared to be more correlated to RMP than to multiple firing (Table 2, Fig. 22).

III.c. Ionic basis underlying the difference between SH and control SCG cultured neurones response to depolarising pulses.

Two main kinds of APs are known in neurones: the so-called sodium- and calcium-spikes, carried respectively by Na^+ and Ca^{++} ions. An inhibitor of fast Na^+ channel, tetrodotoxin (TTX), was, thus, introduced in the recording medium. Whether alone or followed by others (multiple firing), the first AP was partially inhibited by TTX (1 ug/ml) in all strains; in multiple firing neurones, the presence of TTX

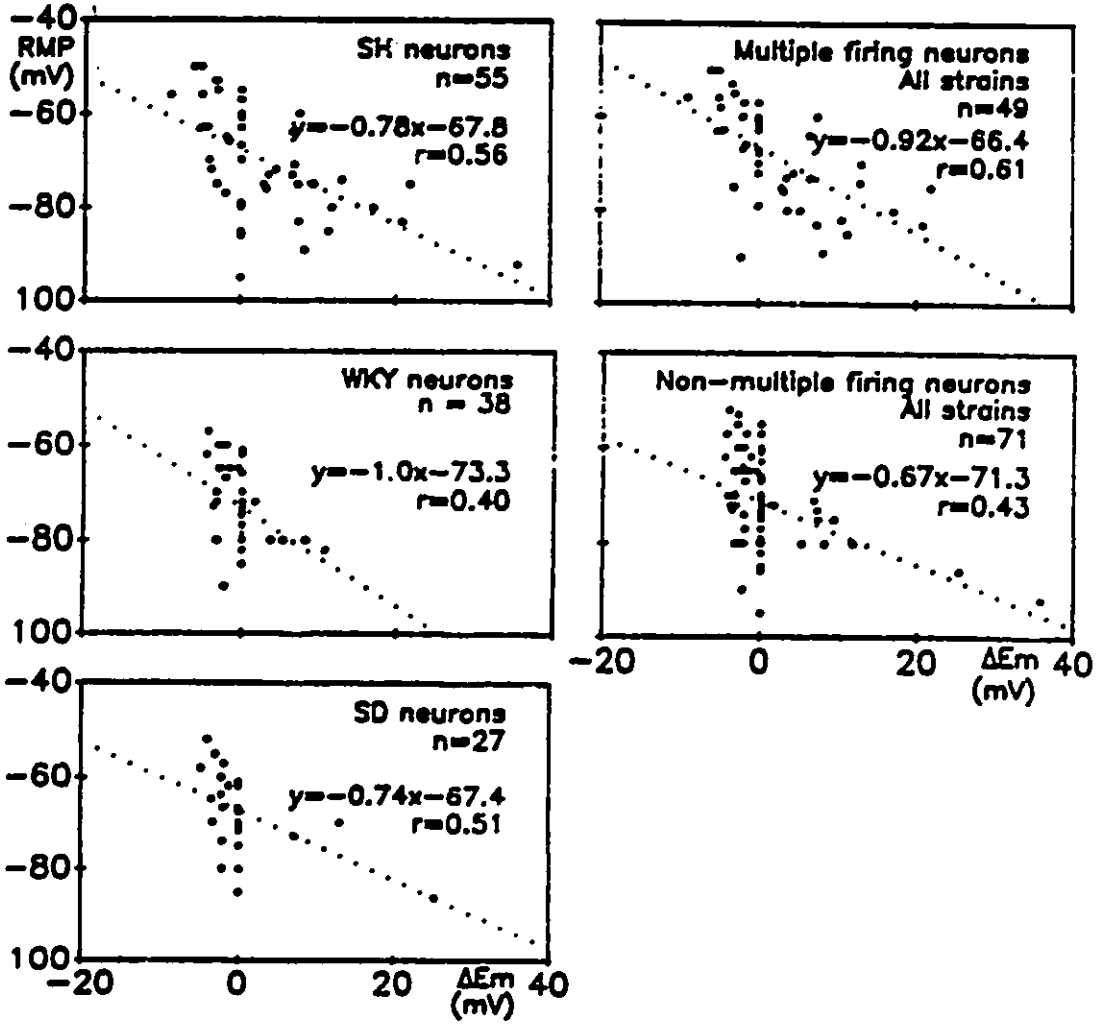


Fig. 22 Relationship between amplitude of afterhyperpolarization (AHP) or afterdepolarization (ADP) and resting membrane potential in neurons obtained from the 3 strains. There is no apparent correlation between multiple firing and AHP/ADP, since neuron's origin (SH, SD or WKY) or membrane response to depolarizing current (multiple or nonmultiple firing) affects only slightly the relation between the 2 variables. RMP, resting membrane potential; ΔE_m = RMP − (maximum E_m during AHP or ADP); ΔE_m is negative for AHP and positive for ADP.

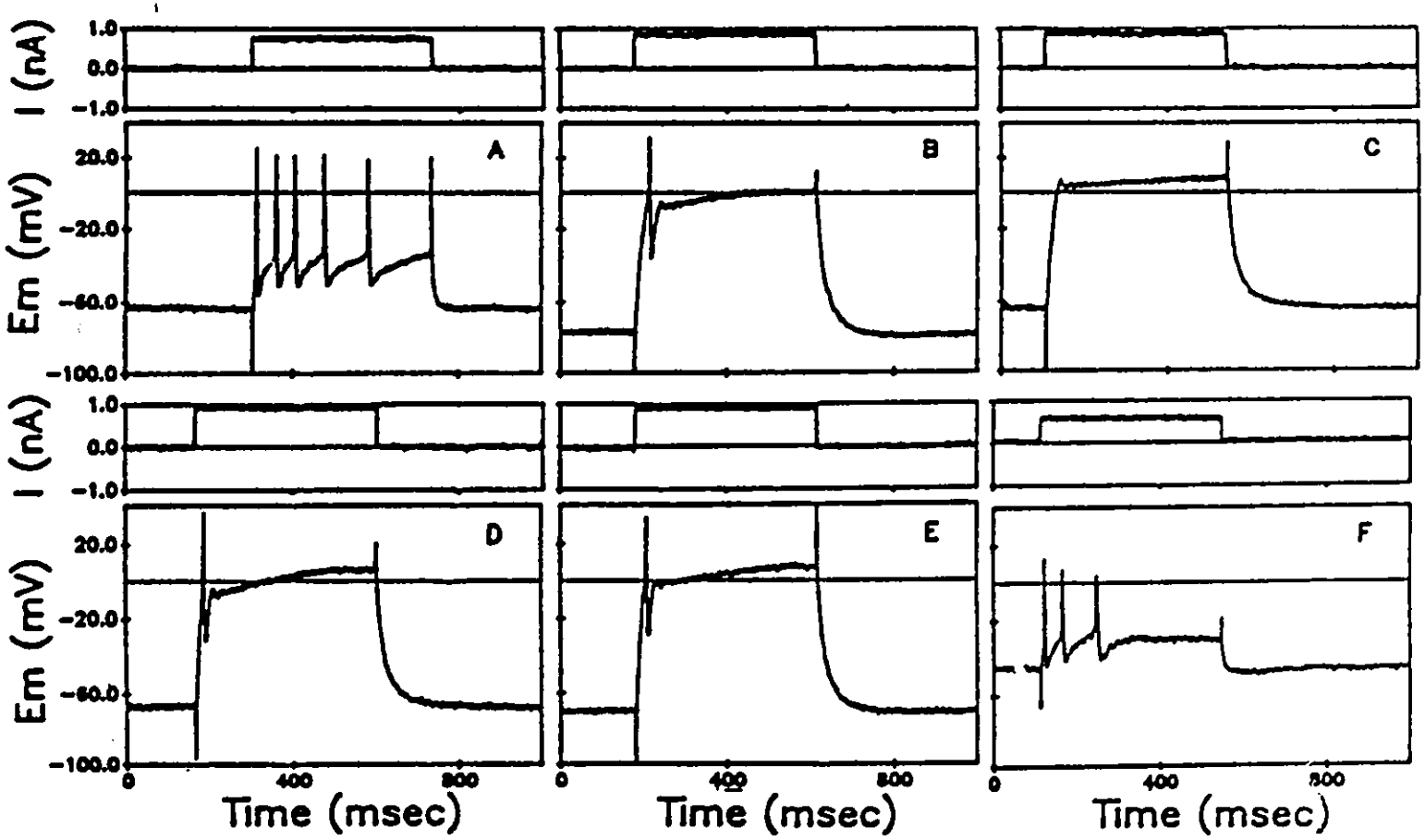


Fig. 23 Membrane responses to depolarizing currents in a multiple-firing neuron before (*A*) and after exposure to low (10^{-4} M, *B*) and high (10^{-3} M, *C*) concentrations of cobalt. Note inhibition of multiple firing (*B*) and initial action potential (*C*) concomitant with a large increase in membrane resistance during the plateau phase. Increasing current intensity (*D*) resulted in recovery of the first action potential that was insensitive to TTX (3×10^{-6} M, *E*). On washout of cobalt (*F*), multiple firing is restored (different cell than in previous panels).

did not prevent the following spikes to take place (Fig. 23 f). Single and multiple spikes have been shown in some other cells to be carried by Ca^{++} (Walsh and Singer, 1980; Yarowsky and Weinreich, 1985; Miller, et al., 1987 b; Alonso and Llinas, 1988; Hilarey, et al., 1988). Assuming, therefore, that the first spike was partially Na^{+}- and partially Ca^{++}-dependent and that multiple firing could be carried by calcium current, the effects of Ca^{++}, Ca^{++} channel activator, and Ca^{++} antagonist were studied.

The inorganic calcium antagonist cobalt (Co^{++}) was introduced in the medium of SH neurone culture. Low concentrations (10^{-5} M) of Co^{++} inhibited multiple firing while preserving the initial spike (Fig. 23 b). However, higher Co^{++} concentrations ($\geq 10^{-3}$ M) inhibited all spike activity (Fig. 23 c). In the presence of Co^{++}, the membrane resistance at plateau (MR_{pl}) was increased (23 b, c). The inhibition of the first AP by higher concentrations of Co^{++} could be partially overcome by raising the intensity of the stimulus (Fig. 23 d). To determine if the absence of multiple firing in SD and WKY neurones was due to low concentration or poor availability of internal calcium, experiments were carried out with high external Ca^{++} (5 and 10×10^{-3} M, as opposed to 3×10^{-3} M in normal medium) and/or Ca^{++}-channel opener Bay-K 8644 (10^{-6} M). The two conditions, separately or in combination, not only failed to generate multiple firing in these neurones, but frequently resulted in an inhibition of the response to a depolarising pulse, i.e. diminished the amplitude of the first AP and inhibited the second one when present

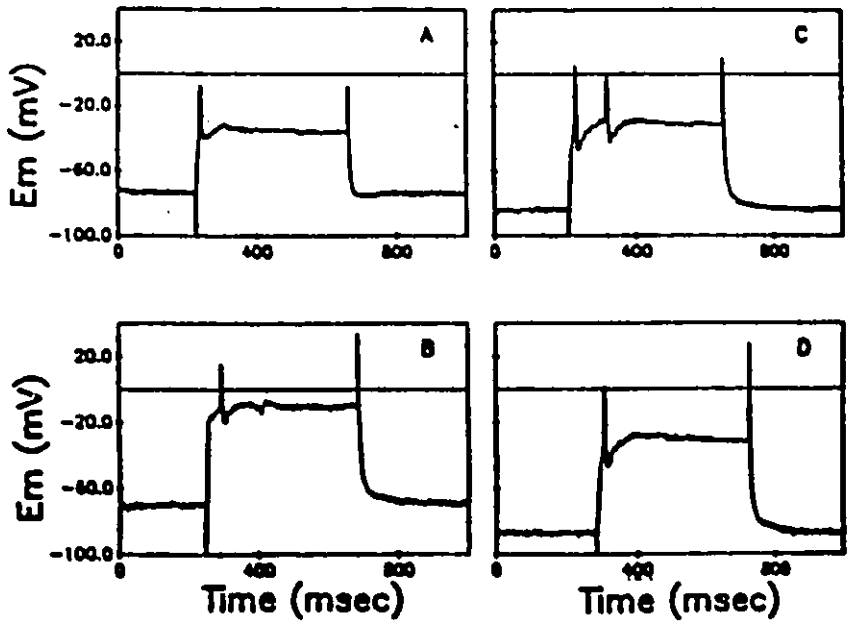
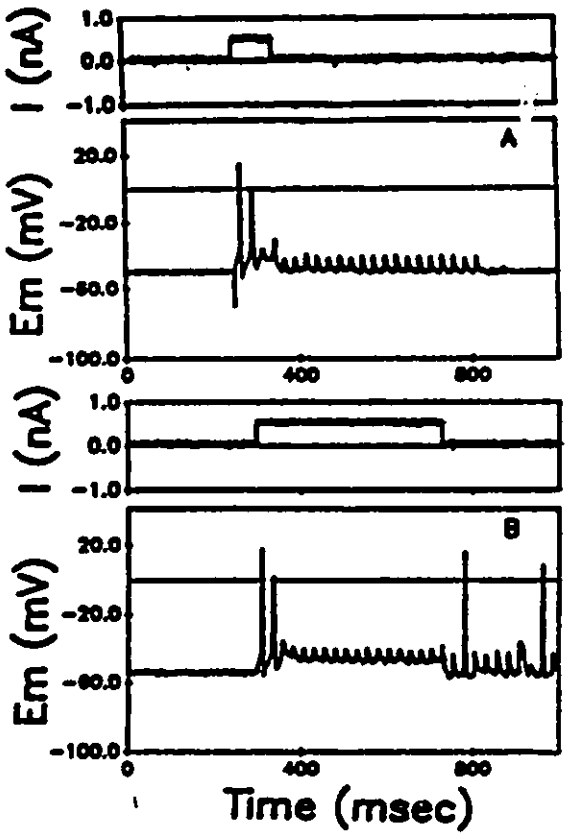


Fig. 24 Effects of high external calcium (5–10 × 10^{-3} M) on non-multiple-firing cell responses to depolarizing current pulses (0.5 nA). Various effects include a delay (*B*) of the single spike (*A*), an increase in membrane resistance during the plateau phase (or an inhibition of the second action potential (*D*) when it is part of the cell response to a depolarizing pulse (*C*); note in *B* and *D* the slight inhibition of action potential. Increased external calcium never induces multiple firing.



