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Electrophysiological characterization of serotonergic neuron activity in the dorsal raphe region of the 5-HT_{1A} receptor overexpressing mouse, an animal model of sudden infant death syndrome

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ABSTRACT

Serotonergic nuclei of the raphe play an important role in psychiatric human pathologies as well as in several physiological and homeostatic functions. Serotonergic raphe neurons discharge at a characteristically slow and regular frequency (1-4 spikes/s) that is generated by mechanisms intrinsic to individual cells. Thus, the regular firing of raphe serotonergic neurons maintains the tonic output of serotonin (5-HT) at terminal level. The activation of inhibitory 5-HT_{1A} (auto)receptors expressed at somatodendritic level by raphe serotonergic cells is responsible for the inhibition of their own firing rate (i.e. *autoinhibition*) and, as a consequence, for a decrease of 5-HT release from serotonergic terminals. The physiological importance of this auto-inhibitory mechanism has remained predictive until it was developed a method to dissect the role of 5-HT_{1A} autoreceptors from that of the heteroreceptors.

The availability of transgenic mice with conditional and tissue-specific knock-in/out of 5-HT_{1A} receptors is now providing efficient tools to study the involvement of 5-HT_{1A} receptor-mediated autoinhibition and the modulatory role of serotonin in cognitive or integrative functions, as well as in behavioural states.

For instance, mice with conditional and selective 5-HT_{1A} receptor overexpression in raphe nuclei ($Htr1a^{RO}$) have been recently developed to study the 5-HT_{1A} autoreceptor function and possible disorders associated with increased autoinhibition of serotonergic system. Quantitative autoradiographic analysis revealed approximately 10-fold Htr1a protein overexpression $Htr1a^{RO}$ mice as compared with control littermates. The selective 5-HT_{1A} receptor overexpression onto serotonergic neurons of the raphe nuclei in $HTr1a^{RO}$ mice was found to cause a severe autonomic dysregulation that often leads to death and shows striking similarities with sudden infant death syndrome (SIDS) events in humans.

The principal objective of the present work was to obtain a more complete functional characterization of serotonergic neuron activity in the dorsal raphe of $HTr1a^{RO}$ mice, to further study the functional role and the analogies between this animal model and SIDS. Serotonergic cells are responsive to changes in pH/CO2 level in blood and brainstem, thereby sensing and responding to hypercapnia with modulatory signals to respiratory nuclei. It has been proposed that one of the

possible causes of SIDS is a failure in resuscitation in response to hypercapnia/hypoxia that follows rebreathing or hypoventilation. Thus, I investigated if chemosensitive responses of serotonergic cells where altered in this mouse model of SIDS, so to extend the characterization of this animal model of human pathology to functional responses that could sensibly be implicated in sudden death.

The research developed on three major intermingled lines, namely: i) assessment of distinctive parameters allowing the electrophysiological identification of serotonergic neurons when recording with advanced extracellular techniques (loose-seal cell-attached patch); ii) comparison of electrophysiological cell characteristics, firing pattern(s) and response to 5-HT_{1A} receptor activation of serotonergic cells, in 5-$HT1A^{RO}$ compared to wild-type mice; iii) study of chemosensitivity of serotonergic cells in wild-type and mutant mice.

The first step of the present work was to develop a method for electrophysiological identification of serotonergic neurons both on whole-cell and in loose-seal cell-attached patch-clamp recordings. Thus, I initially developed and validated in C57bl/6 mice a robust criterion for discriminating serotonergic neurons from non serotonergic cells during recording. This consisted of a "visually guided selection" of large neurons located in raphe midline under InfraRed microscopy, combined with the presence of an electrophysiological criterion, i.e. action potential duration and shape, that was quantified as half-height width -HHW- in whole-cell and Interval between Upstroke and Downstroke –UDI- for action currents in loose-seal cell-attached recordings. Thus, serotonergic neurons show a longer action potential/current duration in respect to non-serotonergic neurons (1.42 ± 0.40 ms, n=84 and 1.67 ± 0.43 ms, n=95, respectively, mean ± SD; $P < 0.001$, Mann Whitney test). When I applied these criteria to identify serotonergic neurons in $HTr1a^{RO}$ and wild type mice I discovered that serotonergic neuron of $HTr1a^{RO}$ mice displayed a shorter action potential/current compared to serotonergic neurons of wild type mice and a smaller cell surface as indicated by cell capacitance. These findings indicate subtle, developmental, modifications of morphology and electrophysiological properties of $HTr1a^{RO}$ serotonergic cells.

The next step was to assess if ten times greater expression of 5-HT_{1A} receptor in $HTr1a^{RO}$mice produces increased electrophysiological responses 5-HT_{1A} receptor activation. For this purpose I measured the inhibition of firing activity of serotonergic cells in loose-seal cell-attached recording and the GIRK-related conductance activation mediated by 5-HT_{1A} receptors in whole-cell patch-clamp experiments. In all tests, I found that dose-response relationships for selective agonist (+)8-Hydroxy-N,N-dipropyl-2-aminotetralin (R-8-OH-DPAT) action were significantly different, but that the effectiveness of 5-HT_{1A} receptor activation was only doubled in $HTr1a^{RO}$ respect to wild type mice. However, $HTr1a^{RO}$ serotonergic cells responded to the activation of the 5-HT_{1A} receptors by endogenous 5-HT as expected for ten folds overexpression of 5-HT_{1A} receptors. Thus, autoinhibition is greatly increased in serotonergic cells of $HTr1a^{RO}$ mice, although this is only partially revealed by synthetic agonists, such as R-8-OH-DPAT.

Finally, I investigated the chemosensitivity of serotonergic cells in $HTr1a^{RO}$ mice and I found that this response, which is physiologically relevant to the respiratory alteration found in SIDS victims, is virtually absent in these mice.

This finding provides a further analogy with human pathology and strengthens the notion that the autonomic dysregulation present in $HTr1a^{RO}$ mice could be used for elucidating functional mechanisms of SIDS-like autonomic deficits.

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ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP	adenosine triphosphate
Ba^{2+}	barium (clorure)
C57bl/6	C57 black mice strain
CC	current clamp
CI	current injection
CO_2	carbon dioxide
5-CT	5-carbossitriptamine
Ctrl	control
D_1	first downstroke of action potential first derivative or of action current
D_2	second downstroke of action potential first derivative or of action current
DOX	$HTr1a^{RO}$ mice fed with doxycicline
DRN	Dorsal raphe nucleus
EC_{50}	half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FR	firing rate
GABA	gamma-aminobutirric acid
G_{Basal}	basal conductance
GIRK	G protein gated inward rectifier potassium channels
G_{R8HD}	R-8-OH-DPAT induced conductance
G_{Slope}	Slope conductance
GTP	Guanosin triphosphate
HCVR	Hypercapnic ventilatory response
5-HT	5-hydroxytriptamine (serotonin)
$5\text{-}HT_{1A}R$	1A 5-hydroxytriptamine receptor
5-HTT	5-hydroxytriptamine transporter
$HTr1a^{RO}$	$5HT_{1A}$ raphe overexpressing mice
HHW	action potential half height width
IV	current-voltage relationship
NMDA	N-methyl-D-aspartic acid
P_{CO2}	CO_2 partial pression
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PhEP	phenylephrine
PND	postnatal die
R8HD	(R)- 8-Hydroxy-2-(di-n-propylamino) tetralin
R-8-OH-DPAT	(R)- 8-Hydroxy-2-(di-n-propylamino) tetralin
RO	$5HT_{1A}$ raphe overexpressing mice
SD	standard deviation
SERT	5-hydroxytriptamine transporter
SIDS	sudden death infant syndrome
TpH2	Tryptophan hydroxylase 2
Trp	Tryptophan
UDI (UD_2I)	upstroke to (second) downstroke interval of action potential first derivative or of action current
VC	voltage clamp
V_{Cell}	cell membrane potential
WT	wild type mice

INTRODUCTION

In human, non human primates, and other mammals, serotonergic signalling is a major modulator of emotional behaviour including fear, anxiety, aggression and integrates complex brain functions such as cognition, sensory processing, motor activity, and autonomic responses. The diversity between these functions is due to the fact that serotonergic system diffusely projects to all brain regions implicated in anxiety and related behaviours as well as to autonomic brainstem nuclei where it coordinates the activity and the interaction of several other neurotransmitter systems. Moreover, serotonin is an important regulator of early brain development and adult neuroplasticity, including cell proliferation, migration, differentiation and synaptogenesis (Di Pino et al., 2004). As it shapes various brain systems during development, it sets the stage for functions that regulate emotions and autonomic responses throughout life.

Indeed, emotional and autonomic dysfunctions in serotonin system appear to be settled during development when serotonin plays a role as morphogenetic factor (Azmitia, 2001; Whitaker-Azmitia, 2001; Gaspar et al, 2003; Gross et al., 2004; Di Pino et al., 2004). Insufficient or excessive levels of serotonin (5-hydroxytryptamine, 5-HT) during postnatal period (i.e. excessive or poor activation of certain serotonin receptors) is believed to produce permanent changes in the formation of selected neural connections (Cases et al., 1996; Upton et al., 1999; Yan et al., 1997; During and Hornung, 2000). For example, the role of 5-HT_{1A} receptors in the maturation of the forebrain seems established. Stimulation of 5-HT_{1A} receptors during early postnatal period is necessary for normal anxiety-like behaviour throughout life (Gross et al., 2002).

Furthermore, as serotonin seems to act as true trophic factor, alterations in its synthesis, metabolism, and functional turnover may profoundly affect morphogenesis of the developing brainstem network.

The availability of serotonin as “trophic messenger”, as well as neurotransmitter, is regulated by synthesizing (L-tryptophan Hydroxylase type 2: TPH_2; Gutchneckt et al., 2009) and metabolizing (monoamine oxidase A: MAO-A) enzymes, and the serotonin transporter (Lesch et al., 2003). The level of serotonin in the synaptic

space at the level of the terminals is regulated by a delicate balance between rhythmic firing of serotonergic neurons and the *autoinhibitory* control exerted by serotonin itself on the firing of serotonergic cells through stimulation of their somatodendritic 5-HT_{1A} receptors.

It was predicted that the manipulation of one of these components would affect extracellular levels of serotonin at the target regions, thus it would help to elucidate the role of serotonin as a morphogenetic factor and as a moderator of autonomic functions and emotionality.

Several studies have shown that serotonergic nuclei play an important role in several physiological and homeostatic functions and there is increasing interest in the possible involvement of genetic/developmental alterations of the serotonergic system in non psychiatric human pathologies.

In fact, serotonergic neurons make connections with multiple nuclei in the brainstem, thereby modulating autonomic functions such as breathing and thermogenesis (Richerson, 2004; Hodges et al., 2008). Recently, it has been proposed (Paterson et al., 2006; Duncan et al., 2010) that dysfunction of serotonin system may represent the underlying vulnerability in Sudden Infant Death Syndrome (SIDS), where rebreathing or hypoventilation result in hypercapnia/hypoxia and failure to arouse (Richerson, 1997; Washburn et al., 2002; Severson et al., 2003; Buchanan et al., 2008; Buchanan and Richerson, 2009).

Accordingly, it has been recently shown that selective overexpression of 5-HT_{1A} receptors by serotonergic neurons during the early postnatal development of mice, produces altered serotonergic autoinhibition and autonomic dysregulation that causes life-threatening bradycardic and hypothermic crisis, having striking similarities with SIDS (Audero et al., 2008).

Functional organization of serotonergic system

In the central nervous system, the serotonergic system is phylogenetically ancient and evolved very early. The basic morphology of serotonergic neurons and their anatomical organization is highly conserved from the simplest to the most complex of vertebrates, implying that the principal functions of this system in physiology and behaviour are also conserved.