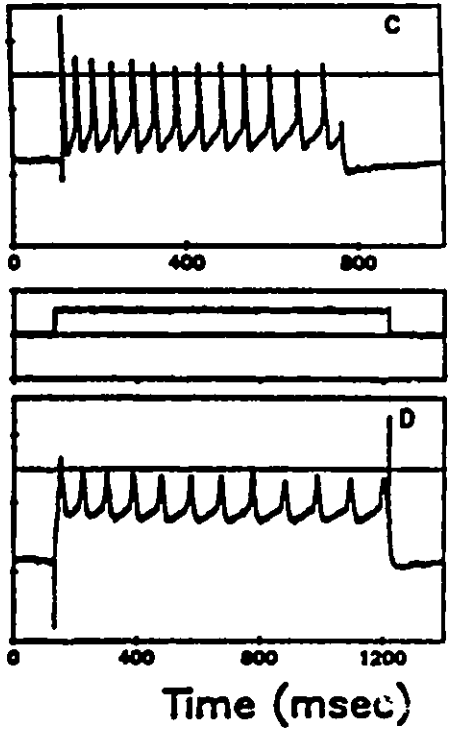


Fig. 25 Membrane responses in non-multiple-firing neurons to depolarizing pulses in the presence of apamin (10^{-4} M, *A–C*) or tetraethylammonium chloride (TEA; 5×10^{-3} M, *D*). In some neurons, apamin induces an increase in background activity following the stimulus (*A*; note short duration of current pulse) sometimes even generating a few action potentials (*B*). In most neurons, apamin generates multiple firing (*C*) similar to native SH neurons (see Fig. 2*C*); note in *C* the duration of the stimulus (660 ms) and presence of AHP. TEA also induces multiple firing (*D*), but with a reduced amplitude and widening of action potentials. Duration of the stimulus (1,114 ms) and the time scale are different in *D*. Refer to text for detailed discussion of the different effects of the 2 K-channel antagonists.

(Fig. 24). Thus, it appeared that even though multiple action potentials were dependent on extra-cellular calcium entry in the cell through voltage-dependent channels, the intracellular availability of calcium was not the primary factor preventing multiple firing.

Since the activation of Ca^{++}-dependent K^{+} channels has been suggested to be the basis of the accommodation to depolarisation in other neurones (see Discussion), the effect of blockers of these channels, such as tetraethylammonium (TEA) and apamin, was studied on non-multiple firing SD and WKY neurones.

After addition of apamin (10^{-6} M), the cells impaled showed consistently multiple firing upon depolarisation (Fig. 25-c), with frequencies similar to native SH multiple firing neurones. The shape of the spikes was not modified and all spikes were similar. Apamin-induced multiple firing showed the same sensitivity to Co^{++} (10^{-5} M) and lack of sensitivity to TTX (1 ug/ml) as native multiple firing neurones. In most cells, apamin not only readily induced multiple firing during the stimulus, but also induced oscillations of the RMP and many generated a few APs and low amplitude spikes after the cessation of the stimulus (Fig. 25 a, b).

In the presence of TEA (5×10^{-3} M), the neurones also showed multiple firing in response to depolarising current pulses. Although this response was qualitatively similar to apamin-induced and native multiple firing, the APs were broadened, their amplitude diminished and their firing frequency lowered (Fig. 25 d).

These results suggested that apamin induced multiple firing

TABLE 4. Membrane electrical passive properties of WKY, SD, and SH superior cervical ganglia neurones in co-cultures with their respective A and MA vascular smooth muscle cells.

Neurone	VSMC	n	RMP ,mV	MIR ,MΩ	MR_{thr} ,MΩ	MR_{pl} ,MΩ	MIR_{pl} ,MΩ
WKY	A WKY	148	-58.6 ±4.1	120.0 ±12.7	88.1 ±5.8	49.3 ±4.6	86.8 ±16.3
	MA WKY	17	-70.9 ±5.7	79.6 ±9.1	88.6 ±11.4	76.1 ±9.1	86.6 ±18.9
SD	A SD	39	-58.0 ±1.6	64.4 ±7.7	75.8 ±8.1	44.3 ±4.7	51.4 ±9.2
	MA SD	39	-61.6 ±1.9	61.3 ±6.0	78.5 ±7.8	41.0 ±4.5	39.8 ±3.9
SH	A SH	66	-63.2 ±2.3	33.9 ±3.3	---	---	---
	MA SH	31	-62.4 ±1.8	76.8 ±4.7	79.8 ±4.4	46.5 ±5.4	45.6 ±3.6

Values are means ± SE; n = number of cells; A, aorta; MA, mesenteric artery; VSMC, vascular smooth muscle cells; RMP, resting membrane potential; MIR, membrane input resistance; MR_{thr}, membrane resistance at threshold for action potential; MR_{pl}, membrane resistance during plateau phase; MIR_{pl}, membrane input resistance during plateau phase. MR_{thr} , MR_{pl} , and MIR_{pl} values are given only for firing neurones. The proportion of firing neurons are as follows: 12/148, 17/17, 23/39, 37/39, 6/66, 31/31.

TABLE 5 Membrane electrical active properties of SCG neurons from WKY, SD, and SH superior cervical ganglia neurones in co-culture with their respective A and MA vascular smooth muscle cells in response to injection of depolarising square pulse of current.

Neurone	VSMC	n	Em thr ,mV	I thr, nA	Rate pp , V/S	AP ampl, mV	Rate of depolarisation	Rate of repolarisation
WKY	A WKY	148	-36.1 ±1.0	0.38 ±0.04	8.08 ±2.02	48.2 ±4.9	8.2 ±1.7	- 3.4 ±0.3
	MA WKY	17	-39.5 ±1.2	0.36 ±0.02	3.96 ±0.57	90.2 ±4.2	25.3 ±3.2	-12.4 ±1.3
SD	A SD	39	-38.4 ±1.6	0.34 ±0.02	3.90 ±0.50	78.6 ±2.7	25.0 ±2.8	-14.3 ±1.6
	MA SD	39	-40.2 ±1.2	0.33 ±0.02	2.61 ±0.32	79.4 ±3.2	26.9 ±3.3	-12.5 ±1.4
SH	A SH	66	---	---	---	---	---	---
	MA SH	31	-38.1 ±1.3	0.34 ±0.02	3.72 ±0.75	85.2 ±2.8	24.5 ±2.8	-12.3 ±1.2

Values are means ± SE; n, no. of cells. Em_{thr} , membrane potential at action potential (AP) threshold; I_{thr}, threshold current to generate AP; $rate_{pp}$, rate of depolarization during the pre-AP; AP_{max} and AP_{ampl}, AP maximum E_m value and amplitude (i.e., AP_{max} - RMP). All values are given for firing neurons only (see Table 4). all values refer to the initial AP.

by selectively inhibiting the Ca^{++}-dependent potassium channels responsible for accommodation to depolarising pulses in the neurone. TEA, being less selective than apamin, inhibited also other potassium channels (not necessarily Ca^{++}-dependent) which regulate other aspects of SCG neurones membrane properties.

IV. Genetic hypertension: comparison of membrane electrical properties of neonatal SCG neurones from SD, WKY and SH rats in irradiated co-cultures with VSMC.

IV.a. Membrane electrical properties and firing characteristic of SCG neurones co-cultured with their respective VSMC. Comparison between A and MA co-cultures and between strains.

Table 4 shows the membrane passive electrical properties for all strains. There is no obvious difference in these properties to be related to the presence of A or MA VSMC, except for WKY neurones in co-culture with A VSMC (diminished RMP and heightened MIR) and SH neurones in co-culture with A VSMC (diminished MIR). Also to be noted, is the fact that there is little variation between strains. Table 5 summarises the membrane active electrical properties of these neurones. These properties, characterised during the response to an intra-cellular pulse of depolarizing current, are remarkably similar between A and MA co-cultures (for SD co-cultures) and between strains (all MA VSMC co-cultures). Only AP characteristics in A WKY co-cultures are modified. This could be related to the very

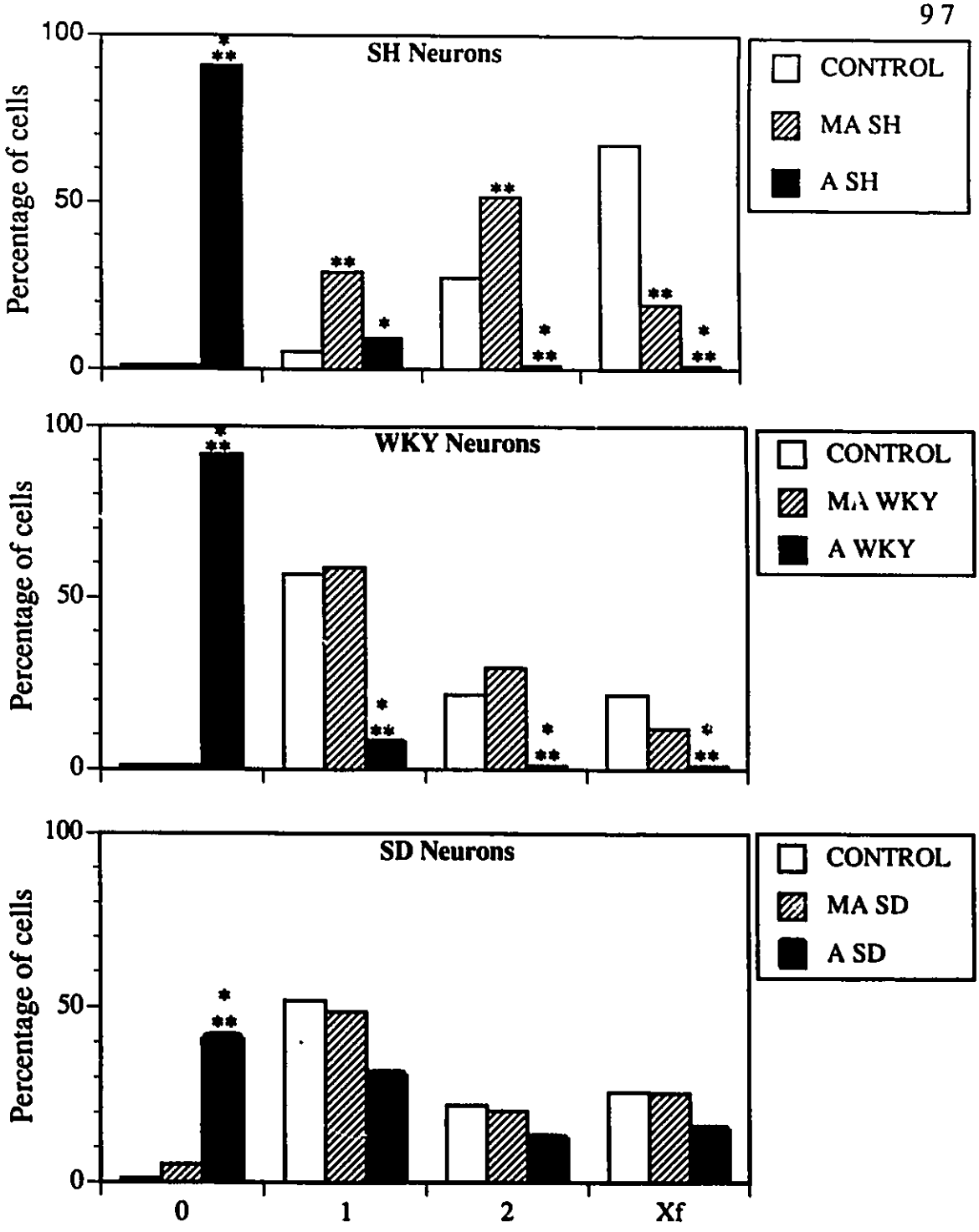


Figure 26: Distribution of firing frequencies of superior cervical ganglia neurones in single (control) and co-culture with their respective vascular cells from aorta (A) and mesenteric artery (MA). In response to long-duration depolarizing pulses, neurons generate no action potential (0), 1 , 2 , or Xf (multiple firing); * : different from MA co-culture ($p \leq 0.05$); ** : different from control ($p \leq 0.05$).

TABLE 6. Membrane electrical passive properties of WKY, SD, and SH superior cervical ganglia neurones in co-cultures with A and MA vascular smooth muscle cells from SD.

Neurone	VSMC	n	RMP ,mV	MIR ,MΩ	MR_{thr},MΩ	MR_{pl} ,MΩ	MIR_{pl} ,MΩ
WKY	A SD	17	-57.9 ±2.7	65.0 ±4.7	86.2 ±2.2	39.7 ±3.1	54.2 ±3.8
	MA SD	13	-59.9 ±4.6	73.7 ±10.0	74.1 ±5.8	52.3 ±6.9	45.3 ±3.5
SD	A SD	39	-58.0 ±1.6	64.4 ±7.7	75.8 ±8.1	44.3 ±4.7	51.4 ±9.2
	MA SD	39	-61.6 ±1.9	61.3 ±6.0	78.5 ±7.8	41.0 ±4.5	39.2 ±3.9
SH	A SD	72	-65.2 ±2.2	76.1 ±6.6	107.9 ±6.0	55.3 ±4.2	43.7 ±6.3
	MA SD	32	-68.0 ±2.1	88.9 ±5.9	122.0 ±9.9	61.7 ±4.9	55. 4 ±6.4

Values are means ± SE; n = number of cells. Legends as in Table 4. Proportion of firing neurones as follows: 14/17, 11/13, 23/39, 37/39, 53/72, 31/32.

low occurrence of firing in this situation (less than 10%) since the difficulty in generating AP is reflected in the lower rate of depolarisation and repolarisation as well as a small AP amplitude.

Neurones firing characteristics: the most striking effect is the near complete inhibition of firing of SH and WKY neurones in the presence of A VSMC from their respective strains (90.1 and 91.9 % respectively) and the less effective but statistically significant inhibition of firing of SD neurones in the presence of A VSMC from SD (41 %) (Figure 26, all panels, class "0", black columns, p-94). In addition, Wistar SCG neurones in irradiated co-cultures also showed a 95% inhibition of firing (n=51). In contrast, the presence of MA VSMC does not prevent neurones from firing in any of the strains tested (Figure 26, class "0", all panels, hatch-crossed columns). When firing is not totally inhibited (SD A VSMC co-cultures) there is no difference in firing frequency distribution between A and MA VSMC co-cultures (Figure 26, black and hatched columns, SD panel).

IV.b. Membrane electrical properties and firing characteristics of SH and WKY neurones co-cultured with SD VSMC.

Passive (Table 6) and active (Table 7) electrical membrane properties of SH or WKY neurones show little differences when they are cultured with A or MA VSMC from SD origin. There is however some inter-species differences in that the values for the passive properties of WKY neurones co-cultured with A or MA VSMC from SD

TABLE 7. Membrane electrical active properties of SCG neurons from WKY, SD, and SH superior ganglia neurones in co-culture with SD A and MA vascular smooth muscle cells in response to injection of depolarising square pulse of current..

Neurone	VSMC	n	Em $_{thr}$,mV	I $_{thr}$, nA	Rate $_{pp}$, V/S	AP $_{ampl}$, mV	Rate of depolarisation	Rate of repolarization
WKY	A SD	17	-43.7 ±0.9	0.26 ±0.01	4.01 ±0.39	75.4 ±3.8	24.9 ±3.2	-11.3 ±1.5
	MA SD	13	-37.6 ±1.5	0.39 ±0.02	4.29 ±0.54	79.3 ±3.6	29.9 ±4.4	-12.8 ±1.5
SD	A SD	39	-38.4 ±1.6	0.34 ±0.02	3.90 ±0.50	78.6 ±2.7	25.0 ±2.8	-14.3 ±1.6
	MA SD	39	-40.2 ±1.2	0.33 ±0.02	2.61 ±0.32	79.4 ±3.2	26.9 ±3.3	-12.5 ±1.4
SH	A SD	74	-37.4 ±1.4	0.31 ±0.02	3.32 ±0.39	86.6 ±2.8	26.1 ±2.7	-10.4 ±1.0
	MA SD	32	-36.5 ±1.1	0.28 ±0.02	3.77 ±0.49	88.5 ±3.0	26.7 ±2.4	-10.9 ±0.7

Values are means ± SE; n = number of cells. Legends as in table 5.

TABLE 8: Comparison of membrane electrical properties of SCG neurones in single culture and in co-cultures with their respective VSMC.

Neurone	VSMC	ΔRMP	ΔMIR	ΔMR_{thr}	ΔMR_{pl}	ΔMIR_{pl}	ΔEm_{thr}	ΔI_{thr}	$\Delta Rate_{pp}$	ΔAP_{ampl}	ΔRate Depol	ΔRate Repol
WKY	A WKY	↓(-14)	*→(+29)	→(-11)	↓(-20)	→(+22)	*→(-4)	→(+2)	↑(+49)	↓(-37)	↓(-12)	↓(-4)
	MA WKY	→(-2)	→(-11)	→(-11)	→(+7)	→(+21)	→(0)	→(0)	→(+8)	→(+5)	→(+5)	↑(+5)
SD	A SD	↓(-9)	↓(-28)	↓(-34)	↓(-16)	→(+5)	→(-2)	↑(+6)	→(+8)	→(-6)	→(-4)	↑(+4)
	MA SD	↓(-6)	↓(-31)	↓(-32)	↓(-19)	→(-5)	→(0)	*→(+5)	→(-5)	→(-5)	→(-2)	→(+2)
SH	A SH	↓(-7)	↓(-58)	---	---	---	---	---	---	---	---	---
	MA SH	↓(-8)	*→(-15)	↓(-39)	→(-10)	→(-11)	*→(-4)	↑(+6)	→(+9)	→(-3)	→(-2)	↑(+3)

Δ = (value in co-culture - value in single culture); all variables defined as in Table 4 and 5. (-) sign for ΔRMP represents a depolarised state compared to resting potential. Numbers in brackets represent values of Δ in the variables respective units. ↑,↓: statistically significant difference from neurones in single cultures. →: not statistically different from neurones in single cultures. *: approaching statistically significant difference ($0.05 < p \leq 0.07$).

are remarkably similar to SD values (Table 6), while SH neurones show a statistically significant tendency to a higher RMP, MIR, MR_{thr} and MR_{pl}. These differences appear to be more pronounced in A VSMC co-cultures. For the active properties (table 7), only AP_{ampl} is statistically higher for SH neurons; all other values are similar between the three species. The firing abilities of SH and WKY SCG neurones cultured with VSMC from SD strain (Figure 27) are not modified to a statistically significant extent by the presence of MA VSMC from SD (Figure 27, Class "0", Crossed-hatched columns). However, A VSMC from SD inhibit SH and WKY neurones firing to a lesser extent (25.7 and 17.6 %, respectively), but similar to the inhibition of native SD neurones (41%) (Class "0", double crossed-hatched columns). Although there appears to be a slight shift in the distribution of firing frequency for WKY neurones between co-cultures with A and MA VSMC from SD, this difference is not statistically significant. Overall, there is no difference in firing frequency distribution of SH, WKY or SD neurones between co-cultures with A and MA VSMC from SD. Also to be noted is a group of multiple firing neurones (10-20%) which do not seem to be affected by target cell presence unless firing is altogether prevented.

IV.c. Comparison of membrane electrical properties and firing frequencies distributions between SCG neurones in single cultures (controls) and co-cultures with VSMC.

The values for all variables used as controls are the values obtained

in single neuronal cultures (see section III). The single and co-culture experiments were done under identical circumstances and time schedule. Table 8 summarizes the differences in membrane properties between co-cultures (same strain) and controls. These values indicate that SCG neurones in the presence of their target cells tend to have diminished passive membrane properties (lower RMP, MIR, MR_{thr}, MR_{pl}), but show little or no difference in most of their active membrane properties, with the exceptions of the current necessary to reach threshold (higher I_{thr}) and the rate of repolarisation (higher Rate Repol). Again, there is no obvious difference between co-cultures using A or MA VSMC as targets. Table 9 shows the same comparison when all neurones are cultured with VSMC from SD. Properties of WKY neurones appear to be modified in a similar manner by the presence SD VSMC as they are by WKY VSMC, but SH neurones seem to be particularly insensitive to the presence of SD VSMC, A or MA.

As previously stated, we classified neurones as multiple firing when they generated 3 or more APs in response to a 400 msec depolarising pulse. The firing frequencies were then separated into 4 classes depending if the depolarising stimulus generated 0, 1 or 2 APs, or multiple firing. The firing frequency distributions of WKY and SD neurones in co-culture are not modified by the presence of their respective MA VSMC (Figure 26, WKY and SD panels, clear and single hatched columns). However, the firing frequency distribution of SH neurones in presence of SH MA VSMC is shifted towards lower

TABLE 9: Comparison of membrane electrical properties of SCG neurones in single culture and in co-cultures with SD VSMC .

Neurone	VSMC	ΔRMP	ΔMIR	ΔMR_{thr}	ΔMR_{pl}	ΔMIR_{pl}	ΔEm_{thr}	ΔI_{thr}	$\Delta Rate_{pp}$	ΔAP_{ampl}	Δ Rate Depol	Δ Rate Repol
WKY	A SD	↓(-15)	↓(-26)	↓(-13)	↓(-29)	→(-11)	↑(+4)	↓(-10)	→(+8)	↓(-10)	→(+5)	↑(+4)
	MA SD	↓(-13)	→(-17)	↓(-25)	↓(-17)	↓(-20)	→(-2)	→(+3)	*→(+11)	→(-6)	*→(+10)	↑(+6)
SD	A SD	↓(-9)	↓(-28)	↓(-34)	↓(-16)	→(+5)	→(-2)	↑(+6)	→(+8)	→(-6)	→(-4)	↑(+4)
	MA SD	↓(-6)	↓(-31)	↓(-32)	↓(-19)	→(-5)	→(0)	*→(+5)	→(-5)	→(-5)	→(-2)	→(+2)
SH	A SD	*→(-5)	*→(-16)	→(-11)	→(-2)	→(-12)	↓(-5)	→(+3)	→+5	→(-2)	→(0)	→+(1)
	MA SD	→(-2)	→(-3)	→(+3)	→(+4)	→(-2)	↓(-5)	→(0)	*→+10	→(0)	→(0)	*→(+2)

Legends as in Table 8. ↑,↓: statistically significant difference from neurones in single cultures. →: not statistically different from neurones in single cultures. *: $0.05 < p \leq 0.07$.

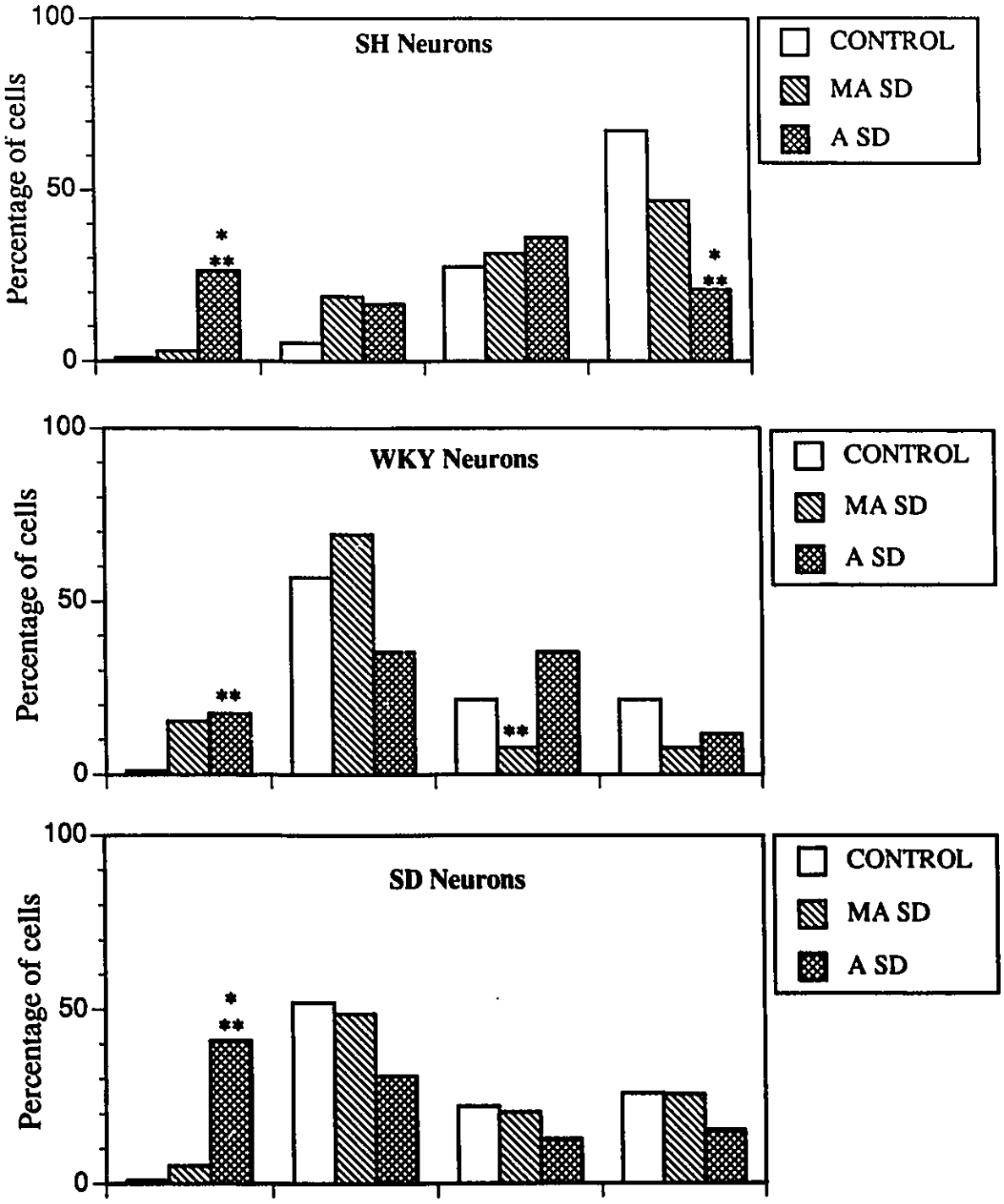


Figure 27: Distribution of firing frequencies of SCG neurons in single (control) and co-culture with SD vascular smooth muscle cells (legend as in figure 26) .

values with a maximum for double spike. This distribution is still different from any distribution in normotensive strains (Figure 26 and 27), whether in controls or co- cultures, for which the main class of response is a single AP (with the exception of SH and WKY A VSMC co-cultures, where firing is altogether inhibited). The results when all neurones co-cultured with VSMC from SD (Figure 27) show that the presence of SD MA VSMC does not statistically modify the control firing frequency distribution of any strain (all panels, clear and single-hatched columns). The firing frequency distribution is shifted towards single and double spikes in presence of A VSMC (Figure 27 clear and double-hatched columns).

IV.d. Summary.

When in co-cultures, the passive properties of VSMC, either A or MA, were not modified by the presence of neurones. The presence of VSMC, however, modified some passive and active membrane properties of the neurones. Some of these effects underlined again that WKY rats are not the best controls for SH rats because they show the different membrane properties when compared to SH and SD. Other effects, however, underlined the unique character of neo-natal SH SCG neurones even in co-cultures. These results, as well as those from previous sections, appear to suggest some inter-species differences and tissue specific (A or MA) mechanisms involved in the effects of the target cells on neurones membrane properties in vitro. Thus, there appears to be three different types of effects: a general

non-descript class of VSMC influences, possibly related to a lowered RMP of neurones in presence of target VSMC; tissue specific (A vs MA), which prevents neurones from firing, and strain specific, which diminishes the expression of multiple firing; these results also appear to define two groups of multiple firing neurones, (10 to 20%) present in all strains showing a multiple firing activity which is inhibited only when firing activity is totally prevented, and a second group (40-50 % of all neurones), specific to the genetically hypertensive state, whose firing activity can be modulated by the target cells.

V. Summary of Results section.

A system of co-culture between SCG from neonatal rats and VSMC obtained by first passage of explanted cells could be studied over time. In this system, stability (constant density of all cell types) over time was obtained by irradiation (5 Krads). Irradiated cultured cells from the vasculature were shown to be VSMC, whose characteristics were similar to in situ and other in vitro non-irradiated VSMC. The RMP of irradiated VSMC was shown to be proportional to cell density at the time of irradiation and independent of time in culture. The study of irradiated cultures of neurones confirmed the fact that irradiation did prevent the division of accessory cells from the ganglia without modifications of nerve cell growth, membrane electrical properties or ability to extent neurites.

Control studies of VSMC from hypertensive (SH) and normotensive (WKY, SD) rats demonstrated no difference between strains. Control cultures of SCG neurones from SH, WKY, Wistar and SD rats showed non-significant differences among neurones from different strains with respect to their membrane passive electrical properties, but showed different responses to long-duration depolarising current pulses. Neurones from genetically hypertensive rats (SH) could not accommodate and fired repetitively (multiple firing). Only 20-30% of SCG neurones from normotensive origin (WKY, Wistar, SD) showed a similar behaviour. Multiple firing was shown to be carried by calcium (insensitive to TTX, sensitive to low cobalt concentration), and could be induced in SD or WKY neurones by blocking $K_{(Ca)}$ channels with its specific blocker, apamin.