In mammals, virtually all brain serotonergic cell bodies are located in the brainstem, especially on or near the midline. As with other brainstem-mediated functions, this implies an involvement in basic processes, especially those associated with axial functions, such as those controlling the proximal limb and trunk muscles, and respiration.

In vertebrates, the cell bodies of the vast majority of brain serotonergic neurons are localized in or near the raphe nuclei (Jacobs and Azmitia, 1992). These midline clusters of serotonergic cells constitute ~1/ 1,000,000 of all CNS neurons. However, their influence on their target sites appears to go far beyond these numbers. In the rat brain it is estimated that there are ~6 $x10^6$ serotonergic varicosities/mm^3 cortical tissue. By extrapolation, this means that each serotonergic neuron projecting to the cortex may be responsible for 5 x 10^5 serotonergic varicosities, that each of their cortical target neurons receives ~200 varicosities, and that serotonergic terminals may account for as many as 1/500 of all axon terminals in the rat cortex (Audet et al., 1989).

This makes the serotonergic system the most divergent between monoamine neuromodulatory systems. Consistent with the stable distribution of serotonergic cell bodies across phylogeny, the projections network also appears to be highly conserved. The serotonergic cell fibres enter the various target structures through similar pathways and appear to innervate the same target neurons with synaptic and non synaptic connections (i.e. volume transmission). Serotonergic cells project along several ascending and descending pathways, most of which are shared with one or more of the raphe nuclei (Fig. 1). The highest levels of serotonergic innervations in the forebrain are seen in limbic areas (temporal lobe, hypothalamic and thalamic nuclei, amygdaloid nuclei, and the hippocampus) and

in sensory areas (visual-calcarina, auditory-superior temporal gyrus, somatosensory-postcentral cortex, olfactory-amygdaloid nuclei and entorhinal cortex, nociceptive-posterior complex). The main targets of the descending pathways are the cerebellum, the lower brainstem, and the spinal cord. Lower, but clearly detectable serotonin-immunoreactive fibres are found in virtually every area of the brain, except in major fibre tracts such as the corpus callosum and optic tract.

Serotonergic neurons also contact non neuronal cells. Serotonergic cells are associated with the ependymal cells of the ventricular system through their dendrites, with large blood vessels through the cell body, or through processes in physical proximity to endothelial cells (Azmitia, 1978). This convergence of neuronal and non neuronal input is also conserved and it provides the raphe cells with a broad sampling of information from many brain and endocrine systems that might have neuronotrophic as well as physiological relevance.

In the human brain, serotonin neurons are more numerous (>250,000) than in any other species and form a tight, small cluster along the midline of the brainstem (Tork, 1990). Projections from these clusters are more restricted than the diffuse projections seen in rodents. The axons in rats and mice are predominately thin, highly branched, and unmyelinated. In primates, highly myelinated fibres are common, whereas in rodents they are rare (Azmitia and Gannon, 1983). Thus, the organization of the serotonin neurons is more evolved in primates indicating that this system, although “ancient”, has evolved during phylogeny and has acquired relevant and more specific neuromodulatory influence on lower and higher brain functions.

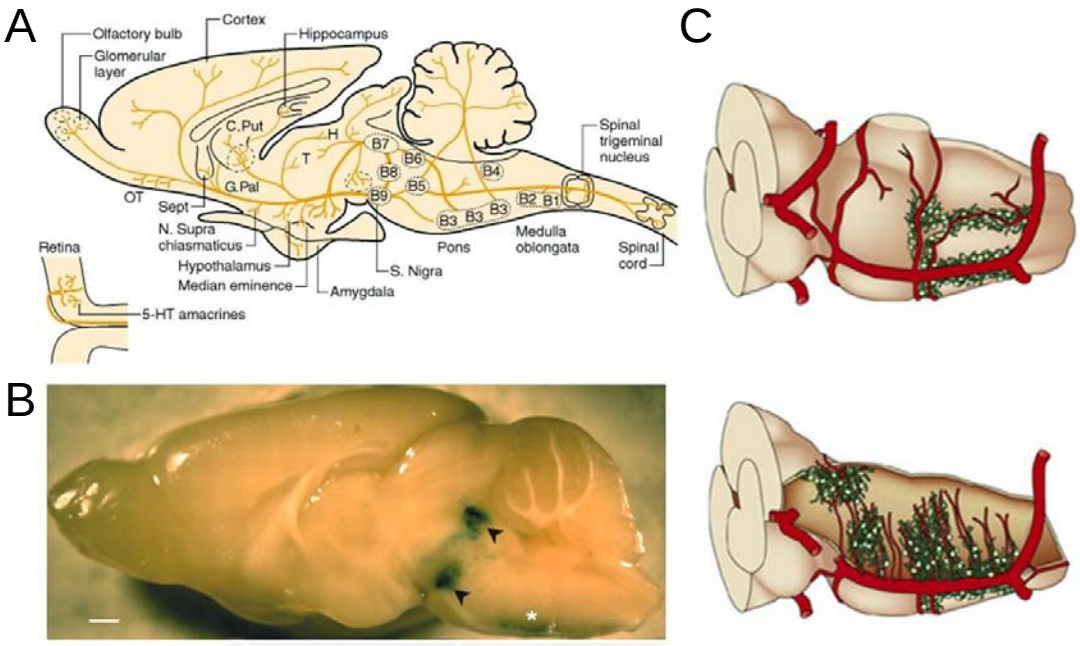

Fig.1. Brainstem raphe nuclei localization, projections and their association with brain arteries. (A) Schematic drawing showing the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections. *OT,* olfactory tuberculum; *Sept,* septum; *C. Put,* nucleus caudate-putamen; *G. Pal,* globus pallidus; *T,* thalamus; *H,* habenula; *S. Nigra,* substantia nigra. *B6-B7*, dorsal raphe; *B8-B9* median raphe; *B3*, raphe magnus and pallidus; *B2-B1* raphe obscurus (Consolazione et al., 1982).

(B) Adult mouse brain in which serotonergic neurons are marked with yellow fluorescent protein, dorsal and median raphe nuclei are indicated with arrows, asterisk indicate medullary raphe nuclei (Scott et al, 2005). (C) Brainstem serotonergic nuclei association with arteries is showed from the three-dimensional reconstruction of immunohistochemical labelling of vessels and neurons (Severson et al., 2003).

Physiology of serotonergic neurons

Serotonin has been involved in a vast array of physiological and behavioural processes in vertebrates, but appears to be essential for none of them (Calas, 1981; Jacobs, 1984; Whitaker-Azmitia, 1990). This apparent contradiction could be explained by the fact that the expansive system of serotonergic neurons exerts a tonic modulatory influence on its widespread targets. This is expressed primarily in association with the organism's motor activity and is in phase with the sleep-wake-arousal cycle. Like most of neurons in the brain, the activity of serotonergic neurons changes significantly across the sleep-wake-arousal cycle and serotonergic system is maximally activated during diurnal wake.

This issue was well examined with extracellular single unit recordings in the dorsal raphe nucleus (DRN) of the domestic cat that gives a general insight on serotonin neuronal activity (action potential firing) in behaving animals.

During the quiet waking state, the activity of DRN serotonergic neurons is slow and highly regular (Trulson and Jacobs, 1979), just as it is when examined under anesthesia and even *in vitro* (Aghajanian et al., 1968; Mosko and Jacobs, 1976). From a firing rate of approximately 3 spikes/s during quiet waking, the activity of serotonergic neurons typically increases by approximately 10%-30% in response to activating or arousing stimuli. Reciprocally, the activity of these neurons declines as the cat becomes drowsy, and becomes even slower upon entering slow-wave sleep. Finally the culmination of this state-dependent decrease in single unit activity occurs as the cat enters rapid eye movement (REM) sleep, when the activity falls virtually silent.

In general, the pattern of activity across the sleep-wake-arousal cycle in the DRN neurons is closely paralleled by serotonergic neurons in other lower groups, the only differences are that the latter display higher spontaneous frequency and that neuron activity is significantly reduced, but not completely suppressed during REM sleep (Fornal and Jacobs, 1988).

Apart from being switched on or off during the sleep-wake-arousal cycle, serotonergic neuron activity is not responsive to sensorial stimuli nor is it changed by a variety of physiological variables.

It is assessed that intense or stressful conditions, especially those that evoke strong behavioural responses and/or physiological changes of sympathetic activation, do not significantly activate serotonergic neuron activity beyond the level normally seen during the undisturbed active waking state (Jacobs and Fornal, 1992). In further experiments it has been observed there is a relationship between a variety of central pattern generator-mediated motor activities such as oral-buccal movements and serotonergic activity. Approximately one fourth of DRN serotonergic neurons increase activity from two to fivefold before the engaging of the repetitive motor behaviour. In contrast, the rest of serotonergic neurons maintain a slow and rhythmic activity.

Contrary to pontine serotonergic nuclei, where few cells respond to central pattern-generator behaviours, most of medullary serotonergic neurons are activated under at least some of these conditions (Veasey et al., 1995). The degree of activation, however, is much less impressive, i.e., 50%-100% above baseline versus 100%-400% above baseline for DRN. There also appears to be at least some degree of response specificity for these neurons. Thus, virtually all medullary serotonergic neurons are activated during treadmill-induced locomotion, 50% of them during environmental cooling, but none of these stimuli activate serotonergic neurons in DRN. Interestingly, subgroups of neurons in both nuclei were found responsive during carbon dioxide hyperpnea (CO_2 inhalation). These neurons seems to function as central carbon dioxide chemoreceptors, thus they have the intrinsic property to sense changes in pH/CO_2 in large blood vessels and to trigger the proper arousal response as well as stimulation of breathing (Nason and Mason, 2006, Richerson, 1995, 1997; Wang et al. 2001; Severson et al., 2003, Hodges 2008).

In summary, the tonic activity of serotonergic neurons, and accordingly the output levels of serotonin at the terminals, appear to vary in a stereotypical manner in association with motor activity and behavioural states and not specifically in association with any processes. The discharge of these neurons appears to reach a “ceiling” during active waking beyond which they cannot be activated, regardless of the impact of the affecting stimulus on the organism. Therefore, the primary role of these neurons in physiology and behaviour may be to coordinate the

activity of target structures (or set the tone for) in conjunction with the organism's level of motor activity and behavioural arousal.

Control of serotonergic neuronal activity

Tonic output of serotonin at the terminal level is maintained by the regular firing of raphe serotonergic neurons. The proportionality between the firing rate and neurotransmitter release at the terminals was confirmed by *in vivo* microdialysis studies in behaving animals (Auerbach et al., 1989; Kalen et al., 1989; Jacobs and Azmitia, 1992). Thus, the unperturbed and highly regular firing activity of raphe serotonergic neurons determines a constant release of serotonin from terminals in the projection areas.

Serotonergic raphe neurons discharge at a characteristically slow and regular frequency (1-4 spikes/s) that is generated by a mechanism intrinsic to individual cells.