The presence of neurones in co-culture modified neither A or MA VSMC passive membrane properties in SH, WKY or SD. In contrast, the presence of VSMC cells in neuronal cultures modified some passive and active membrane properties in all neurones as well as their response to depolarizing pulses of intracellular current. A VSMC prevented firing of almost all neurones (95 to 100%) in SH and WKY co-cultures and of about 50% of neurones in SD co-cultures. MA VSMC did not influence firing of SD and WKY neurones, but displaced the maximum of the distribution of firing from multiple firing to double spikes in SH neurones. Overall, most firing SD or WKY neurones (in single or co- cultures) fired one AP, while most SH neurones fired three APs or more in single cultures and two APs or

more in co-cultures.

Co-cultures between SH or WKY neurones and A or MA VSMC from SD rats, and between Wistar neurones and A WKY VSMC, showed three levels of modulation of neuronal function by VSMC: general, non-tissue specific (A and MA showed the same effect); tissue (A or MA) specific but not strain specific; and strain specific (poor level of interaction between cells from different strains). These results also suggested the presence of a small sub-group of multiple-firing neurones resistant to VSMC influence in SCG from all strains, including normotensive ones.

DISCUSSION

Neurones from neo-natal SCG and VSMC from genetically hypertensive rats (SH) and normotensive (WKY, Wistar and SD) rats were cultured, each separately and in co-culture, and investigated by intra-cellular recording techniques to contrast their membrane electrophysiological properties. It was hoped that changes related to hypertension in situ could still be observed in vitro, and that the identification of these changes could help explain the hyper-reactivity of the neuro-vascular junction described in SH rats (Smith, et al., 1982). These changes were also incorporated in a tentative framework of the hypertensive state and its consequences on the vasculature.

Neurones from neo-natal SH SCG showed a lack of accommodation (multiple firing) in response to a long duration depolarising pulse of current. No other dysfunction of membrane properties could be correlated to the hypertensive state. The cultured VSMC, whether A or MA, showed membrane properties similar in all strains, in single cultures as well as in co-cultures. When in co-culture, however, A VSMC prevented neurones from firing while MA VSMC did not affect noticeably the neuronal response to depolarisation, except for SH neurones. In SH co-

cultures, MA VSMC diminished the proportion of multiple firing. Still, the majority of SH neurones in MA VSMC co-culture fired two or more APs, while most WKY or SD neurones fired a single AP.

In this chapter, I will discuss first the validity of the model used (i.e., irradiated cell culture), i.e. the necessity of using cultures stable over time to study the interaction of the two cell types, and the use of irradiation to that effect; second, the differences observed in this model between cells from hypertensive and normotensive rats; third, the nerve cell multiple firing and its ionic basis; fourth, the effect of VSMC on neuronal firing properties; and, finally, how these findings can be correlated to other modifications of various cell types in the SH model to propose a possible theory about the origin and development of essential hypertension.

In conclusion, studies necessary to confirm that hypothesis will be described and a few considerations related to a possible treatment for genetic hypertension will be made.

I. The model.

To study in vitro the interaction between neurones and VSMC, it was necessary to develop a long-term co-culture model of stable, mature cells, whose intrinsic characteristics would change as little as possible over time; the observed modifications could, thus, be related to the presence of the other cell type and its origin. Few studies examined smooth muscle cell properties in stable long-term

co-cultures. Particularly relevant to our study is the study by O'Lague, et al., (1978), which used irradiated co-cultures of SCG neurones and heart cells; irradiation (5 Krads) was used to prevent cell division without modifying cell membrane electrical properties. The lack of data specifically related to VSMC irradiated cultures meant that an adequate protocol had to be developed.

I.a. Culture of VSMC.

Vascular cultured cells has been and is still a system widely used and yielded numerous results, but the identity of the cells cultured from vessel walls has been questioned (see Introduction, section V.b). However, a study using cloned A VSMC in our laboratory detailed the types and proportion of cells present in the aortic wall (Blennerhassett et al., 1989). It was previously known that VSMC compose up to 98% of cells in the aorta media (in Blennerhassett, et al., 1989). This study showed that the main cell type migrating from vessel wall explant after endothelium and sub-mucosa removal possessed the same morphology, culture appearance and RMP at maximum density as other well-characterized VSMC. The fact that the explanted A VSMC and the A VSMC from the first passage (only cells used in our study), showed similar characteristics (cell morphology, culture appearance and RMP) indicated that cultured cells in our experiment were indeed aortic smooth muscle cells. The MA wall may not have such an important proportion of VSMC but the morphology of the cultured cells (explant and first

passage) and their RMP in high density cultures are similar to cultured A VSMC; the cell type defined by those characteristics also appears to represent a large majority of the cells migrating from MA vessel wall explants. In the case of our explanted cells and first passage A cells, the density augments rapidly until a monolayer is formed, then slows down and reaches a plateau of constant density, where the RMP of the cells is high and similar to the ones observed in other VSMC cultures (Grunwald and Wischer, 1985, Blennerhassett et al., 1987) and to in situ measurements (around -40 mV) (Campbell and Chamley, 1976, Chamley-Campbell et al., 1979, Campbell et al., 1981, Lee et al., 1983, Clegg, et al., 1986; Blennerhassett, et al., 1989; Paquet, et al., 1989).

Cell division and maturation appear to be related in a complex manner, and the transition from multiplication to maturation seems to result from a combination of effects resulting from intrinsic factors, cell-to-cell contact and growth factors. Little is known of each of these factors. Cell division is also halted by the presence of a feeder layer of fibroblasts, cyclic-AMP or endothelial cells (Chamley, et al., 1977). It is also sensitive to various hormones and neurotransmitters such as tromboxane (Ishimitsu, et al., 1988), Angiotensin II, serotonin, and bradykinins (Paquet, et al., 1989). Also, growth factors (FCS, TGF-beta, heparin) are known to potentiate cell division (Tagami, et al., 1986; Majak, 1987; Paquet, et al., 1989) and shift cells behavior from mature to de-differentiated (Tagami, et al., 1986). The reason why FCS regulates cell survival and

differentiation is still unknown, but Tagami, et al., (1986) found that VSMC cultured in absence of FCS are well differentiated and retain their contractile activity, but degenerate after eight days in culture; when cultured in 20% FCS, the cells were totally de-differentiated and actively proliferating, whereas in 10% FCS, they appear to be "just in the process of differentiation" (Tagami, et al., 1986). The cells cultured in 10% FCS also showed many gap junctions (Tagami, et al., 1986; Blennerhassett, et al., 1987), present in differentiated cells (no FCS) but absent from cells cultured in 20% FCS (Tagami, et al., 1986). A 10 % FCS concentration was used in our cultures.

The results summarised above support the use of cultured cells which originated from explants of vascular wall: the cells are mature VSMC, first passage only, density corresponding to the beginning of the plateau phase of the growth curve (between 15 (WKY) and 30 (SD, SH) $x10^4$ cells/cm^2, Blennerhassett, et al., 1989), grown in presence of 10% FCS and 1 ng/ml NGF (so that it is a complete control of the co-cultures, supplemented with NGF to insure SCG neurones survival), and irradiated (with 5 krads).

Irradiation procedures had never been used with VSMC, so that the effect of irradiation on the membrane electrical properties of these cells had to be assessed. The membrane properties and morphology of VSMC from first passage cells in irradiated single cultures are similar to those of cells migrating directly from the explant. As shown in the RESULT section, this experimental protocol generated reproducible, stable cultures in which the irradiated A and

MA VSMC demonstrated mature morphology and membrane properties (RMP and MIR), constant over time and similar to non-irradiated cells in vitro (with similar densities) and within the range of values found for smooth muscle cells in different tissues (Campbell, et al., 1981; Cheung, 1984; Bryant, et al., 1986; Blenner-hassett, et al., 1989; Fujii, et al., 1989; Shimamura, et al., 1989). This protocol also demonstrated clearly that the RMP of cultured VSMC is dependent on cell density and not on time in culture. The irradiated cells were able to survive up to 47 days in culture; this survival time is ample enough to allow the interaction between VSMC and neurones to take place. The limited survival time could be explained either by the limited amount of an unknown factor present in FCS (see differentiation vs. limited survival time in relation to the percentage of FCS in culture medium, Tagami, et al., 1986) and necessary for the survival of differentiated cells, or, more likely, by the turn-over period of a molecule essential to the survival of differentiated VSMC, which would either be lacking from the culture medium or not be synthesised by the irradiated cells.

There is currently no explanation for the different growth patterns noticed between A and MA tissues. One possibility is that fibroblasts are more numerous in MA than in A wall and define areas unfavorable to VSMC adhesion, possibly related to their ability to prevent cell division (Chamley, et al., 1977). What is important, though, is that the difference is noticed in explants as well as in first passage cultures, irradiated or not. This reinforces the fact that

irradiated first passage cells show characteristics (membrane properties and growth patterns) similar to explanted non-irradiated cells.

Also, the VSMC (A, MA, first passage or explanted, irradiated or non irradiated) show a membrane response (depolarisation to low doses and hyperpolarisation, to higher ones) to added exogenous neurotransmitter (norepinephrine) as they do in vivo (Hirst, 1977; Hottenstein and Kreulen, 1987; Hirst and Edwards, 1989; Fujii, et al., 1989). This part of the study was not extensive enough to identify a difference in sensitivity to norepinephrine between A and MA tissue, but there appear to be similar cell responses to similar doses of norepinephrine. One might have expected an augmented response to neurotransmitter in MA, because it is highly innervated in situ whereas A is not. Results from studies of early synaptogenesis (Anderson, 1986; Rotter and Frostholm, 1988) have suggested that neurotransmitter sensitivity is induced by nerve cell presence, activity, and reciprocal interaction with the target cells. If this lack of difference in sensitivity was confirmed by a more extensive study, it could mean that mature VSMC in single cultures would express a basic genetic ability to synthesise receptors (ability to respond to neurotransmitter), but would be devoid of any previous neural influence (changes induced in situ). These nerve-induced changes in VSMC membrane electrical properties would, thus, be labile and disappear during the active cell division phase of the culture.

Thus, using VSMC from the first passage, at confluent density and preventing cell division by irradiation, yielded cells as close to the mature in situ situation as possible, as shown by their morphology, RMP, response to exogenous transmitter, and differential growth pattern.

I.b. Cultured nerve cells.

Irradiation was necessary to prevent VSMC division, but also to prevent the division of SCG accessory cells. In older neuronal cultures and VSMC co-cultures, ganglia accessory cells formed a monolayer which eventually lifted from the bottom of the dish, carrying the nerve cells with them in the process; this confirmed a previous observation by Gilad and Gilad (1987). Irradiated SCG neurones showed membrane electrical properties (MIR, $E_{m.thr}$, AP generation and AP_{ampl}) and a development over time (preferential association with non-neuronal cells, extension of neurites, cell body growth and flattening, nucleoli size and number, and regrouping in pseudo-ganglia structures) similar to non-irradiated neurones and to those described in similar studies of irradiated neurones (Mains and Patterson, 1973; Patterson and Chun, 1977; O'Lague, et al., 1978; Adler and Black, 1985; Furhspan, et al., 1986 a). Interestingly, these characteristics for cultured irradiated SH SCG neurones are also very similar to those described in SH neurones from freshly extracted ganglia (Yarowsky and Weinreich, 1985). The difference in average RMP between the two studies can be accounted for by an easier

access to the cell in culture (cleaner penetration) and possibly to spatial factors (different shape of the cell when isolated than when in a ganglion), although the range of recorded RMPs is similar to the range obtained in other studies of cultured neurones (O'Lague, et al., 1978 a).

The fact that the first AP in cultured neurones is only partially inhibited by TTX and show a strong calcium component confirms previous results in cultured SCG principal neurones (O'Lague, et al., 1978) and agrees with results from studies using other nerve cells (Ahmed, et al., 1983; Hirst and McLachlan, 1986; Moghina and Stjarne, 1989), in which a progressive loss of spike sensitivity to TTX in neurones in situ between days 7 and 14 post-natal was described. It remains to be confirmed that they are not observing a calcium spike, as might be the case in our study; the partial inhibition by TTX demonstrates that the first spike is carried partially by sodium, partially by calcium.

Following the end of a depolarising stimulus, the irradiated cultured nerve cells showed four different membrane responses: AHP (2 different durations), ADP, and simple passive current decay. Cells with a large RMP (-65 mV and lower) tended to show an ADP, while cells with a lower RMP (-55 to -65 mV) tended to show an AHP. Also, the amplitude of ADP and AHP appeared to be related to the RMP. The reversal potential of AHP/ADP occurrence (around -70 mV) is similar to previous observations (around -80 mV), within the range of the equilibrium potential for potassium (O'Lague, et al.,

1978; Brezina, et al., 1987; Christian and Weinreich, 1988; Poulter and Padlen, 1989). Also, in the results of Nagaro and Cooke (1987) with the crab (X organ), one notices that the only ADP observed is in a neurone whose RMP is -87.7 mV, while other neurones show lower RMPs. White, et al., (1989) notice a small ADP (DRG neurones) whose amplitude is greatly increased when the cell holding potential is switched from -60 to -80 mV. Thus, most studies show that potassium ions are likely to carry the current involved in ADP/AHP.

The term AHP appears to cover a complex situation with many variations between different neurones and animal models. Three broad categories of AHPs can be described: a brief undershoot (AHP proper, approximately 50 msec duration, 4 to 10 mV max. amplitude), a long duration AHP (LHP, up to 500 msec, same maximum amplitude) and a very long after-hyperpolarisation (VLHP, one second or more, same maximum amplitude) (O'Lague, et al., 1978; Brezina, et al., 1983; Yarowsky and Weinreich, 1985; Weinreich and Wonderlin, 1987; Christian and Weinreich, 1988; Poulter and Padjen, 1989). Two problems are related to the analysis of this result: the three types of hyperpolarisations (AHP, LHP and VLHP) may occur simultaneously and the amplitude and duration of hyperpolarisation might be reduced (or modified) in vitro (regenerating B cells, bull frog sympathetic ganglion, Kelly, et al., 1988).

The duration of VLHP seems to be Ca^{++}-dependent, but its maximum amplitude is insensitive to TEA (Brezina, et al., 1987).

VLHP is inhibited by histamine, prostaglandins or cyclic-AMP, which augment the cell excitability to successive stimuli (Brezina, et al., 1987; Weinreich and Wonderlin, 1987; Christian and Weinreich, 1988). It is also temperature-sensitive and disappears between 35 and 24 degrees Celsius (Christian and Weinreich, 1988), and, thus, was not recorded in our preparation (room temperature). The **LHP** duration, however, is sensitive to inhibitors of $K_{(Ca)}$-channels: apamin reduces it in neurones of the locus coeruleus (Osmanovic, et al., 1990) and the motor cortex of the cat (Szente, et al., 1988); TEA has the same effect on frog myelinated axon after multiple APs (Poulter and Padjen, 1989) (but changes AHP to ADP after a single AP (Poulter, et al., 1989)). **AHP** was shown to result from the activity of a Na^+-dependent potassium conductance (Poulter and Padjen, 1989); it was not inhibited by apamin or TEA in our study (see results section), confirming the results of other studies (Osmanovic, et al., 1990; Poulter and Padjen, 1989). One would have to assume, then, that the first period (approximately 50 msec) of an after-hyperpolarisation always include the AHP, whether or not LHP or VLHP are present.

In summary, the after potential hyperpolarisation seems to include $K_{(Na)}$ (AHP), apamin sensitive $K_{(Ca)}$ (LHP) and cyclic-AMP modulated $K_{(Ca)}$ (VLHP) channels.

Overall, the general properties of cultured nerve cells and the identity of their neurotransmitters (catecholamines, acetylcholine, serotonin or purines) have been shown to be nearly identical to their

in situ counterpart (Mains and Patterson, 1973 a, b, c; Bunge, et al., 1974; O'Lague, et al., 1978 b, c; Potter, et al., 1983; Nishi and Willard, 1985; Willard and Nishi, 1985 a, b; Feldstein, et al., 1986; Furshpan, et al., 1986; Kelly, et al., 1988; Sah and Matsumoto, 1987).

As for VSMC, part of neuronal maturation appears to depend on cell density (Mains and Patterson, 1973 c. p.364), possibly through cell to cell contact (Adler and Black, 1985). Some modifications of the membrane properties of cultured neurones have been shown to depend on the presence or absence of ganglionic accessory cells; even though they do not develop as close a contact as in situ (O'Lague, et al., 1978 a), they may influence the main neuro-transmitter status (Mains and Patterson, 1973 a, b, c; Patterson and Chun, 1977) and they seem to modify the metabolism of neuropeptides such as substance P and neuropeptide Y (Kessler, et al., 1984; Freidin and Kessler, 1989), which are known modulators at the neurovascular junction (Brattstrom, et al., 1986; Westfall, et al., 1987; Scott, et al., 1990). Also, they prevent the induction of acetycholine receptors in nodose ganglia neurones (Cooper, 1987) and their absence is correlated with the appearance of multiple firing in SCG principal neurones (O'Lague, et al., 1978 a). Because accessory cells are part of the ganglion in situ and because their absence modifies the membrane electrical properties of the neurones and their neurotransmitter status, the presence of accessory cells in culture was insured by irradiating all cultures only three to five days after plating; if irradiated before, the neurones grew in the virtual

absence of accessory cells, which correlates with the appearance of multiple firing (O'Lague, et al. 1978 a). Irradiation allowed accessory cells present in the dish to survive but prevented them from overtaking the cultures (covering the neurones and ultimately lifting them from the bottom of the culture dish), thus allowing long-term studies of VSMC-neurone interaction.

In summary, even if irradiated cultured cells (VSMC and neurones) show some slightly modified properties when compared to their in situ counterparts, their characteristics appear to be mainly defined by their genetic make-up and are not induced by the irradiated culture status. These properties remained stable for 6-7 weeks. Thus, their study will give valuable information on their normal properties as well as the modifications brought about by a genetic disease.

I.c. Co-culture.

All the interactions described above between glial cells, target cells and neurones in co-culture are well-established as early as two weeks after plating (O'Lague, et al., 1978 a; Potter, et al., 1983; Furshpan, et al., 1986 a). Thus, even if the development of the neuro-effector junction or the status of neurotransmitter were not assessed, the relationship between neurones and VSMC and its possible effect(s) on the membrane properties of the two cell types were well established by the time our observations began, two weeks after plating, as confirmed by the neuro-vascular junctions

noted in the electronmicrographs of the co-cultures. This period of time also corresponded to the time course of neurones maturation in vitro, which is similar in co-culture and single cultures (see previous section).

II. Cells from hypertensive animals in culture.

II.a. VSMC.

Several studies demonstrated the requirement of functional peripheral sympathetic nervous system in the development of hypertension (Johnson and Macia, 1977; Lee, et al., 1986; Mulvany, 1986; Bhalla and Sharma, 1986) and in the expression of post-junctional adrenoceptors mediating the pressor effect of noradrenaline (Folkow, et al., 1972; Yamori, et al., 1972; Johnson and Macia, 1979). Some studies even suggested a greater innervation and neuronal uptake of noradrenaline in the SH rat vasculature, compared to WKY's (and WKY only) (Mulvany, et al., 1980; Laher and Triggle, 1984). Laher and Triggle suggested that the sympathetic nervous system was responsible for the elevated vascular NA sensitivity shown in some vascular beds from SH rats since it could not be demonstrated in poorly innervated tissue such as the aorta (Laher and Triggle, 1984). One aspect which has not been considered in these studies is that the modifications could originate from the VSMC alone, even if the presence of the nervous system is necessary to reveal it. Indeed, VSMC from SH rats have demonstrated different

properties than VSMC from normotensive animals (Suzuki and Twarog, 1982; Cheung, 1984; Westfall and Meldrum, 1985), including a hypersensitivity to neurotransmitters (Tsuda, et al., 1984, 1988 a; Phelan and Simpson, 1986; Nickols, et al., 1986; Bhalla and Sharma, 1987; Cline Jr and Yamamoto, 1987; Fouda, et al., 1987; Yamamoto and Cline Jr, 1987). Also, these modifications could possibly eliminate the cell sensitivity to a relaxing co-neurotransmitter (or neuro-modulator) of a non-adrenergic non-cholinergic nature (i.e. peptidergic or purinergic). A non-adrenergic non-cholinergic vasodilatation has been reported in various blood vessels (Hughes and Vane, 1970; Kalsner, 1974; Duckles, 1979), using such mediators as vasoactive intestinal peptide (VIP) and opioids (Gaddis and Dixon, 1982; Kuriyama and Suyama, 1982; Crowe, et al., 1983; Illes, et al., 1985 a, b; Shepherd and Vanhoutte, 1985; Westfall and Meldrum, 1985; Kannan and Seip, 1988). Another possible explanation for a modified neuro-vascular junction is the production of trophic factors which could modify the nerve cell physiology (see previous section).

VSMC from SD, WKY or SH origin did not show any obvious differences in their membrane properties. There was also no difference between A and MA VSMC. Sensitivity to NE was also apparently not affected by the origin of the cells. This would be in disagreement with experiments showing a hypersensitivity to neurotransmitter in vivo (Ekas and Lokhandwala, 1981; Masuyama, et al., 1986; Suzuki, et al., 1986; Bhalla, et al., 1987; Cline Jr and Yamamoto, 1987; Yamamoto, et al., 1987; Ek, et al., 1989), unless the

augmented contractility does not translate into augmented depolarisation. It would agree, however, with studies which attribute the hyperactivity of the neuro-effector junction to the hyperactivity of the neurones accompanied by an increase of neurotransmitter release (Ekas and Lokhandwala, 1981; Tsuda, et al., 1984; Masuyama, et al., 1986; Zhang and Westfall, 1986; Kawasaki, et al., 1987; Nyborg and Bevan, 1988; Westfall, et al., 1987; Tsuda, et al., 1990, a, b), or hyperinnervation (Shimamura, et al., 1987, 1989; Cassis, et al., 1988). The study of norepinephrine sensitivity, however, was not extensive enough to make definite conclusions; the objective was only to show that irradiated cells were sensitive to neurotransmitters. Also, all cultures are supplemented with NGF, to insure nerve cell survival, and FCS: all the effects of NGF or FCS on VSMC are not known, some of them could protect, mask or modify some cell membrane properties.

In our experiment, neurones in co-culture seemed to mature and extend their neurites faster than in single cultures, but this result was impossible to quantify and simple physical factors (such as adherence) might explain that difference. There were no marked differences in most membrane properties between SCG neurones from SH, WKY and SD, with one exception: multiple firing was the only SH neurone property which could be related to the genetic hypertensive origin of the neurones. It is discussed in the next paragraph.

II.b. Neo-natal SCG neurones from SH, SD and WKY.

Most cultured SCG neurones from genetically determined hypertensive (SH) neonatal rats show a lack of accommodation (i.e., show multiple firing) to a long duration depolarising pulse. This result is in agreement with the results of Yarowsky and Weinreich (1985) in the young adult freshly extracted whole SCG, and suggests that it is a fundamental genetic modification of the nerve cell membrane properties and not an adaptive modification brought about by an increase in BP (this will be discussed in a further section).

This is the only difference between SH and WKY and SD neurones which can be related to the hypertensive state. The differences noted between strains for the $Rate_{depol}$ and $Rate_{repol}$ (WKY < SD = SH), MIR_{pl} (WKY > SH > SD) and I_{thr} (WKY > SD = SH) cannot be correlated to the hypertensive state; in fact, they show that WKY is the strain different from the others for these variables. This emphasises again the fact that WKY is not an absolute control for SH and that a third (genetically unrelated) strain, such as SD, has to be included to be able to suggest a correlation between cellular modifications and the hypertensive state. Previous studies already stressed that fact in relation to growth rate (Blennerhassett, et al., 1989) and efficacy of transmission of impulses in SCG (Yarowsky and Weinreich, 1985). Two possible reasons can explain why WKY physiology and cell characteristics might differ from SH as well as the general rat populations: first, the technique of genetic

inbreeding, necessary to select a homogeneous population of recessive genes (such as the ones of hypertension) tends to also co-segregate genes which are expressed together but still are unrelated to hypertension per se (such as salt sensitivity and hypertension) (Watt, et al., 1985; Falkner, et al., 1986; Harrap, et al., 1986; Horan and Lovenberg, 1986; Horie, et al., 1986; Schlager, et al., 1986; Schofield, et al., 1986; Luft, et al., 1987; Williams, et al., 1987; Youngue and Myers, 1988; Folkow, 1989); second, the individuals from WKY strain (but not the SH strain) have been shown to be genetically heterogeneous (DNA printing), including a sub-group with elevated BP, (Kurtz, et al., 1989) and to demonstrate various basal cardiovascular patterns (Henry, et al., 1990). To remedy that situation, one should either study the transmission of the changes and its correlation with BP using F2 generation and backcross breeding (Harrap, 1986; Bruner, et al., 1986) or include a third, genetically unrelated strain, which was the solution chosen in this study.

Thus, the presence of multiple firing in WKY neurones should not be surprising, if one is to assume that a strong possibility exists to find (bordeline) hypertensive individuals among the WKY substrains (see previous paragraph). Also, ganglia from one, two or even sometimes three litters were pooled for each nerve cell extraction. The proportion of multiple firing cells may, therefore, reflect the proportion of hypertensive pups, as suggested by Yarowsky and Weinreich (1985). The presence of a similar

proportion of multiple firing neurones in SD nerve cell cultures suggests that either all strains of so-defined normotensive rats have a similar proportion of naturally occurring spontaneously hypertensive individuals or that there are two populations of neurones in rat SCG, one with normal firing properties, the other showing a lack of accommodation (in view of the co-culture results, this conclusion is favored).

In the latter case, one would then have to infer two different mechanisms to generate multiple firing: one for the sub-population and one for the genetic variation in SH neurones; this hypothesis is supported by different firing frequencies and different sensitivities to the presence of A VSMC (see section IV.).

Another possibility is that more of the neurones in this sub-class would be allowed to survive in the SCG of the SH strain, to the detriment of normally accommodating neurones, therefore reversing the respective proportion of the two populations. The existence of two subclasses of neurones with different survival rates in the SCG of the SH and WKY or SD rats is possible since: 1) a hyperinnervation of the blood vessels has been suggested in the SH rat (Shimamura, et al., 1987, 1989; Cassis, et al., 1988), with a possible increased survival rate of neurones from the multiple firing subclass; 2) this, in turn, would imply the existence of a permissive (or protective) factor protecting specifically these cells from death in the SH rats; it could be a circulating factor secreted by a) the parathyroid gland (Zachariah, et al., 1988, Kaneko, et al., 1989; Pang and Lewanczuck,

1989), b) the kidney (Retting, et al., 1989), or c) the target cells (VSMC) (Bhatnagar and Meaney, 1989); 3) target cells, VSMC in this case, have been shown to produce NGF (Donohue et al, 1989), and, thus, be able to influence nerve cells survival, but also membrane characteristics and activity, although no known mechanism is currently postulated (Aletta, et al., 1988, see also previous section); 4) anti-NGF antibodies have to be used in addition to guanethidine to obtain a complete sympathetic denervation in the SH rat, suggesting an additional protection by NGF against guanethidine for a sub-group of SH sympathetic neurones (Johnson Jr and Macia, 1979; Lee, et al., 1986; Mulvany, 1986; Bhalla and Sharma, 1986); and 5) the protective effect could be achieved by any factor increasing internal calcium, since the beneficial effect of potassium on neurones survival was shown to depend in fact on the increased internal calcium concentration that it generates (Collins and Lile, 1989).

However, the reversal of the proportion of multiple firing and non-multiple firing neurones is such that it cannot easily be explained by the action of a permissive/protective factor. The co-culture studies show no overt modification of neuronal electrical activity (by MA VSMC), neither did it induce massive cell death (by MA or A VSMC) so that the potential influence of the VSMC on neuronal population would have to take place before birth and be permanent. In addition, the SNS is immature at birth (Judy, et al., 1976; Smith,et al., 1982; Smith, 1985; McCarthy, 1986) and some organs (such as the heart) are not innervated yet (Owman, et al.,

1972; Smith, et al., 1982). The blood vessels are also immature during that period and the nerve cells terminals are still located far away from the layers of differentiated VSMC (Lee, et al., 1988), making a drastic regionalised interaction between VSMC and sympathetic neurons less likely. In addition, the maturation of the SNS takes place only during the post-natal period (Purves, et al., 1986; Creif and Flaherty, 1988) and indeed nerve cell activity is vastly modified by the overall (pre- and post-synaptic) activity of the network they belong to (Creif and Flaherty, 1988); however, the neurones used in our study are less than a day old (i.e. long before the maturation process takes place in situ), grown in vitro for two weeks in single cultures, and still demonstrate multiple firing. Finally, studies on pre-natal animals show that only 10% of SCG neurones (Bunge, et al., 1974) and 5% of myenteric neurones (Willard and Nishi, 1985) show multiple firing, which suggests that multiple firing is not an embryonic characteristic carried over to the neo-natal period and selectively protected in the SH strain. If this was the case, SCG neurones from all strains would show the same proportion of multiple-firing neurones. The possibility remains that the membrane properties or the maturation process observed in vitro are modified during the embryonic period; only patch-clamp studies of embryonic non-cultured neurones could determine that fact.