Direct evidence for the pacemaking activity of brain serotonergic neurons was presented in a study employing intracellular recordings *in vivo*. These experiments revealed that *serotonergic* cells displayed a large afterhyperpolarization (AHP), followed by a gradual depolarization, and finally a spike (Aghajanian and Vandermaelen, 1982). This pattern of repetitive events was also found in subsequent intracellular analysis of serotonergic unit activity recorded in brain slices containing DRN (Crunelli et al., 1983; Vandermaelen and Aghajanian 1983; Allers and Sharp., 2003). When recorded intracellularly, these neurons show very high membrane input resistance and a long time constant. Further studies in DRN slices led to the following proposed sequence of membrane events generating the characteristic slow rhythmic activity (Aghajanian, 1985; Burlhis and Aghajanian, 1987; Segal, 1985): During an action potential, calcium ions enter serotonergic neurons through high-threshold calcium channel. This is followed by a large (15-20 mV) AHP generated by a calcium-activated potassium conductance. This AHP results in a long relative refractory period, thus preventing action potential discharge in bursts and ensuring slow rates of firing. As the AHP decreases (due to sequestration and/or extrusion of calcium), the relative depolarization

deinactivates a low-threshold calcium current and an early transient outward potassium current (I_A). The currents generated by the activation of these two voltage-dependent channels are opposed, with I_A tending to slow the rate of depolarization and the low-threshold calcium conductance increasing it. Under normal conditions, the calcium conductance is stronger, thus leading to a shallow ramp depolarization, which ultimately reaches threshold, guarantees an action potential, and, as the calcium enters the cell, re-initiates the sequence of events. The slope of this ramp is what determines the rate of discharge of serotonergic neurons. This sequence of events is similar to that seen in other pacemaker cells studied in both vertebrate and invertebrate preparations (Llinas, 1988). It should be noted, however, that this classical representation of ion channel activation underlying pacemaker activity is rather simplistic and does not take into consideration the possible contribution from other voltage-activated channels specific to serotonergic cells (Penington and Fox, 1995, Bayliss et al. 1997).

In addition to such intrinsic ionic mechanisms, it appears established that the noradrenergic system exerts a tonic facilitatory modulation of this intrinsic activity during wake and that the GABAergic system is tonically inhibitory on serotonergic cells during sleep (*see* in Adell, 2002). Glutamatergic input from cortical areas in response to phasic sensory input can transiently increase serotonergic neuron firing, but a blockade of glutamate receptors does not appreciably affect constant discharge of serotonergic cells.

Noradrenergic input is believed to drive changes in serotonergic activity across the sleep-waking cycle, since both neuronal groups are wake-on and REM-off. During waking state, noradrenergic input to raphe nuclei produces a tonic and maximal activation of serotonergic neurons (Baraban and Aghajanian, 1980; Adell., 2002; Haddjeri et al., 2004). In fact, an iontophoretic application of an α1-adrenergic agonist (phenylephrine) or noradrenaline does not alter spontaneous activity of serotonergic cells during wake. In contrast, GABAergic system seem to be involved in an inhibition of serotonergic activity during sleep, as an iontophoretic application of the GABA antagonist bicuculline during sleep restores activity of the DRN serotonergic neurons to near the waking level. However, the same antagonist applied during waking state does not change

serotonin neuron activity (Levine and Jacobs, 1992). These results indicate that a GABAergic input to brain serotonergic neurons becomes activated during sleep and exerts a powerful inhibitory influence exclusively during that state.

In conclusion, apart from being switched off or on by two opposing systems during the sleep-wake cycle, it seems that regular firing of serotonergic neurons is not perturbed significantly by any afferent neurotransmitter system. Instead, there is a local mechanism that appreciably regulates the firing of serotonergic neurons during wake and allows for transient changes in firing activity that can be observed during specific behaviours.

This is a *tonic inhibition* of serotonergic neuron activity, exerted by serotonin itself through activation of 5-HT_{1A} (auto) receptors, expressed on the soma of serotonergic neurons. This feedback control is called *autoinhibition* and is the most important mechanism that modulates the serotonergic firing rate in raphe nuclei and the consequent release of serotonin at terminals.

Autoinhibiton is therefore crucial for serotonergic system functioning and dysregulation of this fundamental physiological mechanism is suspected to be involved in several pathological states from anxiety to aggression (Audero et al., 2007), vulnerability to stress, and depression (Richardson-Jones et al., 2010) and SIDS (Audero et al., 2008).

A SUMMARY REVIEW of synthesis, release, receptor actions and re-uptake of serotonin can be found at the end of the Introduction section

5-HT_{1A} autoreceptors and heteroreceptors

Dorsal raphe nucleus together with hippocampal formation are the brain areas most enriched with 5-HT_{1A} receptors (Chalmers et al., 1991; Kia et al. 1996; Miquel et al., 1991; Pompeiano 1992).

In the dorsal raphe, 5-HT_{1A} receptors are expressed mainly by serotonergic neurons and distributed in their plasma membrane of perikarya and dendrites (Kia et al. 1996; Riad, 2000).

This localization is also typical of some forebrain areas such as the septum or the cortex, whereas in the hippocampal formation, 5-HT_{1A} receptors appear to predominate on the dendritic spines (Kia et al. 1996). It is therefore clear that 5-HT_{1A} receptors have a role of autoreceptors in dorsal raphe and a role as heteroreceptors in forebrain areas. Both receptor populations are coupled to Gi/o proteins, and their activation results in membrane hyperpolarization and decreased neuronal excitability. Thus 5-HT_{1A} receptors in raphe serotonergic neurons mediate the mechanism of autoinhibition whereas in the forebrain region they inhibit the activity of the target neurons.

Regional differences in Gα subunit coupling and downstream effectors of 5-HT_{1A} receptors have been demonstrated.

In dorsal raphe, 5-HT_{1A} receptors seem mainly coupled to Gαi3, whereas in the hippocampus they are coupled to Gαo, and in the frontal cortex to Gαo and Gαi3 G protein subunits. In addition, heteroreceptors seem to inhibit adenylate cyclase, while no coupling with this enzyme has been demonstrated in raphe neurons (Hensler, 2003; Lanfumey and Hamon 2004).

Although there is clear difference in their functional activity and associated transducing systems, all 5-HT_{1A} receptors have very similar pharmacological profiles. Therefore, it has been difficult to infer which receptor population is responsible for the effects of agonists administration *in vivo*.

In the rat, administration of R-8-OH-DPAT and other 5-HT_{1A} receptor agonists causes a wide range of behavioural and physiological effects including the induction of the serotonergic behavioural response, hyperphagia, hypothermia, decrease in heart rate, altered sexual behaviour, and nociceptive response (Green and Grahame-Smith, 1976; Green and Heal, 1985; Glennon and Lucki, 1988; Millan et al., 1991; Lucki, 1992). In addition, there is a large amount of literature on basic and clinical data attesting to the anxiolytic and antidepressant activity of 5-HT_{1A} receptor agonists.

In rats, intra-raphe injection of 5-HT_{1A} receptor agonists evokes hypothermia (Higgins et al., 1988; Hillegaart, 1991), however inhibition of 5-HT synthesis and serotonin system lesions do not prevent hypothermia when the agonists are injected systemically (Bill et al., 1991; O'Connell et al., 1992; Millan et al.,

1993). In comparison, in the mouse, lesions of the serotonergic system abolish the hypothermic response to 5-HT_{1A} receptor agonists (Goodwin et al., 1985; Bill et al., 1991).

Therefore, it appears that differences exist across species in the mechanism(s) underlying the hypothermic effect of 5-HT_{1A} receptor agonists; in the mouse it appears that this effect is due to inhibition of serotonergic neuron firing by activation of somatodendritic receptors (i.e presynaptic), whereas in the rat the hypothermic effect seems to be mediated via both pre- and postsynaptic mechanisms (presumably involving different neural circuits).

Studies of the mechanisms underlying the anxiolytic properties of 5-HT_{1A} receptor agonists were in favour of presynaptic action, at least in some models, although an involvement of postsynaptic mechanisms could not be ruled out (Handley, 1995; DeVry, 1995; Jolas et al., 1995).

Even if a considerable amount of information on the roles of the 5-HT_{1A} receptor populations has been gathered, our present knowledge on the multiple roles of 5-HT_{1A} receptor-mediated regulation of mood and autonomic function(s) is elusive and warrants the development of new strategies to dissect the effects of their function *in vivo*.

The generation of transgenic mice has greatly expanded the possibility to obtain new information on this issue. It is well known that 5-HT_{1A} receptors are involved in anxiety-related behaviour, as 5-HT_{1A} receptor knock-out mice display anxiety-like end-phenotype in response to novel cues (Gross et al., 2004; Olivier et al., 2001). However only with the development of conditional knock out/in it was possible to establish a clear participations of one 5-HT_{1A} receptor population in the regulation of emotions and their disorders. Hippocampal specific and conditional 5-HT_{1A} receptor-rescue mice were used to show that the control of forebrain activity by postsynaptic 5-HT_{1A} receptors, during early postnatal period, is necessary to establish normal anxiety-like behaviour in the adult, while raphe 5-HT_{1A} receptors are not involved in this process (Gross et al., 2002).

In contrast, 5-HT_{1A} receptors in raphe nuclei have a wider range of functions as they control serotonin neuron activity and therefore serotonin release from terminals onto almost all brain regions.

Indeed, the level of expression of 5-HT_{1A} receptors in raphe nuclei has been correlated to a panel of pathological manifestations in mice. Thus, selective overexpression of 5-HT_{1A} receptors in raphe nuclei changes the level of autoinhibition and leads to an aggressive endophenotype accompanied by profound dysregulation of autonomic system (Audero et al., 2008), whereas selective decrease in the expression of these receptors produces vulnerability to stress and loss of response to antidepressant drugs (Richardson-Jones et al., 2010).

The 5-HT_{1A} receptor and autoinhibition

The intrinsic negative feedback mediated by 5-HT_{1A} receptors is the major mechanism that controls the activity of brain serotonergic neurons. Thus, as the levels of brain serotonin increases, e.g. following administration of its precursors, L-tryptophan or L-5-hydroxytryptophan, the activity of brain serotonergic neurons correspondingly decreases (Aghajanian, 1972; Trulston, 1975). It is presumed that this neuronal decrease is a homeostatic response that acts to compensate for increases in synaptic levels of serotonin.

Converging experimental evidence showed that the nature of this feedback is a short local loop, rather than a long loop, involving the response of postsynaptic target neurons. Studies with iontophoresis demonstrated that serotonergic neurons are directly responsive to the application of serotonin and that drugs which release serotonin from endogenous stores, or block its reuptake, depress serotonergic unit activity in the DRN, independent of any feedback from the forebrain (Aghajanian, 1972; Mosko et al., 1977). These neurophysiological findings were coupled with anatomic evidence that serotonergic neurons make dendrodendritic and dendrosomatic connections, with serotonergic neurons in the same, as well as in other, raphe nuclei (Mosko et al., 1977; Kapadia et al., 1985; Wiklund et al., 1981). Increased levels of synaptic serotonin appear to inhibit serotonergic neurons by a direct action at somatodendritic serotonin receptors (the 5-HT_{1A} autoreceptors). Thus serotonin 5-HT_{1A} receptor agonist drugs such as 8-OH-DPAT strongly inhibit neuronal firing.

8-OH-DPAT is highly potent and selective at 5-HT_{1A} site, it displays high affinity (Ki=~2nM) and selectivity (>400-fold) relative to other 5-HT binding sites (5-$HT_{1B,1C,1D,}$5-HT_2, or 5-HT_3) (Van Wijngaarden et al., 1990). In intracellular studies 8-OH-DPAT mimicked the hyperpolarizing action of serotonin on serotonergic neurons at concentrations approximately 1000 time lower than that of serotonin (Williams et al., 1988). This compound inhibits the serotonergic firing rate also in vivo in dose-dependent manner (Fornal et al., 1994a). The onset of 8-OH-DPAT action is rapid (20 s) and the complete suppression of neuronal activity is observed at low doses (5-20 ug/kg). The inhibitory action of 8-OH-DPAT is blocked by several non-selective antagonists, including spiperone (Lum and Piercey, 1988; Blier et al., 1989, 1993a; Escandon, 1994) and (-)-tertatolol (Jacobs et al., 1992; Jolas et al. 1993; Lejeune et al., 1993; Prisco et al., 1993), and by more selective 5-HT_{1A} receptor antagonists as WAY100635 (Fletcher et al. 1995; Corradetti et al., 1996; Fornal et al., 1996).