Notwithstanding the cause, it is clear, however, that most SH neurones have lost their accommodative properties and show systematically a higher frequency of firing during the whole length

of the stimulus, whereas most neurones from normotensive strains accommodate to the depolarising pulse. It is surprising that the proportion of multiple firing neurones in normotensive strains has not been noted in many other studies. First, few studies used long-duration depolarising pulses, and so would not be able to detect multiple firing. Second, this could possibly be because even when SD or WKY neurones are classified as multiple firing in our study, their firing frequency remains much lower than for SH neurones, most frequently showing only three APs; thus, they could have been included in these studies in the extreme range of cell responses. The following quotes support this hypothesis: "Neurones grown in L-15 Air, ... , usually fired repetitively ... while neurones grown in the CO_2 media ... **usually** responded once or at most a few times ..." (O'Lague, et al., 1978) and "The ability to discharge repetitively ... was ... observed in 5 cells [out of 57] ... The other 4 cells responded only 2-4 times ..." (Bunge, et al., 1974).

III. Ionic basis of SH neurones membrane properties.

The results of my study showed that following the initial AP, the SH neurones do not accommodate the long-duration depolarizing pulse and a series of voltage-dependent calcium-spikes is generated. Voltage-clamp experiments on freshly extracted SCG ganglion from adult normotensive rats showed that the upstroke of the AP (the first AP the series in multiple firing neurones) results mainly from

the activation of a fast transient voltage-dependent Na current, I_{Na}, accompanied by a small, slowly inactivating Ca current, I_{Ca} (Belluzzi and Sachi, 1986). The resulting depolarisation activates a large, fast, transient, voltage-dependent K outward current, I_A, which is mainly responsible for the AP downstroke, is inactivated around -50mV after maximum activation, and needs MP larger than -70 mV to be fully activated (Belluzzi , et al., 1985a, 1988). During the same time period, a delayed K conductance, generating a rather small outward current, I_K, and a Ca-dependent K conductance, $I_{K(Ca)}$, generating a larger outward current, are both activated, tending to repolarise the neurone (Belluzzi, et al, 1985 a, b). This latter conductance was shown to be of approximately 200 pS in the rat SCG neurons (Smart, 1986), and thus correponds to I_c or BK in other nomenclatures (Smart, 1986, see review by Castle, et al., 1989). The interaction of these conductances in the generation of AP was studied in detail in the normotensive rat SCG neurones (Galvan and Seidlmeir, 1984; Belluzzi, et al., 1985; Belluzzi and Sachi, 1988, Nerbonne and Gurney, 1989). In addition, an outward K conductance, activated at RMP and inhibited by muscarine, hence the name I_M current, was also shown to be present in these neurones (Brown et al, 1989).

In my results, the first AP in cultured neurones is partially inhibited by TTX or high concentration of cobalt, thus shows a strong calcium component in addition to I_{Na}. This confirms previous results in SCG principal neurones in culture (O'Lague, et al., 1978 a) or in freshly extracted ganglia (Yarowsky and Weinreich, 1985),and agrees

with results from studies using other nerve cells (Ahmed, et al., 1983; Hirst and McLachlan, 1986; Brock and Cunnane, 1987; Moghina and Stjarne, 1989). Also, Ahmed et al.(1983) observed a progressive loss of spike sensitivity to TTX in neurones in situ between days 7 and 14 post-natal. TTX insensitive Na channels have been identified, but Belluzzi and Sacchi (1986) did not find TTX insensitive Na currents in rat SCG neurones. Under TTX, at normal sweep speed, one can observe an apparently normal spike; however, if the tracing is observed at fast sweep speed, an aborted spike can be noticed (shoulder on the tracing), and the fast ascending part of the (first calcium) spike (of a multiple firing sequence) is initiated only towards the end of the abortive spike, when the potential starts to decrease again. This is rarely observed in non-multiple firing neurones (no activation of the regenerative Ca^{++}-current, no calcium spike) but one can still observe a slower developing AP of diminished amplitude if the intensity of the stimulus is raised (our results, O'Lague, et al., 1978 a, Nerbonne and Gurney, 1989). This also agrees with the result of Belluzzi et al. (1985 b) in rat SCG, which showed the presence of a Ca current simultaneous to I_{Na} but of smaller magnitude, longer duration and slowly inactivating.

During a long-duration depolarising pulse, most neurones ultimately accommodate and generate a "plateau phase" following the initial spike(s). Neuronal MIR is lower during that phase, which mirrors the opening of channels. Multiple firing (i.e. regenerative spikes during the plateau phase of a single stimulus) represents a

disturbance of accommodation. Which conductance are involved in accommodation? The ability to generate firing during the plateau by superimposing a small intensity depolarisation over the first stimulus or over the electrotonic potential generated by a subthreshold depolarising pulse suggests that accommodation does not result from a voltage- , calcium-, or time-dependant inactivation of Na^+-conductances .

Could any of the conductances (or lack thereof) described in the first paragraph be involved into the lack of accommodation of SH neurones?. I_A is probably not involved for the following reasons: first, it is only partly effective in repolarising the cell at RMP between -40 and -60 mV (Belluzzi and Bianchi, 1988), which is closer to the neurones RMP in my experiments; it needs a hyperpolarising pulse (such as the AHP) to remove the channel inactivation; finally, if this conductance was inhibited, the duration of the AP, $Rate_{repol.}$ and MIR during the plateau would be higher in SH than in SD or WKY neurones: this is not so. Also, this fast conductance is likely to be largely de-activated around plateau potential, i.e. not very active during multiple firing. The lack of a role for I_A also agrees with the conclusions of Nerbonne and Gurney (1989). Following an AP, this conductance may help determine the level of excitability of the cell to a following impulse (i.e. modulate the synaptic input) by driving the MP to a more or less depolarised state (in combination with I_K $I_{K(Na)}$ and $I_{K(Ca)}$), but this is different than multiple firing during the plateau phase, especially considering the large time-scale of the

repetitive firing related to I_A conductances (1 AP/100 msec.) (Cassel and McLachlan, 1986), much more than firing frequencies observed in multiple firing (Yarowsky and Weinreich, 1985, results section). The delayed rectifier (I_K) helps bring the MP down and very likely participate to the plateau phase, and also participate in LHP. If it does, this conductance does not appear to be responsible for the multiple firing in SH neurones, since SD and SH SCG neurones show similar values for MIR_{pl}, in agreement with other studies (Yarowsky and Weinreich, 1985; Poulter, et al., 1989). Also, since AHP characteristics are similar between the three strains, one can assume that I_C and I_k currents are similar in all neurones, therefore not responsible for the lack of accommodation. The I_M current, if inhibited or absent, could depolarise the cells and help generate multiple firing; the fact that the RMP, MIR, and MIR_{pl} are similar in all strains argues against that possibility. In addition, Yarowsky and Weinreich (1985) tested the effect of muscarine on SCG neurones from hypertensive rats and found them to possess a normal complement of muscarinic receptors.

A calcium-activated potassium ($K_{(Ca)}$) conductance, different from BK, has been proposed to be responsible for accommodative properties of other types of neurones (Tillotson, 1979; Szente, et al., 1988; Alkadhi, et al., 1989; Alkadhi and Hogan, 1989; Poulter, et al., 1989; Osmanovic, et al., 1990). This is in agreement with the ability of TEA and apamin to inhibit accommodation (i.e. induce multiple firing) in cultured rat SCG neurones (our experiment) (see following

discussion). However, Smart (1987) did not find apamin-sensitive $K_{(Ca)}$ channels in rat SCG main neurones; apamin is specific for SK, which is the smallest $K_{(Ca)}$ conductance; it is possible that its detection was difficult in their experimental set-up.

Multiple firing is inhibited by cobalt and insensitive to TTX in our experiment, thus results from the activity of regenerative calcium currents. Three types of calcium channels --T, L and N, according to the nomenclature proposed by Nowycky, et al. (1985)-- are thought to be present in nerve cells (Nowycky, et al., 1985; Miller, 1987 b; Catterall, 1988; Krueger, 1989).

Under high cobalt (inhibits the first spike), only the electrotonic plateau is observed, and the Na^+-conductance is not activated (no sodium spike), either because the potential threshold is not reached or because it is dependent on calcium activation. A similar separation between low and high threshold calcium spikes and the electrotonic plateau was observed in mammillary neurones by Alonso and Llinas (1988).

Thus, it suggests that at least two different types of Ca^{++}-channels are present in cultured rat SCG neurones: one which has a low sensitivity to cobalt, a low threshold of activation (between RMP and $E_{m.thr}$) and helps bring the membrane potential from resting level (RMP) to the threshold of the sodium spike, and the second which has a higher sensitivity to cobalt, a higher threshold of activation (close to the potential of the plateau phase) and which carries the regenerative calcium current underlying multiple firing

(may be a T type, although it should be noted that, in opposition to other studies - see previous paragraphs- Nerbonne and Gurney (1989) could not detect T-type channels in whole-cell patch clamp studies of cultured SCG rat neurones..

If I_{KCa} (SK) is responsible for accommodation in rat SCG main neurones, what happens when it is not active?. Spikes can be generated during the plateau by superimposing a small intensity depolarising current on the long-duration pulse, supporting a voltage-dependent activation of Ca channels. The one or two AP induced during the plateau phase of non-multiple firing neurones are of similar amplitude to the initial (Na) spike; however, in the case of multiple firing neurones, multiple APs induced during the plateau phase usually are of somewhat smaller amplitude and seem to exhaust faster; this can be explained if, in addition to the voltage inactivation, a slow feedback inactivation of the Ca^{++}-channel by internal calcium in the proximity of the channel is effective and can be sometimes overridden by raising the stimulus intensity. These characteristics of fast transient, voltage-dependent activation and Ca^{++}-/voltage-dependent inactivation of some calcium channels types have been identified previously in various cell types (Tillotson, 1979; Standen, 1981; Lee, et al., 1985; Nowycky, et al., 1985; Dupont, et al., 1986; Alonso and Llinas, 1989; Satin and Cook, 1989; Sherman, et al., 1989; Kramer, et al., 1991). These results, thus, suggest that multiple firing is carried by a regenerative Ca^{++}-current, possibly through voltage-activated and voltage/calcium-inactivated T Ca^{++}-channels.

This hypothesis is consistent with the tendency of high external calcium or Ca^{++}-channel activator (Nowicky, et al., 1985; Bean, et al., 1986) to partially inhibit the APs generated by the depolarising pulse, since both tend to raise internal calcium; also, Yarowsky and Weinreich (1985), could inhibit multiple firing with Nickel, which is more specific for T channels than for L or N (Tsien, et al., 1987). Multiple firing is common, as found in studies on neurones and other cell types, and is generally thought to involve calcium channels of the "T" type (Yarowsky and Weinreich, 1985; Miller, 1987 b; Nagano and Cooke, 1987; Hilarey, et al., 1988). With very few exceptions, all neurones ultimately accommodate to the depolarising pulse.

The effects of two $K_{(Ca)}$ channel blockers, apamin and TEA, in non-multiple firing neurones were studied. These compounds were able to generate multiple firing in WKY and SD non-multiple firing nerve cells with cobalt, calcium, and (lack of) TTX sensitivities similar to native multiple firing SH rats SCG neurones. In another study, an inorganic potassium antagonist (cesium) induced a bursting activity in guinea-pig and rat desheated ganglia which was blocked by hexamethonium (a ganglionic-blocker); this implies that the bursting activity depended on the depolarisation brought about by the first spike (as in native SH neurones) and was not generated spontaneously in the post-synaptic neurone (Alkadhi, et al., 1989). The firing frequency induced by the specific channel blocker apamin is higher than seen in the native multiple firing neurones; this compound also induces a destabilisation of the membrane, powerful

enough to generate a few APs and some low amplitude spikes after the cessation of the stimulus. Similar observations were made by Szente, et al. (1988), using apamin intracellularly on neurones from the motor cortex, and by Poulter et al. (1989) with Dendrotoxin and TEA on frog myelinated axons. Apamin did not modify the shape of the APs, as anticipated (Castle, et al., 1989). It blocked specifically the $K_{(Ca)}$ channels (SK) involved in the generation of the plateau phase, not the $K_{(Ca)}$ channels (BK) involved in AP repolarisation. A similar selectivity for $K_{(Ca)}$ channel subtype (Kl, not Ks) and the extra-cellular side of the membrane, was suggested for TEA, which inhibits preferentially the $K_{(Ca)}$ channel subtype involved with the repolarisation phase of the AP (Bolton, 1979; Inoue, et al., 1985; Castle, et al., 1989; Gola, et al., 1990), itself not affected by apamin (Castle, et al., 1989). Indeed TEA was able to generate multiple firing (see RESULTS section) probably by blocking all I_K and $I_{K(Ca)}$ channels, but also broadened the APs and lowered the amplitude and frequency of multiple firing (when compared to apamin-induced or native multiple firing). TEA is known to block a multitude of channels (I_K, I_{IR}, I_A, BK, I_M, $I_{K(Na)}$, etc.) (see review by Castle, et al., 1989), but also to interact with other receptors, such a dopamine receptors (Alkadhi and Hogan, 1989; Drukarch, et al., 1989). Finally, multiple firing observed in our preparations appeared to be different than the one observed by O'Lague, et al. (1978 a), in nerve cell cultured in L-15 Air (no accessory cells), in that the firing frequency they observed was much higher than ours under apamin, and

multiple firing was associated with the absence of VLHP, which is absent from all our cultures conditions, because the study is carried at room temperature (see section I.c., this chapter).

In summary, multiple firing in SH neurones is probably the result of the activity of regenerative calcium currents carried by T channels; this activity is allowed to take place because of a lack of activation of $K_{(Ca)}$ (SK) conductance, which is not due to a diminished intracellular calcium availability to stimulate the $K_{(Ca)}$, but more likely to a failure of the activation process through a protein modulatory to the channel.

At the end of a stimulus, the membrane potential returns to RMP, directly or with an AHP or ADP. The after potential hyperpolarisation can be classified in three groups: AHP, LHP, and VLHP. VLHP was never observed in WKY, SD or SH cultured neurones. Only AHP and LHP were observed and cultured SH neurones did not show a different LHP/AHP ratio than SD or WKY neurones. A small increase in AHP amplitude and duration was observed in neurones showing multiple firing, related to the number of APs generated during the stimulus. This is in agreement with the results of Nishi and Willard (1985) and Poulter and Padjen (1989). It suggests that the activation of $K_{(Ca)}$ channel generating LHP are voltage dependent and may involve high Ca^{++} threshold (multiple firing increases more internal calcium than a single spike). The amplitude of AHP was proportional to RMP, with a similar reversal potential to ADP, just as with neurones from normotensive strains.