5-HT_{1A} receptor activation inhibits neuronal firing mediating the hyperpolarization of cell membrane by increasing membrane conductance to potassium and decreasing calcium conductance (Williams et al., 1988). The activation of 5-HT_{1A} receptors leads to the dissociation of Go/Gi-protein, the βγ subunit activates an inwardly rectifying potassium conductance (GIRK), and the α subunit inhibits the adenylate cyclase. The G protein is directly responsible also for inhibition of N-type Ca^{++} channels and $I_{ca\cdot Raphe}$ channels (perhaps a P/Q type channels) for slowing the activation of both currents to a similar extent (Penington and Fox, 1995, Bayliss et al., 1997). Together, these two mechanisms may provide a feedback inhibition of raphe neuronal activity and thus limit the serotonergic tone imposed on neurons in target areas. According to this scheme *1*) activation of inwardly rectifying K/ channels by serotonin would inhibit the firing of raphe neurons, thereby decreasing activity-dependent serotonin release, and *2*) inhibition of calcium current by serotonin, if present at serotonergic terminals, would directly decrease calcium-dependent transmitter release (Bayliss et al., 1997).

Apparently, this feedback exerts a tonic influence on serotonergic activity since administration of 5-HT_{1A} receptor antagonists increases the firing rate, and

regularizes the discharge pattern, of serotonergic neurons in behaving animals (Fornal et al., 1988, 1989). Furthermore, as expected of a negative feedback system, the autoreceptor-mediated mechanism appears to be engaged when neurons are firing at a relatively high firing rate and disengaged at lower firing rates. Thus, under conditions where serotonergic neurons are known to be relatively inactive, e.g. during sleep, autoreceptor blockade has little or no effect on neuronal activity. However, under conditions where serotonergic neurons are activated and serotonin release would be expected to exert feedback inhibition, e.g. during arousal, autoreceptor blockade is manifested as a significantly larger increase in the activity of serotonergic neurons (Fornal et al., 1988, 1989; Jacobs and Fornal., 1997). These results provide evidence for the physiological significance of this feedback inhibition.

Indirect evidence also suggests that the density of cell body autoreceptors on individual serotonergic neurons may be a factor in determining both their level of spontaneous activity as the degree to which they respond to 5-HT_{1A} receptor agonists (Heym et al., 1982; Jacobs et al., 1983). Thus neurons with few autoreceptors would be firing with faster rate, due to less feedback inhibition, and less responsive to 5-HT_{1A} receptor agonists, due to fewer sites for drug-receptor interactions. Neighbouring serotonergic neurons in the DRN can exhibit a variety of interactions such synchronization of activity, inhibition, or mutual inhibition of each other (Wang et al., 1982). These data are consistent with the aforementioned concept that serotonergic neurons influence the activity of other serotonergic neurons.

In summary, several studies provide the evidence that serotonin autoreceptor are activated under normal conditions by endogenous serotonin release, and that the level of autoinhibition varies as a function of basal firing rate and behavioural state of the animal.

One physiological role of these receptors is therefore to function as sensors which regulate serotonin neuron that respond firing according to extracellular serotonin level in raphe thereby setting the steady-state concentration of serotonin released at terminal synapses.

Alterations in normal autoreceptor function may lead to pronounced changes in central serotonin neurotransmission and may play a role in the etiology of various autonomous and affective disorders (e.g., SIDS, anxiety, aggression) in which serotonin has been implicated.

A mouse with a selective overexpression of 5-HT_{1A} receptor on serotonergic raphe neurons as a model of the functional study of increased autoinhibition and its consequence *in vivo*: the sudden infant death syndrome (SIDS).

As already mentioned, the 5-HT_{1A} autoreceptor is responsible for the mechanism that regulates the firing rate of serotonergic neurons and thus thereby release of serotonin at terminals (i.e. *autoinhibition*).

The physiological importance of this regulating mechanism has remained predictive until it was developed a method to dissect the role of 5-HT_{1A} autoreceptor from that of the heteroreceptors. Transgenic mice with conditional and tissue-specific knock-in/out of 5-HT_{1A} receptors are an efficient tool to study 5-HT_{1A} receptor-mediated autoinhibition and the modulatory activity of serotonin at postsynaptic sites, then their role in cognitive or integrative functions, as well as behavioural states.

Mice with conditional and selective 5-HT_{1A} receptor overexpression in raphe nuclei ($Htr1a^{RO}$) were developed recently (Audero et al., 2008) and they has been used as powerful tool to study autoreceptor function and the possible disorders associated to excessive autoinhibition. These mice showed 5-HT_{1A} receptor protein overexpression in raphe nuclei of the mid- and hindbrain. Quantitative autoradiographic analysis reveals approximately 10-fold more receptor protein in $Htr1a^{RO}$ mice as compared with control littermates.

Unexpectedly, while mice completely lacking serotonin (Richer et al., 2002) or serotonin neurons (Taylor et al., 2005; Urbain et al., 2006) are viable, these mice exhibited autonomic crises that occurred during a limited developmental period and frequently progressed to death. While baseline heart rate and body temperature were normal, consistent with robust homeostatic control of autonomic activity, the altered serotonin neurotransmission led to sporadic crises

characterized by decreases in heart rate and body temperature that persisted for several hours and frequently recovered only after several days. In 37% of the observed crises severe bradycardia and hypothermia were irreversible and progressed to death. Death was most frequent between PND25 and PND80, and only approximately 30% of $Htr1a^{RO}$ mice survived beyond PND120. Death could be prevented by treating $Htr1a^{RO}$ animals continuously with doxycycline which suppresses 5-HT_{1A} receptor overexpression in raphe, or by delivering WAY100635 with osmotic minipumps. Furthermore, overexpression of 5-HT_{1A} receptor beginning at PND60 led to significantly fewer deaths than overexpression beginning at PND40 confirming the existence of a sensitive developmental period for the death phenotype.

Autonomic crises in $Htr1a^{RO}$ animals appear to share critical features with sudden infant death syndrome (SIDS). SIDS is a condition characterized by the unexplained death of an otherwise healthy infant aged between a month and a year. Typically, the infant is found dead in the early morning after a sleep period.

A rapidly growing number of studies indicates that genetic, developmental, or functional alterations of the serotonergic system may contribute to SIDS (Okado et al., 2002; Sawaguchi et al., 2003; Paterson et al., 2007; Rand et al., 2007; Hodges et al., 2008; Morley et al., 2008; Thach, 2008). SIDS victims have been found to have abnormalities in the brainstem serotonergic system (Paterson et al., 2007; Duncan et al., 2010) including an increase in the number of serotonergic neurons in the medulla with immature and granular cell morphology, which suggests a failure or delay in the maturation of these neurons in SIDS infants.

It has been proposed that the mechanisms leading to SIDS represent a failure in the neural integration of the cardiovascular and respiratory systems, with a concomitant failure to arouse from sleep (Harper et al., 2000; Horne et al., 2004).

The involvement of a defect of serotonin system in SIDS would not be surprising given the modulatory actions of serotonin on many physiological functions (e.g. arousal, sleep, cardiorespiratory, and thermoregulatory functions) that resulted anomalous or impaired in newborns that succumbed to SIDS.

Although it appears that SIDS infants do not exhibit increased Htr1a autoreceptor expression, it is possible that they have functionally equivalent deficits in

serotonin homeostasis that include alterations in local serotonin release, changes in intrinsic electrophysiological and chemosensitive properties of serotonin neurons, and deficiencies in autoregulatory feedback networks, such as those involving noradrenaline (Haddjeri et al., 2004) or γ-aminobutyrric acid (GABA) (Celada et al., 2001).

The triple-risk model of SIDS (Filiano et al., 1994) argues for the simultaneous occurrence of an intrinsic susceptibility, an exogenous stressor, and a critical developmental time period. Studies on $Htr1a^{RO}$ animals showed that altered serotonin homeostasis alone is sufficient to precipitate catastrophic autonomic failure and death in the absence of overt stressors.

Indeed, it was proposed that serotonergic system dysfunction would be responsible for apnea and death in SIDS, because chemosensitive serotonergic neurons would fail to activate the arousal response and to induce dyspnea following P_{CO2} increase during sleep (Buchanan and Richerson, 2009).

The present study on $Htr1a^{RO}$ mice has takes advantage of the availability of mice with altered expression of 5-HT_{1A} receptors to extend our knowledge on cellular mechanisms of 5-HT_{1A} receptor-mediated autoinhibition and to investigate the possible altered sensitivity to CO2/pH in this animal model of SIDS.

SUMMARY REVIEW

Synthesis, storage, and release of serotonin.

Serotonin synthesis

The initial step in the synthesis of serotonin is the facilitated transport of the amino acid L-tryptophan from blood into brain (Fig. 2). L-tryptophan is an essential aminoacid assumed with diet proteins and competes with other neutral large amino acid to enter the cytoplasm. In the serotonergic neuron L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme L-

tryptophan hydroxylase type II (TpH 2), the brain isoform. This enzyme is synthesized only in serotonergic cell bodies of the raphe nuclei and its distribution in brain is similar to that of serotonin itself. The enzyme requires both molecular oxygen and a reduced pteridine cofactor, such as l-erythro-tetrahydrobiopterin (BH_4), for activity. In the enzymatic reaction, one atom of oxygen is used to form 5-HTP and the other is reduced to water. The pteridine cofactor donates electrons, and the unstable quinonoid dihydrobiopterin that results is regenerated immediately to the tetrahydrobiopterin form by a NADPH-linked enzymatic reaction:

L-tryptophan $+BH_4+O_2 \rightarrow$ L-5-HTP+quinonoid $BH_4 + H_2O$

The affinity of partially purified L-tryptophan hydroxylase for L-tryptophan is extremely low, approximately a Km of 30 to 60 μM (Friedman et al., 1972), a concentration comparable to that of L-tryptophan in brain. If the concentration of L-tryptophan in serotonergic neurons is assumed to be comparable to that in whole brain, the enzyme would not be saturated with substrate and the formation of serotonin in brain would be expected to rise as the brain concentration of L-tryptophan increases. Conversely L-tryptophan depletion *in vivo* leads to a large drop in extracellular levels of serotonin and metabolites in the dorsal raphe nucleus (Bel and Artigas, 1996).

Relevant to studies in slices is the fact that *in vitro* L-tryptophan bioavailability is lower, due to the impossibility for cells to synthesize or derive this aminoacid. *In vitro* preparations lacking L-tryptophan also have low concentration of serotonin that is rapidly depleted, partly during cutting/maintenance of brainstem slices and partly by pharmacological activation of serotonergic neurons (Mlinar et al., 2005). Supplementation of L-tryptophan to *in vitro* preparations was found the only way to restore the serotonin turnover and the regulatory mechanisms of firing rate observed *in vivo* (Liu et al. 2005, Mlinar et al., 2005; Evans et al. 2008).

The other enzyme involved in the synthesis of serotonin, the aromatic L-amino acid decarboxylase (AADC), is a soluble pyridoxal-5′-phosphate-dependent enzyme which converts 5-HTP to serotonin. Even this enzyme has a Km of 10 μM, much higher than the concentration of substrate 5-HTP and contrary to TpH2, it is contained also in catecholaminergic neurons.

Serotonin storage

Neosynthesized serotonin is stored in vesicles through its active transport from the cytoplasm. The Vesicular Mono-Amine Transporter type 2 (VMAT-2) uses the electrochemical gradient generated by a vesicular H^+-ATPase to drive transport, such that a cytoplasmic amine is exchanged for a luminal proton; that is, uptake of serotonin is coupled to efflux of H^+ (Erickson et al., 1992). Vesicles storing serotonin contain a specific protein that binds serotonin with high affinity and virtually no ATP is present. This protein binds serotonin with high affinity in the presence of Fe^{2+} and is secreted along with serotonin by a calcium-dependent process.

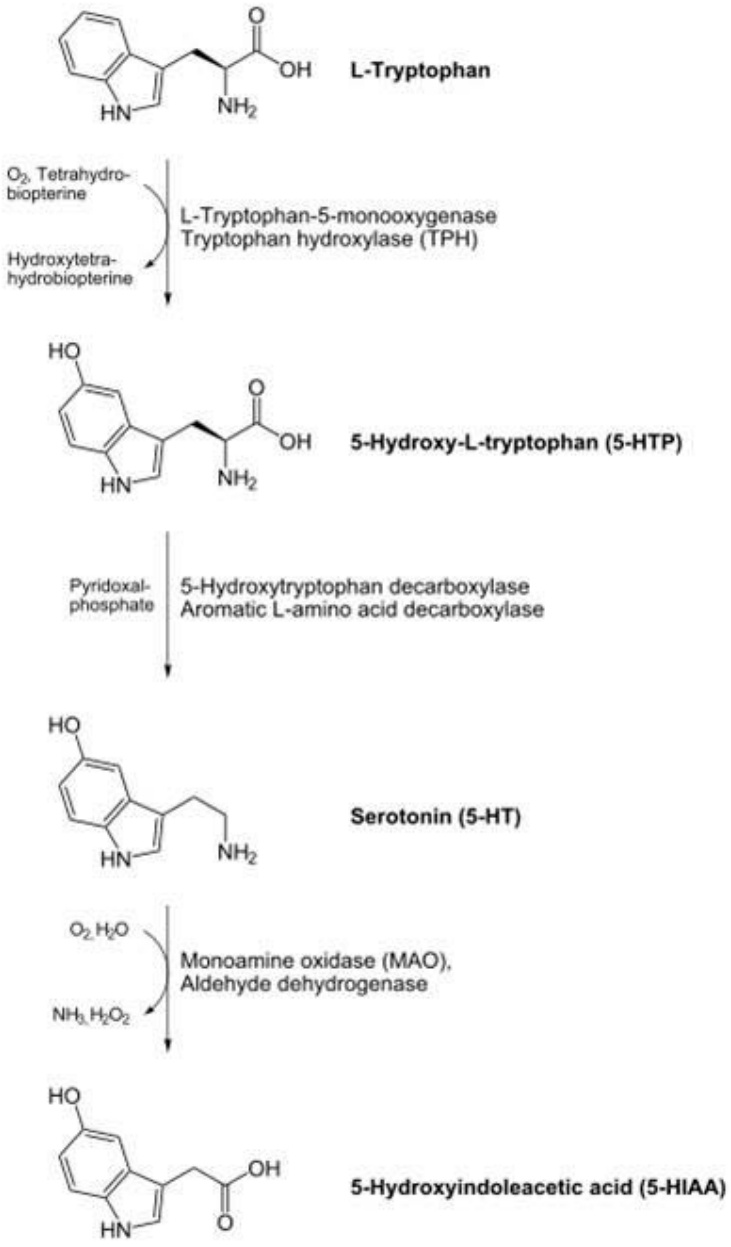

Fig.2. The biosynthesis and catabolism of serotonin.

Serotonin release

The release of serotonin in raphe nuclei originates from somatodendritic area, and presumably from the recurrent axon collaterals, in the efferent projections from axon terminals. The nature and regulation of serotonin release from these sites may be different.

There is considerable evidence that the release of serotonin occurs by exocytosis at axon terminals and axon collaterals, that is, by the discharge from the cell of the entire contents of individual storage vesicles. More discussed is the nature of serotonin release in the somatodendritic area. The dendrites of serotonergic cells have spines and are poorly branched, and generally remain within the neuropil of raphe nuclei (Adell et al., 1993, 2002). These dendrites synthesize serotonin, but those which contain vesicles are scarcer and their presence in the DR of the rat is equivocal (Chazal et al., 1987; Descarries et al., 1986). Moreover, studies in which extracellular concentration in the DRN was directly measured with microdialysis *in vivo* gave contradictory results on the nature of release.

Thus, following these findings, it was suggested a different mode of storage and/or release of serotonin in these dendrites also exist, primary from cytoplasmatic pool and independent from neuron firing (Adell, 2002; Harsing, 2006; De kock et al., 2006).

The rate of serotonin release at axon terminals is dependent on the firing rate of serotonergic soma in the raphe nuclei. Numerous studies have revealed that an increase in raphe cell firing enhances the release of serotonin in terminal fields. The opposite effect is observed when raphe cell firing decreases (Bosker et al., 1994; Kalen et al. 1989). This means that a mechanism that changes the firing rate of serotonergic neurons modifies the release of serotonin as well. An important mediator of such mechanism is the somatodendritic autoreceptor 5-HT_{1A}, which, as discussed elsewhere, is the receptor subtype that is responsible of autoinhibition.

Serotonin receptors

Serotonin (5-HT) acts at the projection area through at least 14 subtypes of receptors that are divided into seven major classes, all are G-protein coupled

receptors except 5-HT_3 that is ionotropic. For a detailed review of serotonin receptor subtypes and their functional roles see Barnes and Sharp (1999).

The subtype 5-HT_{1A} is responsible for some of the best known postsynaptic inhibitory actions of 5-HT, especially in the hippocampus and cortex. 5-HT_{1A} receptors on serotonergic cell bodies mediate tonic autoinhibition of their action potential discharge.

5-$HT1_B$/5-$HT1_D$ receptors (5-$HT1_D$, in humans) act as autoreceptors at axon terminals in rats and primates and their activation inhibits serotonin release from axon terminals. These receptors can also act as presynaptic heteroreceptors and inhibit the release of other neurotransmitters (e.g. glutamate).

5-$HT_{2A,C}$ receptors are expressed postsynaptically and are associated with the fine serotonergic fibers in the middle layers of the cortex. They are responsible for many of the behavioural effects of serotonin and have been shown to be involved in the action of major hallucinogenic drugs.

5-HT_4 5-HT_6 and $5HT_7$ are coupled to Gs proteins, and activate cAMP at postsynaptic level. All these receptors are suspected to be involved in synaptic plasticity.

5-HT_5 receptors are highly expressed in hippocampus, but their transduction mechanisms and functional role remain elusive.

Finally, 5-HT_3 is the only ionotropic serotonin receptor. Opening of the channel produces a cationic current that depolarizes the neurons. This receptor is found in limbic areas, where it may serve a role in anxiety and psychosis; in the substantia gelatinosa, where it may modulate sensory input; and in the area postrema, where it may stimulate emesis.

This large variety of serotonin receptors may account for the multifarious action of serotonin and may explain how serotonergic neurons, that show a rather simple pattern of firing and communicate through unmyelinated fibres impinging onto non specialized postsynaptic sites, could activate specific responses in neighbouring cells. In fact, different neurons would manage to decipher the "uniform" serotonin message into specific physiological responses through the expression of different panels of receptors.

Termination of serotonin action.

The principal mechanism that terminates the actions of serotonin on postsynaptic sites is the re-uptake of the neurotransmitter by the serotonin transporter (SERT) located in the plasmamembrane of serotonergic neurons. Activity of SERT regulates the concentration of serotonin in the synapse, thereby influencing synaptic transmission. The uptake system for serotonin is saturable and of high affinity, with a K_m value for serotonin of approximately 0.2 to 0.5 μM. Uptake of serotonin is an active process that is temperature-dependent and has a requirement for external Na^+ and Cl^-; it is inhibited by metabolic inhibitors as well as by inhibitors of Na/K ATPase activity. From these and other data, it has been inferred that the energy requirement for serotonin uptake is not used directly to transport serotonin but, rather, is necessary to maintain the gradient of Na^+ across the plasma membrane, upon which serotonin uptake is dependent. The current model of transport has one Na^+, one Cl^- and one protonated serotonin binding to the transporter extracellularly prior to translocation to form a quaternary complex that subsequently undergoes a conformational change to release the neurotransmitter and the ions into the cytoplasm. The conformational change may involve the "opening" of a pore formed by some portion of the transmembrane domains of the SERT. In the cytoplasm, K^+ associates with the SERT to promote reorientation of the unloaded carrier for another transport cycle. In addition to facilitating the removal of transmitters from the synapse, plasma membrane transporters function under certain circumstances to release transmitter by a non-Ca^{2+}-dependent, that is, non-exocytotic, process. The role of such transporter-mediated release in physiological circumstances is speculative (Sitte et al., 2000, 2001). However, certain drugs elicit the release of serotonin, at least in part, through such a mechanism. For example, both fenfluramine and MDMA act as substrates for the SERT and not only inhibit the transport of serotonin into the cell but also facilitate its outward transport. The release of serotonin caused by drugs such as fenfluramine is prevented by inhibitors of the SERT (Hilber et al., 2005).

Once transported inside the cell, serotonin is substrate of the enzyme monoamino oxidase that catalyzed the first path of serotonin catabolism. Monoamino oxidase (MAO) converts serotonin to 5-hydroxyindoleacetaldehyde, and this product is

oxidized by an NAD^+-dependent aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA). The intermediate acetaldehyde also can be reduced by an NADH-dependent aldehyde reductase to form the alcohol 5-hydroxytryptophol. Whether oxidation or reduction takes place depends on the ratio of NAD^+ to NADH in the tissue. In brain, 5-HIAA is the primary metabolite of serotonin.

There are at least two isoenzymes of MAO, referred to as MAO-A and MAO-B. These isoenzymes are integral flavoproteins of outer mitochondrial membranes in neurons, glia and other cells. Evidence for the existence of isoenzymes was based initially on differing substrate specificities and sensitivities to inhibitors of MAO. For example, both serotonin and noradrenaline are metabolized preferentially by MAO-A. Selective inhibitors of each form of MAO exist: clorgyline or moclobemide for type A and deprenyl for type B.

Serotonergic cell bodies contain predominantly MAO-B, for which serotonin is not a preferred substrate. The low level of MAO-A in serotonergic neurons suggests that glial cells might have an important role in degrading the neurotransmitter serotonin. Indeed, inhibitors of MAO-A but not MAO-B have been shown to increase brain content of serotonin and to have antidepressant activity. Thus, serotonin may well be oxidized preferentially by MAO-A *in vivo,* as it is *in vitro,* even though serotonergic neurons do not contain much of this form of the enzyme. The principal role of MAO-B in serotonergic neurons might be to eliminate foreign amines and minimize their access to synaptic vesicles. In conjunction with membrane and vesicle 5-HT uptake systems, this could contribute to the purity of serotonin delivered to the synaptic cleft.

AIM

The selective overexpression of 5-HT_{1A} receptors on serotonergic neurons of the raphe nuclei in transgenic mice was found to cause severe autonomic dysregulation that often lead to death. This is the unique lethal phenotype and involves one protein modification in the serotonergic system.

The main aim of the present work was to study the modification in electrophysiological properties and responses of serotonergic neurons caused by the selective 5-HT_{1A} receptor overexpression in the dorsal raphe nuclei.

In particular, we were interested in characterizing the modifications in functional responses to 5-HT_{1A} receptor activation and in the main autoregulatory mechanism mediated by this receptor (*autoinhibition*).

In addition, the pathological profile caused by serotonergic cells 5-HT_{1A} receptors overexpression shares feature in common with SIDS events.

Therefore we studied the possible modifications induced by 5-HT_{1A} receptors overexpression on serotonergic neuron properties as chemosensitivity, that is suspected to be involved in physiological alterations leading to SIDS and that could contribute to produce SIDS-like events in $Htr1a^{RO}$ mice.

EXPERIMENTAL PROCEDURES

All animal procedures were conducted according to the European Community Guidelines for Animal Care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence.