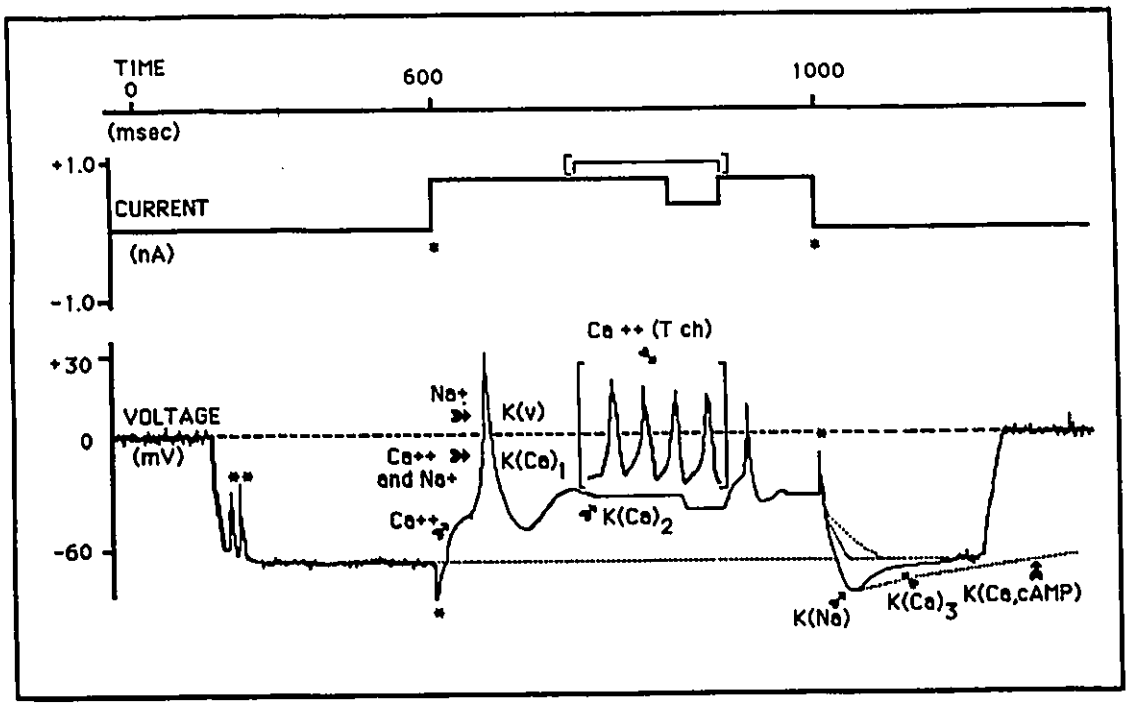


Figure 28. Hypothetical role of the different channels possibly present in cultured SCG neurones from neo-natal rats. Ca++: Calcium channels (low (Ca++ and Na+) and high (T ch) sensitivity to cobalt); Na+: fast sodium channels, TTX sensitive; $K_{(V)}$: voltage-activated potassium channels; $K_{(Ca)1}$: calcium-activated potassium channels, TEA sensitive; $K_{(Ca)2}$: calcium-activated potassium channels, apamin >> TEA sensitive; $K_{(Na)}$: sodium-activated potassium channels; $K_{(Ca,\ cAMP)}$: cAMP-modulated calcium-activated potassium channels. See text for details.

A low threshold Ca^{++}-current has been suggested to carry the ADP (dorsal root ganglia) (White, et al., 1989). A correlation between the amplitude or duration of AHP or ADP and multiple firing could not be demonstrated. The little or no effect of apamin/ TEA on AHP/ ADP, agrees with Yarowsky and Weinreich (1985) that the two events are unrelated and possibly depend on different Ca^{++} activated channel activities.

Summary: Thus, it appears that, in normotensive rat SCG principal neurones, accommodation is brought about by the activity of a $K_{(Ca)}$ channel which keeps the membrane potential of the neurones below the threshold of activation of a class of voltage-dependent, high threshold calcium channels. The modification of SH neurones membrane appears to be at the level of a sub-group of $K_{(Ca)}$ channels which are either absent or inactivated. This dysfunction is mimicked by apamin, which blocks the channels and leaves the membrane potential of the cultured neurones after an AP above the level of activation of the regenerative Ca^{++}-current. This induces multiple firing in normally accommodating neurones, with the same characteristics as native SH neurones. TEA, blocking non-specifically many Ca^{++}-dependent and voltage-dependant K^+ channels, induced multiple firing, but with broadened spikes, lower $AP_{max.}$ and a lower frequency. Because of these differential effects and since neither apamin or TEA appear to have a strong effect on AHP/ADP occurrence, (at least) three different $K_{(Ca)}$ channels are hypothesised to be present in the rat SCG principal neurones. These

channels would be involved, respectively, in the spike repolarisation (TEA sensitive), the plateau phase (apamin >> TEA sensitive) and the LHP. The possible role of the different channels in the neuronal response to depolarising pulses is summarised in figure 28. Only voltage-clamp experiments in SCG neurons from SH rats could define with more precision the specific abnormal conductance(s) responsible for multiple firing.

IV. The effect of target cells on neurones firing activity.

It is difficult to explain the effect of A and MA VSMC on the firing properties of the neurones. The in vitro co-culture system between neurones and their targets has been widely used over the years and has yielded numerous information about the interactions between SCG neurones and cardiac muscle cells (reviewed by Wollenberg, in 1985), striated muscle cells (Crain, 1970; Nurse, 1981 a, b; Haimovich et al, 1986), spinal cord neurones (Bunge et al, 1974; Ko et al, 1976) and also between red nucleus and substancia nigra neurones (Konig et al, 1989). This study presents evidence of modifications of neurones electrical membrane properties induced by the target cells in the context of genetic hypertension.

Even though the resting membrane potential value of neurones cannot be an absolute measurement, the difference between single and co- culture measurements is likely to be significant because the possibility of getting artifactual results in identical experimental

conditions is very similar. It is interesting that the presence of target cells depolarises the neurones towards RMP values (Yarowsky and Weinreich, 1985) and firing frequencies (Chan, et al., 1990) closer to values obtained in situ or in freshly extracted cells, and that these effects are also similar to the effects of high external calcium (see Result section: IIc.).

The modifications of neuronal electrophysiology induced by the presence of target cells have to be discrete, because they do not translate into important modifications of membrane properties. The end-result of these modifications, however, is important: VSMC from the aorta (a poorly innervated vessel in situ) prevent neuronal firing, while VSMC from the mesenteric artery (highly innervated) do not modify neuronal firing as it is expressed in single neuronal cultures. It is worth noting that this is another instance where WKY tissues behave in some respects more like SH than SD. Neuronal activity (depolarisation with subsequent rise in internal calcium concentration) has been shown during the neonatal period or in culture to be important for neuronal survival (Collins and Lile, 1989), neurotransmitter phenotype and field arborisation (Bunge et al, 1974; Black et al, 1984; Kessler et al, 1984; Sretavan et al, 1988), and also regulation of membrane proteins (Greif and Flaherty, 1989; Linderman and Greif, 1990). It is possible that, in situ, the aorta ends-up non-innervated because its smooth muscle cells (or a factor produced by these cells) prevent neuronal firing and, thus, cause their degeneration in the long term or put them at a disadvantage

during competitive elimination. In that respect, it would be interesting to see if there is a difference of innervation between SD and WKY rat aortas, both normotensive but with different A VSMC inhibition of neuronal firing. All our cultures are supplemented with NGF, so that the survival of SCG neurones is insured; in this respect, our results may show an intermediate step explaining the results of Creedon and Tuttle (1988) relative to the inability of A VSMC to support the survival and growth of ciliary ganglion neurones in vitro, and suggest a factor different than lack of NGF, since aortic tissue has been shown to produce large quantity of NGF, especially in young hypertensive animals (Donohue et al, 1989). Also, the VSM referred to in Pun and Ferguson's study of in vitro synaptic transmission (Pun and Ferguson, 1990) are MA VSMC and no results could be generated for A VSMC co-cultures (personal communication). Our results would suggest that it would indeed be impossible to detect synaptic transmission if the nerve cell was not firing. The results obtained with VSMC from SD cultured with WKY and SH neurones show that some effects are strain dependent and that different mechanisms are likely to be involved because neuronal firing is prevented equally in the three strains by VSMC from SD, while only WKY and SD neurones membrane properties are affected.

With respect to genetic hypertension, the presence of MA VSMC in co-culture from normotensive strains does not modify their response to long-duration depolarisation (50% or more neurones fire a single action potential). In the case of SH neurones, however, MA

VSMC shift the response of these neurones to a majority of double spikes. It is of interest to note that a similar difference in firing frequency was observed in medullary neurones in situ (i.e. with target cells present) by Chan et al (1990), who observed a majority of neurones generating single spikes in WKY while most neurones in SH would generate 2 spikes. The shift in firing frequencies distribution could be related to the rate of repolarisation of the action potential in the SH neurone in co-culture: it is augmented when MA VSMC from SH are in co-culture (with a corresponding shift towards double spikes), but it is not when MA VSMC from SD are present (with no shift in firing frequencies distribution). This is also in agreement with our suggestion that multiple firing in SH neurones is dependant on a deficient calcium-activated potassium conductance allowing the expression of a regenerative Ca^{++}-current, because potassium conductances are known to participate in spike repolarisation. Thus, it is possible that, in SH neurones, an augmented potassium conductance during spike repolarisation in the presence of MA VSMC partly compensates for the lack of calcium-activated potassium conductance, insuring a faster accommodation. This would not happen with SD and WKY neurones because of their already normal potassium conductance activity. Alternatively, if calcium indeed stabilises cell membranes, and if SH cells bind less of it (Cirillo, 1990; Bohr, 1989; Tsuda, et al., 1990 b) and if NGF (or NGF-like factor), produced by MA SH VSMC, inactivates some Ca^{++}-

currents (Rausch, et al., 1990; Streit and Lux, 1990), then SH MA VSMC would be able to diminish Ca^{++}-dependent multiple firing.

It is quite difficult to explain the inhibition of neuronal firing by A VSMC. Whether complete (SH, WKY) or partial (SD), it seems to be present in all strains. In addition to the results described in detail for SH, WKY and SD rats, Wistar SCG neurones in co-culture with WKY A VSMC also showed a 95% inhibition of firing (n=51). To our knowledge, it is the first time that such an inhibition is reported. It cannot be due to the cell depolarisation at rest, because there is no difference in neurones RMP between A and MA VSMC co-culture, so it is likely to involve a different mechanism. It appears to be specific for action potential generation, since all other variables measured for the action potential (of the remaining firing neurons) present in A VSMC co-cultures are similar to MA VSMC co-cultures or neuronal single cultures. It appears to involve a mechanism different from the inhibition of multiple firing, because there is no shift in distribution of firing frequencies when the inhibition is partial (as in co-cultures of neurones from any source with A VSMC from SD). Finally, it appears to be strain-specific, since A VSMC from SD inhibit SH and WKY neurones differently than their respective A VSMC do, but produce an inhibition similar to that seen in SD neurons. The shift in frequency and the inhibition of firing could be attributed to selective cell death, but we do not believe it to be the case because there was no marked difference in cell densities between the different co-cultures, no traces of active cell death were noted, and

finally, the non firing neurones had a healthy appearance and a normal RMP, as well as an apparently normal supply of neurites. One hypothesis is that cultured A VSMC generate a negative growth factor, direct inhibitor of spike generation (aortic cells inhibitory factor, or ACIF). Another possibility is that NGF (or NGF-like factor) may induce in neurones alteration in Ca^{++}-channels such that they are inactivated to a greater extent than usual, in a manner similar to the effect of NGF on PC12 cells (Rausch et al, 1990; Streit and Lux, 1990). If NGF was produced in excess by A VSMC (unlikely) or if A VSMC could modify the NGF receptor on the neurones (augment their affinity, for example), and taking into consideration that the first spike generated by depolarising pulses is partly sodium and partly calcium dependent, it is possible that firing be inhibited over time through an NGF-mediated inhibition of calcium channels. Because of the similar effect of A VSMC from SD on SH, WKY and SD neurones and because A WKY also completely inhibit firing in Wistar neurons, the sensitivity to this hypothetical factor does not apparently lie in the neurones, but appears to be dependent on A VSMC. In summary, there is currently no available explanation in the literature for the effects of A or MA VSMC on neurone firing in culture.

All the results combined tend to describe a sub-population of multiple firing neurones (10-20%) which is present in all strains. It could represent a normal cell lineage present throughout the body in various proportions, such as 10% (spinal cord) (Bunge et al, 1974), 2% (RVL Medulla) (Chan et al, 1990) or 5% (neonatal myenteric neurons)

(Willard and Nishi, 1985). Thus, there would be two populations of multiple firing neurones in the SCG of the SH rat: 10-20% normally occurring and 40-55% of genetically abnormal neurones, for a total of 50-80 % of multiple firing neurones. These results also show different effects of the target cells on neurone physiology which are general (all VSMC target cells), tissue specific (A vs MA VSMC), and strain-specific. More studies are needed to determine the exact nature of the ionic change(s) underlying the changes in membrane electrical properties, which channels are affected, and the nature of the diffusible or membranar VSMC factor(s) which are responsible for these changes.

V. Multiple firing in the SH rat and relation to hypertension therapy.

The modification of the membrane electrical properties expressed in irradiated cultured neo-natal SH neurones is similar to those of neurones of freshly extracted whole ganglia from young adults SH rats (Yarowsky and Weinreich, 1985), although no mechanism for the generation of Ca^{++}-dependent multiple firing was suggested in that study. These results suggest that multiple firing in these SH neurones result from a genetic modification, present before the establishment of hypertension. How can this modification of the electrophysiology of the principal SCG neurones be related to the other cellular modifications observed in the SH rat?.

First, a set of results give direct support to similar modifications observed in the rostro-ventro-lateral medulla, where there is an important shift in the spontaneous activity of WKY and SH neurones: 98% of WKY neurones spontaneously generate single isolated spikes, while the remaining 2% generate double spikes; the proportions in SH are 45% and 55%, respectively, and the spontaneous firing is more frequent and more regular (Chan, et al., 1990). This is important because the medulla is thought to be directly involved in BP regulation and this change in firing frequency could be amplified along the BP regulation network.