Animals

Mice C57bl/6, were purchased from Harlan (Harlan Italy s.r.l., Milano). Both male and female offspring with mother were received when aged 17 days postnatal (postnatal day – PND 17) and were used for whole-cell experiments at PND 21-28 and for loose-seal cell-attached experiments when adult.

Mice with conditional over-expression of 5-HT_{1A} receptor in serotonin neurons of the raphe nuclei (Htr1a $^{\text{Raphe Overexpressing}}$ - $Htr1a^{RO}$) were developed and kindly provided by Dr. Enrica Audero and Dr. Cornelius Gross, EMBL, Monterotondo. These transgenic mice were produced by driving expression of 5-HT_{1A} receptor under the control of the serotonin transporter gene (5-HTT, SERT or Slc6a4) promoter using the tetracycline-off (tet-OFF) system (Fig. 3) (Kistner *et al.*, 1996; Gross *et al*., 2002). Further details for their production have been published and are summarized in Box 1.

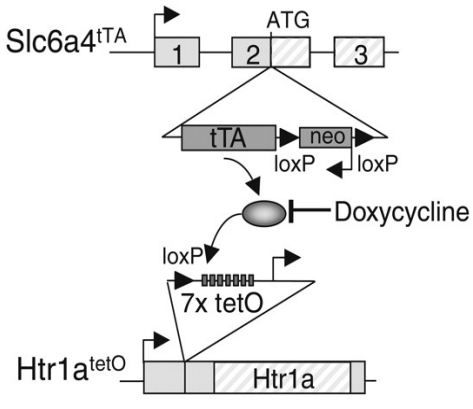

Fig.3. Schematic representation of the genetic construct used to obtain $Htr1a^{RO}$ mice. Mice in which tTA-binding sites (7x tetO) and a minimal cytomegalovirus promoter were engineered into the 5' untranslated region of Htr1a ($Htr1a^{tetO}$) were crossed with mice in which the tTA coding sequence was inserted at the start codon of the serotonin transporter ($Slc6a4^{tTA}$).

In situ hybridization and ligand autoradiography confirmed the $Slc6a4^{tTA}$ allele to be a null allele of the serotonin transporter (from Audero *et al.*, 2008).

Transgenic mice with reversible overexpression of 5-HT_{1A} receptor in serotonin neurons showed doxycycline-reversible overexpression of 5-HT_{1A} receptor

protein exclusively in raphe nuclei, as shown by ([^{125}I]p-MPPI autoradiography, when compared with littermate control mice (Fig. 4). When necessary, experiments were carried out on Htr1a^{RO} mice treated with doxycycline (which suppress Htr_{1A} receptor OverExpression) to assure that the results observed are not caused by deficiency of 5-HTT function.

Transgenic mice were coded with ear tags at postnatal day 18-20 and genotyped (see infra for details) and separated from mothers at PND21.

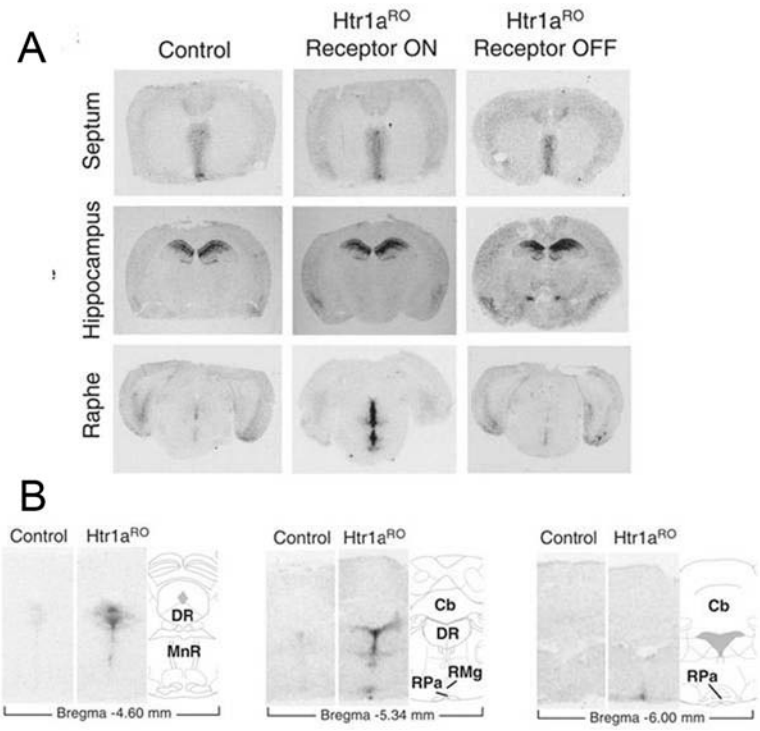

Fig. 4. Htr1a^{RO} mice have selective and reversible overexpression of Htr1a in serotonin neurons. (**A**) Htr1a^{RO} mice show doxycycline -sensitive overexpression of Htr_{1A} protein exclusively in raphe nuclei, as shown by 125I-MPPI–4-(2'-Methoxy phenyl)-1-[2'-(n-2'' pyridinyl)-[^{125}I]-iodo benzamido] ethyl piperazine)autoradiography, when compared with littermate control mice (**B**) Htr1a^{RO} mice show overexpression of Htr1a protein in the dorsal raphe (DR), median raphe (MnR), raphe magnus (RMg), and raphe pallidus (Rpa). Cb, cerebellum. [adapted from Audero *et al*., 2008]

Mice were group housed (3-5/cage), Htr1a^{RO} and wild type together, on a 12:12 light/dark cycle (lights off at 7:00 p.m.) in a temperature-controlled environment (21°C). Food and water were provided ad libitum. When required, doxycycline was given to the animals or pregnant mothers in the form of food pellets (40 mg/kg food).

BOX 1- TRANSGENIC MICE DEVELOPMENT

Mice carrying the gene encoding the tetracycline transactivator (tTA) under the control of the endogenous serotonin transporter promoter ($Slc6a4^{tTA}$) were crossed with mice carrying a tTA-inducible allele of the endogenous gene encoding Htr1a ($Htr1a^{tetO}$) (Gross *et al.*, 2002). $Slc6a4^{tTA}$ mice were produced by replacing portions of exon 2 of the serotonin transporter gene starting at the initial ATG (*1*) with the coding sequence of the tetracycline transactivator protein (tTA2) (*2*) with optimized mammalian codon usage followed by bGH polyA sequences and an FRT-flanked pGK-neo cassette using gene targeting in W9.5 ES cells. $Htr1a^{tetO}$ mice were produced by removing the loxP-flanked pGK-neo transcriptional stop cassette from $Htr1a^{STOP\text{-}tetO}$ knockout mice (*3*) by crossing to a germline Cre-deleter strain. $Htr1a^{tetO}$ allele carried a tetO-CMV promoter (4) targeted into the 5'-UTR of the endogenous Htr1a gene and showed tissue expression of Htr1a that was indistinguishable from wild-type littermates, although with ~20% less binding (data not shown) presumably due to less efficient transcriptional activity of the modified promoter. $Htr1a^{RO}$ and control littermate mice were produced by breeding +/+; $Htr1a^{tetO}$/$Htr1a^{tetO}$ mice with $Slc6a4^{tTA}$/+; $Htr1a^{tetO}$/$Htr1a^{tetO}$ mice [for further details see Audero et al., 2008].

Preparation of brainstem slices

Mice were deeply anaesthetized with isoflurane in a tightly-closed plastic chamber (0.5 mL isoflurane, until breathing slowed down to approximately one breath per second, and stimulation of the limb withdrawal reflex no longer elicited a response), then decapitated using a pair of surgical scissors.

Dissection procedure

In different series of experiments, we have used mice of different age i.e. 20-28 PND-old mice (young) for patch-clamp recordings, or PND 30-80 mice (adult) for loose-seal cell-attached recordings (see below). Conditions for isolation, dissection and slicing of the brain from adult (>50 PND old) mice required some

adjustments to increase the rate of success, as described in the dedicated paragraph at the end of this section.

The dissection was carried out in continuously oxygenated (95%O2: 5% CO2) ACSF solution (124 mM NaCl, 2.75 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose pH 7.3 with NaOH, 320-330 mOsm) cooled with ice blocks of frozen ACSF.

All procedures to obtain the brain were done quickly (30-50s). The head was dipped in ice cold ACSF to clean from the blood and placed in a petri dish with ice cold oxigenated ACSF containing blocks of frozen ACSF.

The skull was exposed by cutting the skin with a small pair of surgical scissors from the back of the skull to the nose. Then with the same scissors three other cuts were made. The first was along the occipital suture. The second was a caudal-to-rostral cut along the sagittal suture. The third was across the skull, near the rostral end of the second cut. These cuts leave two plates of skull covering each hemisphere (Fig. 5A). A small pair of forceps was used to grasp the back of each skull flap and lift firmly upward and outward to remove the skull covering each hemisphere. This was done carefully to avoid that the temporal lobe got damaged by the ventral portion of each plate of the skull. Any extra pieces of skull and muscles residuals that did not come off cleanly were quickly removed to prevent damage during the brain extraction. After the brain was fully exposed, a smooth spatula was inserted along the lateral portion of the brain. Holding the head nearly upside down, the spatula was slid under the brain and all cranial nerves were severed.

The brain was then pushed out of the skull cavity onto a piece of filter paper inside an ice-packed glass petri dish and dipped in ice cold oxygenated ACSF so that dissection occurs at low temperature to slow down tissue metabolism and reduce the damage produced by manipulating the brain and slicing. Once chilled (1 min), the brain was hold on the parietal lobes (upside down) with the aid of a forceps and two coronal cuts were made with a cooled blade. The first cut was at

the level of posterior inferior cerebellar artery. The second cut was under the level of internal carotid artery (mid-circle of Willis) (Fig. 5B).

Preparing brain slices from adult mice (PND 50-80) required particular attention to perform the dissection because at this age the skull of the animal is much thicker and may result more difficult to remove. Therefore it is necessary to use thicker forceps to grip the two plates of the skull to have the necessary force to remove them entirely without damaging the tissue. Also, the brain of an older animal is much less resilient. To improve the quality of the slices we introduced a preincubation stage in which the slices were placed in a holding chamber filled with preheated ACSF at 33°C (see below: maintenance of slices before recording).

Slicing procedure

The midbrain block was carefully taken out of the petri dish with a spatula, the caudal part of the block was quickly dried on a paper filter and it was glued (cyanoacrylate) on the vibratome tray (vibratome DSK1000, Dosaka). Then the tray was placed in the ice cold oxygenated ACSF of vibratome chamber and secured. Keeping the brain tissue as cold as possible during slicing is important because the vibrating blade penetrates more easily and smoothly when brain tissue is made denser and firmer by reducing lipid membrane fluidity. To keep the lowest temperature (~4 °C) constant in the cutting chamber of the vibratome, some frozen ACSF cubes were left to defreeze into the cutting solution.

The brainstem region containing raphe nuclei (between -4.24 mm and -5.20 mm with respect to Bregma rostrocaudally, according to the atlas of Franklin and Paxinos, 1997), was cut coronally with a blade (Biological Instruments SNC, Varese, Italy) into three to four slices of 200 μm (Fig. 5C).

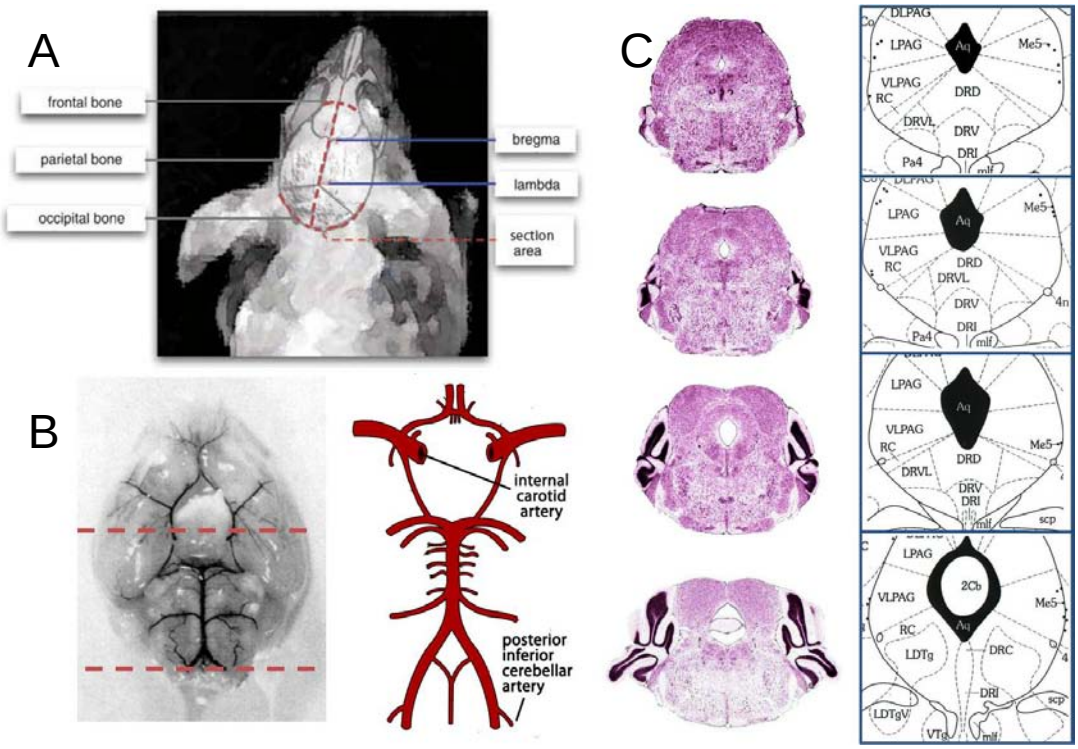

Fig. 5. Mouse Craniotomy and brain dissection. (A) Shows the three cuts made on the skull to expose the brain: the cut along the occipital suture, the caudal-to-rostral cut along the sagittal suture and the cut across the skull, near the rostral end of the second one. (B) shows the level of the cuts for isolation of the brainstem region containing raphe nuclei. The points of reference for the cuts were indicated on the simplified scheme of arterial brain system. (C) Shows four Nissl stained slice of 200 µm, corresponding to those obtained for electrophysiological recordings and next to each, the relative atlas plate (Franklin and Paxinos, 1997). (Upper right), rostral to caudal (from bregma): plate 69, -4.60 mm; plate 70 -4.72; plate 71, -4.84; plate 72, -4.96.

DLPAG dorsolateral periacqueductal grey (PAG); LPAG lateral PAG;VLPAG ventrolateral PAG; RC raphe cap; Me5 mesencephalic 5 nu; DRD dorsal raphe nu; dorsal part; DRVdorsal raphe nu ventral part; DRVL dorsal raphe nu ventrolateral part; DRI dorsal raphe interfascicular; DRC dorsal raphe caudal; Aq acqueduct; LDTgV LDTg laterodorsal tegmantal nu; mlf medial longitudinal fasciculus; SCP superior cerebellar peduncle; Pa4 parattrochlear nu.

The angle of the vibratome blade over the tissue surface was ~ 15 degrees which is optimal for the blade to progress into the tissue without crushing the surface of the brain tissue block or pushing aside the block itself during cutting.

Maintenance of slices before recording

Some laboratories have observed that when the slice are cut at ice-cold temperature, placed into an incubation chamber at a temperature below 10°C and allowed to warm slowly to room temperature *nearly every cell is dead* (Moyer and Brown, 1998). Based on similar observations, a preincubation of slices at temperature warmer than room temperature has been suggested to increase the probability to obtain healthy cell for patch recording (Borst et al., 1995; Hestrin and Armstrong, 1996; Jung et al., 2001; Magee and Johnston, 1995a, 1997).

Therefore, we have applied the following procedure to help recovery of slices from the ice-cold temperature that is most appropriate for cutting the brain tissue with a vibratome. Immediately before the first slice was cut, a multiwell incubation chamber was filled with oxygenated (95%O2: 5% CO2) ACSF solution at 33°C, that was then left to cool down to room temperature (~24 °C) once slices were put inside. As each slice was obtained, it was removed from the vibratome chamber-bath using a wide-bore (~1 cm diameter) plastic dropper and placed with the caudal part face-up (also the recording side) in one well of the slice incubation chamber. Slices were maintained at room temperature for 1-6 h before being transferred to the recording chamber at 32-35 °C.

Superfusion of slices and drug application

For each recording session, one slice was transferred in a custom-made recording chamber positioned under an upright microscope (Zeiss Axioskop) equipped with x4 air and x60 water immersion objectives (Carl Zeiss, Gottingen, Germany), infrared filtered light, Differential Interference Contrast (DIC) optics, a Hamamatsu C2400-07 infrared-sensitive charge-coupled device (CCD) camera (Hamamatsu Photonics, Hamamatsu, Japan) and a video enhancement device, connected to a Hamamatsu thermic-paper printer.

The slice was continuously superfused with ACSF at a flow rate of 2–3 ml/min. Superfusion was fed by a peristaltic pump that deliver continuously oxygenated (95%O2: 5% CO2) ACSF solution from 100-500 ml flasks to the recording chamber, via a small tubing, to reduce the dead-volume. The solution was drained from the recording chamber by suction, *via* a small channel.

Drugs were applied by changing the flask feeding the peristaltic pump inlet and the desired concentration in the flask was obtained by addition of aliquotes of drug at appropriate concentration. Aliquotes were either applied directly into the graduate flasks containing known volumes of oxygenated ACSF and allowed to dilute for 3-4 minutes or diluted in ACSF and placed to oxygenate into the flasks at least 15 minutes before being used for superfusion.

The time required by the solution to reach the recording chamber was 1 minute and 20 seconds. The solution was warmed by an inline heater (Warner Instruments). The temperature of the solution was kept constant by a temperature controller (TC-324, Warner Instruments) connected to the inline heater and receiving input from a thermistor placed in contact with the solution within the recording chamber.

Recording setup

All recordings were done using an EPC10 amplifier (HEKA, Germany). The pipette was held in place by an electrode holder containing an AgCl-coated silver wire and equipped with a side port to allow application of positive or negative pressure to the interior of the pipette. The pipette holder was plugged into the headstage of the EPC10 amplifier. The headstage amplifier was attached to a keyboard-controlled motorized micromanipulator (Luigs and Neuman, Ratingen, Germany) allowing movements of the electrode in three directions (X/Y/Z). Signals were digitized using an analog-to-digital data acquisition device on EPC10 amplifier and recorded on a PC using Patchmaster software (HEKA Germany).

Electrophysiological recording techniques

In my experiments I used two different configurations of patch recording (i.e. whole-cell and loose-seal cell-attached, (Fig. 6), which allowed obtaining different types of information. These configurations will be described in detail separately to highlight the principal characteristics of each technique.

Whole-cell recording

Rationale

The *whole-cell patch-clamp* is a recording technique that provides a powerful approach for investigating the intrinsic electrical properties of neurons and the functional aspects of selected populations of cell membrane channels (BOX 2).

In this patch-recording configuration, the use of *current-clamp* mode allows to gather crucial information that can be used for the electrophysiological identification of a recorded neuron. At the beginning of recording, the pipette solution does not alter substantially the intracellular content of the neurone. Thus, cell characteristics can be assumed to be still preserved and it is possible to obtain reliable measurements of the spontaneous firing pattern and action potential waveform of the recorded neuron. Further information can be obtained using protocols in which steps of constant current are injected into the cell through the recording electrode. Recording cell behaviour during hyperpolarizing or depolarizing current steps allows obtaining additional measures of cell membrane characteristics (e.g. cell membrane input resistance) or firing behaviour (e.g. maximal firing frequency, burst firing at depolarized cell membrane potentials).

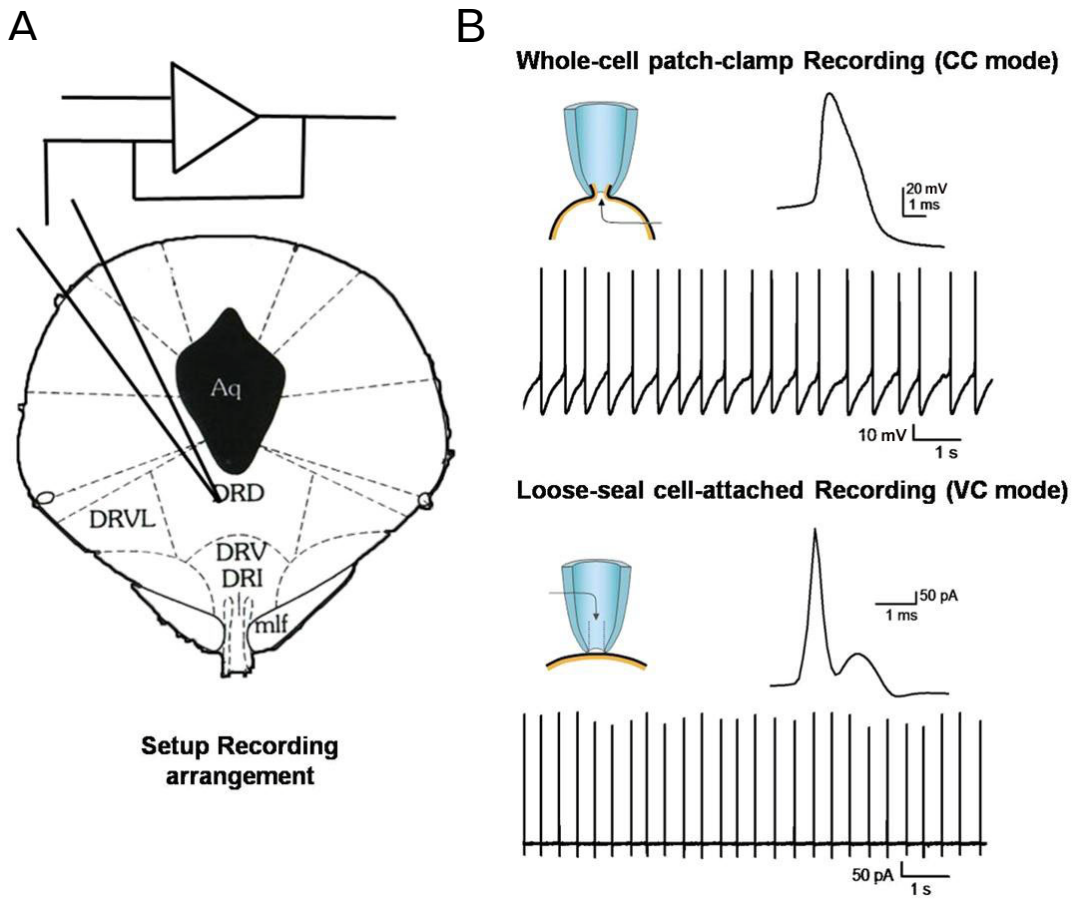

Fig. 6. Whole-cell and loose-seal cell-attached patch-clamp recording configurations. (A) shows a patch-clamp electrode in a typical position to record from cells of the dorsal raphe region. (B) shows the configuration of the electrode in the two different techniques and the respective firing activity recorded from serotonergic neurons. Aq acqueduct, DR dorsal raphe, mlf medial longitudinal fasciculus.