Iijima et al. (1990) detailed how acetylcholine could act on (rabbit) SCG principal neurones: low concentrations act on M1 muscarinic receptors, stimulate calcium release from intracellular store, which activates a $K_{(Ca)}$ channel, which, in turn, generates an outward potassium current (hyperpolarising the cell); higher concentrations act on M2 muscarinic receptors, which activate cations selective channel, generating large inward sodium and calcium currents (depolarisation). If the $K_{(Ca)}$ channel is inoperant, the small doses of acetylcholine (such as released by spontaneous activity of the pre-synatic neurone) would not hyperpolarise the neurone (it may even depolarise it slightly) and would raise cytosolic calcium, making the neurone hyper-reactive to the (following) higher concentrations of acetylcholine and, in the long term, modifying the ionic homeostasis of the cell. The nature of the neurotransmitter at the neuro-vascular junction (and thus the final effect of

neurotransmission) has also been shown to be dependant on neuronal firing frequency (Hottenstein and Kreulen, 1987). An exaggerated multiple firing-like activity is identified in SH portal vein, which normally shows spontaneous rythmic activity (Shimamura, et al., 1989).

If one was to summarise the different cellular and organ malfunctions identified in the introduction, one could say that the common problem is centered around calcium, and that the malfunction is either hyperactivity, hyperreactivity (see Introduction), or synthesis malfunction (Koutouzov, et al., 1987; Akbar, et al., 1989; Bennett, et al., 1989) (in the parathyroid, the "HPF" (hypertensive parathyroid factor) described by Pang and Lewanczuck (1989) could easily be an immature form of parathyroid hormone). Calcium is a common factor to all these cellular dysfunctions, so that a single, genetically defined alteration of one Ca^{++}-dependent molecule could be enough to explain most of the varied cellular dysfunctions present in hypertension. The expression of the cellular dysfunction would vary with the presence or absence of that molecule in the cell and the use of that molecule in the cell function. The suggestion by Dupont, et al. (1986), that all calcium channels might be regulated by a single calcium-binding protein, such as calmodulin/troponin C (possibly modulating fast-activated K current in molluscan neurones) (Muller, et al., 1989) would support that hypothesis. Along the same reasoning, Chao and Chao (1988) found a total absence of a kallikrein binding protein from many

organs (as different as the CNS, heart, thymus, lung, liver, prostate, kidney, pancreas, Cowper gland and adrenal gland) of SH rats; or, at the least, the protein is modified in a way that it can not be detected by the same means as its regular form. Such a generalised absence could mean that the same malfunction is present in all cell types usually synthesising this molecule.

How would an altered $K_{(Ca)}$ channel activity modify the different elements of the cardiovascular system?. First, the hypothetical common (deficient) protein would interact with different calcium dependent intra-cellular pathways. The functional expression of the dysfunction, thus, would vary with the cell use of that pathway combined with the cell's overall function and its current state of activity. SH SCG neurones, for example, would not behave differently from WKY neurones if the plateau phase was the result of a sodium conductance inactivation instead of an activation of $K_{(Ca)}$ conductance; also, multiple firing cannot be detected if the cell is at rest or depolarised with a short (20 milliseconds or less) duration pulse.

This hypothetical common dysfunction could, thus, explain the varied cell physiology abnormalities in SH. Can multiple firing explain genetic hypertension?.

Let us consider the path between the CNS and the blood vessel. All CNS nuclei involved in BP regulation show an already described multiple firing activity in SH or the presence of $K_{(Ca)}$ channels (apamin sensitive), which could be deficient in SH. In the RVL, $K_{(Ca)}$

activity is modified so that the cells spontaneously fire more frequently and more regularly (Chan, et al., 1990); in the **NTS**, 60% of the $K_{(Ca)}$ current is insensitive to the blocker 4-aminopyridine, yet there is a small apamin sensitive $K_{(Ca)}$ current and a large charybdotoxin sensitive one (Moak and Kunze, 1989); and the **locus coeruleus**, where intra-cellular apamin augments the spike frequency (Osmanovic, et al., 1990).

The augmented response of these central neurones to their pre-synaptic input would be the first level of amplification of the original signal (assuming for now that the signal is one of normal amplitude).

This amplified signal would be further amplified by stimulating an hyper-reactive sympathetic neurone (Debinsky and Kuchel, 1989) which does not accommodate (our results, Yarowsky and Weinreich, 1985). The silent period in ganglionic transmission is diminished (Schramm and Barton, 1979), the post synaptic neurone fires more often and in bursts; Cesium (inorganic potassium channel antagonist) induced multiple firing (in the SCG) which generated large compound APs in the post-synaptic neurones following pre-ganglionic stimulation (Alkadhi and Hogan, 1989). The ganglion would be the second level of amplification.

Further amplification occurs at the neuro-effector junction. Large compound APs in the sympathetic nerves would in turn induce giant EJPs (VSMC) (Moghina and Stjarne, 1989) because of a frequency dependent, TTX insensitive, augmented release of

neurotransmitter (Brock and Cunnane, 1987; Moghina and Stjarne, 1989). This firing in burst has been shown to be more efficient to induce vasoconstriction and changes in blood flow/volume than a continuous firing with the same average frequency; ultimately, it is more efficient to induce vessel wall hypertrophy (Lacroix, et al., 1988; Flemming and Yuan-Yuan, 1989; Baumbach and Heistad, 1991). Also, the nature of the neurotransmitter(s) released may vary (See section I.2., this chapter). This would be the third level of amplification.

The augmented release of neurotransmitter would act on an hyperreactive blood vessel wall, because of VSMC hyperplasia and/or augmented sensitivity to neurotransmitters (see Introduction). The sustained calcium current which provides more of the calcium movement in SH VSMC (Rusch and Hermsmeyer, 1988) could be explained by a lack of potassium outward current similar to the deficiency of $K_{(Ca)}$ current which induces regenerative Ca^{++}-current in multiple firing neurones. This would be the fourth level of amplification.

This pattern of activities would explain the increased norepinephrine overflow observed in young hypertensive animals (Ekas and Lokhandwala, 1981; Esler, et al., 1989) as well as the elevated blood pressure and hypertrophied blood vessel without the need of a general reflex hyperactivity of the sympathetic nervous system due to hyperactive afferents. Only a single cellular dysfunction is necessary to generate such a pattern.

Furthermore, if the genetic change is extended to all neurones, it could explain the confusing results relating to the CNS. In some cases, a frequency-dependent (bursting activity) inhibition of nerve conduction (block) has been demonstrated (Hilarey, et al., 1988, McCormick and Feeser, 1990). It was shown not to be the case in the PNS (previous section). Thus, an increased firing ability might silence some part of the central network, possibly explaining the lack of, or the difficulty in, detecting a sympathetic hyperactivity.

Finally, if the dysfunction is generalised to the organism and considered over a long period of time, the complexity of the pathology becomes staggering. Ionic imbalance in the cell could be a side-effect of the original default but would become a complicating factor over time, changing cell reactivity, membrane fluidity, homeostasis and metabolism. Hyperactive nuclei in the CNS could also modify the CSF composition, either by "dumping" neurotransmitters or their metabolites in it, or by modifying its ionic content. This might represent a further complication by tuning up or down the overall activity of neighbouring nuclei or the spinal reflexes. Also, secretory cells could see their synthesis and secretory processes disturbed; in that category, two main elements of BP control should be singled out: the parathyroid and the kidney. The parathyroid could produce a modified or immature PTH because of a secretory process accelerated by high cytosolic calcium and/or modified membrane fluidity; this factor would correspond to the PHF described by Pang and Lewanczuck (1989). The kidney has been

shown to secrete thromboxane in excess (Purkenson, et al., 1986), which is known to stimulate cell VSMC division (Ishimitsu, et al., 1988), not to mention a plethora of other possible hormonal actions by the kidney and the adrenal glands.

This model can also explain why calcium handling anomalies have been detected in numerous cell types of SH rats, some of which have apparently no relation to BP regulation (Lovenberg, 1986; Rapp, et al., 1986; Hermsmeyer, 1987; Lucas, et al., 1989; Cirillo,1990; Drueke et al, 1990).

How this theoretical framework fits the developmental aspect of hypertension ?.

First, the cardiovascular system is minimally challenged in utero, so that each cell type shows an actvity which is mainly the result of intrinsic genetic and circulating factors. The nervous system is immature at birth and its maturation is altered in SH rat (McCarthy, 1986; Smith, et al., 1982); some organs (such as the heart) are devoid of adrenergic innervation until 5 wks post-natal (Owman, et al., 1971 [in Mark, et al., 1973]). The shift to mature SCG adult trans-synaptic pattern occurs during the first week of life (Creil and Flaherty, 1988), but plastic changes still occur over three months and possibly throughout life (Purves, et al., 1986). The rise in BP noted by Gray (1984) during the early pre-hypertensive phase of hypertension can be explained by a hyper-reactivity of the artery wall to stretch (genetic calcium metabolism modification) and/or by

occasional multiple firing neurone activity (more neuro-transmitter liberated more often). The rise is so small that it can be detected only by using a very sensitive method, such as intra-arterial canulae. Lee et al (1988) showed that the vessel wall of newborn SH rats was not hypertrophied yet, while it is at 3-5 weeks old (Lee, 1985). During the first days of life, the SNS will mature and it is known that sympathetic nerve activity influences the VSMC proliferation (Bevan, 1975) and maturation (Smith, 1985; Anderson, 1986; Vita, et al., 1988), and that, in turn, VSMC influence neurones maturation (Rotter and Frostholm, 1988).

The heightened BP, initially the result of VSMC hyper-responsiveness to stretch, would then be induced, more powerfully, by an hyperactive young sympathetic system (hightened release of norepinephrine) (Collis, et al., 1980 [in Ekas and Lokhandwala, 1981]; Tsuda, et al., 1984; Debinsky and Kuchel, 1989; Esler, 1989; Stephenson, et al., 1989). Other cellular defaults are also noted in young SH rats only (Ek, et al., 1985; Ozaki, et al., 1986). After that initial rise in SNS activity, the change tapers off around 24 weeks (Judy, et al., 1976), probably because of self-regulatory processes, such as the resetting of the cardio-vascular and sino-aortic reflexes (see dection II.c.2., Introduction), and renal compensation.

In SH rats, BP measured by the tail-cuff method starts to rise significantly above the WKY level around the fourth week. This is considered the beginning of the young hypertensive stage. This rise is likely to result mainly from baroreceptor dysfunction, the full

effect of a more mature SNS and the beginning of BP-induced compensatory modifications (hypertrophy, hyperplasia, athero- and arterio-sclercsis, etc.). Also likely to participate in BP rise are deficient regulatory hormones produced by the kidney and the original genetic modification as well as co-segregated ones (like salt sensitivity) present in many cell types (red blood cells, enterocytes, fibroblasts, etc.).

A balance is achieved during the established phase of the hypertension (16 wks and older in SH rats) to the detriment of a high BP, and its long-term consequences on numerous organs. Whether the SNS tone remains high during that period is still a matter of controversy (Takeda and Bunuac, 1978; Judy, et al., 1976). By then, the mix of initiating factor(s), compensatory reactions (resetting of reflexes and blood volume regulation) and damages (endothelium injury, vasculopathy, cardiac hypertrophy, etc.) is such that it is difficult to separate them.

How well does this model fit the therapeutic efforts?. Any medication which diminishes the activity of any part of the regulatory system of BP will improve the hypertensive state and the subsequent damages to the organs. However, most therapeutic efforts address the end-result (high BP), not the cause, their efficacy is limited, and their side effects important. Limiting sodium intake is very indirect and possibly dangerous, and helps only patients with the added problem of sensitivity to salt intake. All adrenoceptor agonists (alpha) and antagonists (beta), as well as ganglionic blockers

will prevent SNS activity (which would includes multiple-firing) and will thus reduce BP. However, their scope of action is large (numerous side effects) and does not include other defective cells (in kidney, for example) in the therapy. Diuretics, the first line treatment for hypertension are effective, but they also show numerous side-effects; also, if the kidney is to be defective, it seems dangerous to tax an already deficient organ. According to the model proposed, calcium antagonists and potassium efflux activators offer more promises because they act directly to inhibit (calcium antagonist) or compensate for (potassium efflux activators) the multiple firing generated by $K_{(Ca)}$ channel deficiency and carried by a regenerative calcium current. If it could be tailored to target cells with the deficiency, the compound would be specific and would address **all** deficient cells in the organism.

Lastly, what a subtle genetic modification of intra-cellular calcium homeostasis would do at an early developmental stage is unknown. It can only be hypothesised that it is not life threatening, since neither hypertension in human nor available animal models show obvious pathologies, congenital malformations, or mortality rate associated with genetic hypertension. The developmental effects are probably minimal, but one should not dismiss the much lower average size of SH litters and the higher incidence of maternal cannibalism. Obviously, maternal cannibalism is of no concern to humankind, but one can only regret the near total absence of clinical

data concerning pathologies possibly associated with genetic hypertension.

Since the regulation of BP is such a complex phenomenon, it is obvious that the model can not exclude any other associated deficiency in other BP regulatory organs (such as the kidney) or the possible poly-genetic character of the illness. However, this model reduces notably the necessity of different combination of genetic factors to explain the variations noted in the population.

In **summary**, the model proposed for genetic hypertension is of a single genotypic modification with multiple cellular phenotypes, some of them interacting, some apparently unrelated. It would explain why genetic hypertension appears as a multi-factorial complex phenomenon for which it is difficult to isolate a single initial malfunction. It would also explain some contradictory results of some studies, since the identification of the default depends on the technique used (cytosolic vs membrane bound vs total intracellular calcium, total vs active kallikrein, etc), the level of activity (one does not see multiple firing if there is no pre-synaptic activity or long-duration stimulus, etc), the age of the animal, etc. Ultimately, it depends on the role(s) of the $K_{(Ca)}$ channel (or the Ca^{++} binding common (hypothetical) molecule) affected by the genetic mutation in the cell function.

Last word : the next steps.

To confirm this model, many possible routes can be taken. One would be to do patch clamp studies on SH SCG neurones and analyse in more details the currents and channels involved in multiple firing. Another important step would be to take another cell type, wherever in the body, which is known to have a high $K_{(Ca)}$ channel activity and verify if the activity of this cell type is also deficient in SH rats. Also, one would like to identify the factor produced by A VSMC which inhibits neuronal firing.

I chose to study neural crest embryonic cells colonising the digestive tract in SH rats. If the dysfunction is genetic and non specific to the SCG, it should be present in these cells. Tracking them during development might allow the detection of specific digestive tract impairments in genetically hypertensive individuals.

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