BOX-2 **WHOLE-CELL PATCH-CLAMP TECHNIQUE - AN OVERVIEW.**

Whole-cell configuration allows application of voltage clamping (VC) to small cells with high resolution of recorded currents. Under VC condition membrane current is directly proportional to the conductance, i.e. channel activity of interest. The principle of the method is to isolate a patch of membrane electrically from the external solution (cell-attached recording configuration) and then break the patch membrane under the pipette to obtain electrical access to the cell interior. This is achieved by pressing a fire polished glass pipette, which has been filled with a suitable electrolyte solution, against the surface of a cell and by applying light suction to form a seal of 10 GΩ resistance. A high seal resistance is needed for two reasons. First, higher is the seal resistance, the more complete is the electrical isolation of the membrane patch, i.e. the cell. Secondly, a high seal resistance reduces the current noise of the recording, permitting good time resolution of currents.

Whole-cell patch-clamp configuration is then obtained by destroying the membrane patch using suction. The cell comes into contact with the solution in the pipette and could be voltage or current-clamped. As the cell contents will equilibrate over time with the pipette solution, it is advisable to use this kind of recording over short periods to avoid the loss of responses of interest (Fenwick, Marty and Neher, 1982). The result is equivalent to impaling a cell with a microelectrode. However, the access resistance is typically much lower than what one would obtain with an appropriately narrow conventional microelectrode, and the induced leakage conductance is also lower. These features allow one to obtain lower noise and higher-fidelity recordings with the whole-cell patch technique. The whole-cell voltage-clamp technique has the advantage of the nearly complete control of membrane voltage of the cell or current through it, thanks to the access of the recording pipette to the cell interior. This recording condition however is also the major limit of this technique.

In fact the intracellular content is diluted over time by the solution contained in the pipette and some important constituents of the cytoplasm could be removed or degraded. As a consequence, important features such as electrophysiological behaviour and basic properties of the recorded neurons can change over time as well. To overcome this problem the recording must be relatively brief and the pipette solution composition has to be carefully designed to avoid the loss of ATP and GTP dependent processes and more in general to delay the inevitable dialysis of cellular content.

These responses could be used to functionally discriminate different populations of neurones as they represent the integrative result of the activity of specific channels selectively expressed in different neurones. Further details on the parameters that can be obtained with this approach are given below.

The *voltage-clamp mode* directly measures the current flowing through cell membrane while controlling the voltage. Within the biophysical limits of the technique (see BOX 2), the possibility of closely controlling cell membrane potential becomes crucial for the study of characteristics and responses of voltage-gated ion channels. Even ion channels gated by ligands, including the 5-HT_{1A} receptor-sensitive that are gated by a G-protein and therefore nominally "voltage-independent", a voltage dependence is often seen and electrical measurements are much easier to interpret if the voltage is held constant. Furthermore, for both voltage-gated and ligand-gated channels, the voltage sets the driving force for current through the membrane, so that when the voltage is fixed, the current reflects the number of open channels. Thus, by applying this technique combined with the use of pharmacological tools, the activity of specific groups of channels can often be studied in detail.

Experimental procedures

Intracellular patch pipette solutions

Throughout the present study, pipette solutions were designed to preserve as far as possible the physiological composition of the intracellular "milieu". However, some adjustments were introduced to the composition of the solution when required by experimental needs.

In a first set of experiment carried out at 28°C, patch-pipette solution was comprised of (in mM): $KMetSO_3$ 120, KCl 15, HEPES free acid 10, $MgCl_2$ 2, GTP-Na_3 0.3, Na_2-Phosphocreatine 10, ATP-Mg 4 (pH 7.3 with KOH, osmolarity 320-330 mOsm).

However, when we performed experiments at temperature above 31 °C (31-35°C), we observed that the patch was subject to re-sealing and recordings resulted of lower quality.

Therefore in the following set of experiments, in which the temperature of the superfusing ACSF was set above 30 °C (typically 33-35 °C) we added a low concentration of EGTA (50 µM) to a very similar pipette solution (in mM): $KMetSO_3$ 120, KCl 15, HEPES free acid 10, $MgCl_2$ 2, EGTA-K_4 0.05, GTP-Na_3 0.3, Na_2-Phosphocreatine 10, ATP-Mg 4 (pH 7.3 with KOH, 320-330 mOsm). A small amount of EGTA buffers calcium ions stabilizes the membrane patch and improves quality of recording at temperature higher than 28°C, while introducing undetectable, if any, alteration of electrophysiological cell behaviour.

When the protocol required labelling of the recorded neuron with a fluorescent dye to allow subsequent identification of the cell by means of immunohistochemical methods (see dedicated section below) either lucifer yellow CH potassium salt 2 mM or Alexa Fluor 488 hydrazide sodium salt 300 µM were added to a solution comprised of (in mM): $KMetSO_3$ 120, KCl 15, HEPES free acid 10, $MgCl_2$ 2, EGTA-K_4 0.05, GTP-Na_3 0.3, Na_2-Phosphocreatine 10, ATP-Mg 4 (pH 7.3 with KOH, 320-330 mOsm). These solution, allowed marking the recorded neuron with a high concentration of the fluorescent dye, which

facilitated subsequent *post-hoc* identification of the recorded neuron in the thick (200 µm) slice.

Finally, we found that the substitution of $KMetSO_3$ with K D-Gluconate as impermeant anion in the pipette solution increased the duration of stable recording and therefore we adopted the following pipette solution as a standard (in mM): K D-Gluconate 120, KCl 15, HEPES free acid 10, $MgCl_2$ 2, EGTA-K_4 0.1, GTP-Na_3 0.3, Na_2-Phosphocreatine 10, ATP-Mg 4 (pH 7.3 with KOH, 320-330 mOsm). The use of this pipette solution allowed stable and reliable recordings for longer than one hour at temperature near 33°C.

Patch pipettes

Patch pipettes were fashioned with a four stage process on a P-97 micropipette puller (Sutter Instruments, Novato, CA) using borosilicate glass capillary tubing (1.5 mm o.d., 1.17 mm i.d.; Clark Electromedical Instruments) and had resistance of 2–3 MΩ when filled with pipette solution (previously filtered with 0.2 µm syringe filter).

Placement of the slice in the recording chamber and selection of recording field under visual guidance with IR-DIC microscopy

One slice was taken from the holding chamber and placed in the recording chamber and held in place by a net on top of the slice. This securing net was made with five single strands of nylon hosiery glued across a platinum U-shaped wire using cyanoacrylate. When placed on the slice, the nylon hosiery serves to anchor the slice around the region of interest.

After securing the slice, the dorsal raphe region was positioned in the center of the microscope field of view using the low magnification (x4) objective. Subsequently, the slice was examined using a water immersion objective (x60) and the neuron was selected for recording, based on its appearance under IR-DIC microscopy. Dorsal raphe neurons were identified as being probably serotonergic by their location along the midline, ventral to the Aqueduct of Sylvius and by their size and shape (medium to large, i.e.15–20 µm to 20–30 µm, bipolar or

multipolar cells). An apparently healthy neuron laying at 30-90 μm from the surface of the slice was then chosen and targeted for recording.

Patch-clamp procedure

After connecting the pipette holder to the amplifier headstage, a positive pressure was applied to the pipette by blowing air (~4 ml) with a syringe connected to the pipette holder via a Tygon or Nalgene (Fisher Scientific, Pittsburgh, PA) tube plugged into a three-way valve. Then, pressure was mantained by closing the three-way valve.

The electrode was placed in the bath solution and the potential difference between the tip of the electrode and the Ag/AgCl reference electrode (in contact with the bath solution) adjusted to zero. In voltage clamp mode, with the command potential set to 0 mV, 5 mV hyperpolarizing voltage steps were applied. Based on the measured step current, the pipette resistance is calculated from Ohm's law. Once the neuron of interest was visualized, the pipette was lowered under the objective and positioned near the selected neuron using the micromanipulator. Then the tip of the pipette was directed towards the cell until a dimple appeared on the surface of the neuron. At this point the positive pressure was released and the electrode retracted slightly. When the maneuvre was successful the electrode resistance increased dramatically, either spontaneously or with the aid of gentle suction applied to the interior of the pipette, i.e. a gigaohm seal formed between the electrode and the cell membrane.

The command potential of the amplifier in voltage clamp mode was maintained at -65 mV. Capacitive transients due to the electrode capacity were cancelled using the C_{fast} dial on Patchmaster software. Brief pulses of suction were applied to the interior of the pipette to break the cell membrane under the pipette and to obtain access to the whole-cell. The access resistance was monitored for judging the stability of recording and the capacitive current transient due to the membrane of the cell was cancelled using the C_{slow} dial on Patchmaster software.

After waiting for 3–5 min, to allow for neuron dialysis with the pipette solution, we transiently switched to a current-clamp configuration to record the membrane

potential and to confirm the identity of serotonergic neurons by recording responses to negative and positive current pulses and subsequently to monitor the action of tetrodotoxin (TTX) when applied.

Series resistance was assessed from the peak value of current produced by an application of small (-10 mV) voltage steps according to Ohms Law (R=V/I).

Recordings were accepted if the series resistance was $\leq$ 20 MΩ and did not change during the experiment. If either the amplitude or shape of the hyperpolarization-evoked transients changed abruptly, the experiment was discarded. The liquid junction potential was not corrected.

At the end of recording, the position of recorded the cell was photographed using the Hamamatsu infrared CCD camera and the image taken as photomicrograph. The cell position relative to midline acqueduct was measured from these images. All the cells recorded were found between 600 µm from aqueduct along the midline and 300 µm maximum lateral to midline.

Whole-cell recording: current-clamp and voltage-clamp protocols

In whole-cell recordings we collected a series of parameters useful to characterize the recorded cell and its activity under control conditions and during the application of drugs.

Since one of the aims of the present work was to identify electrophysiological parameters which would permit to distinguish between serotonergic and non serotonergic neurons on the basis of their spontaneous or pharmacologically-stimulated activity, the information obtained in current-clamp is of great interest. In fact, using appropriate protocols, it would be possible to explore some integrated functional characteristics (e.g. action potential characteristics, maximum rate of action potential discharge) that may result specific of each type of neuron, and that cannot be recorded in voltage-clamp conditions. Thus, the electrophysiological protocols applied were designed to measure different parameters in current-clamp or voltage-clamp conditions, as described below. Whenever required, drugs were bath applied.

Current-clamp

In current-clamp the spontaneous discharge of action potentials (when present) was recorded for 1-3 min to permit analysis of the firing frequency and to obtain a sufficient number of spontaneous action potentials for measurement of their relevant parameters (e.g. action potential amplitude, duration). Occasionally, when the cell was not spontaneously active, a small positive current was injected through the recording electrode to elicit action potential firing. Appropriate protocols were applied to investigate the total input resistance (Rin) of cells and their membrane I/V response. Rin and the I/V relationship were measured by applying negative constant current steps of increasing intensity and 1-3 s duration, and measuring the resulting hyperpolarization of the cell at the end of the current steps.

The negative current steps were programmed to hyperpolarize neurons up to a membrane potential of -115/120 mV (if allowed by the characteristics of recording electrode). At this membrane potential, the presence of a sag which develops during the step is interpreted as a relative depolarization produced by the activation of a voltage- activated cationic current (I_H). To accurately detect the presence of a depolarizing sag and measure its amplitude, we acquired a series (n=5-7) of negative current steps (typically -20/-40 pA) adjusted so that the negative peak of the cell membrane potential during the step reached -115 to -120 mV from a cell membrane potential held at -70 mV. Series of square positive current steps of 1-3 s duration and increasing intensity were injected in recorded neurons to elicit action potential firing. The intensity of the depolarizing current was increased at each following step until the firing rate of the recorded cell reached a maximum, became irregular or stopped.

A further parameter investigated in most of the recorded cells was the sensitivity to 5-HT_{1A} receptor activation. The typical electrophysiological response to stimulation of 5-HT_{1A} receptors is the opening of G protein-coupled inwardly rectifying potassium (GIRK) channels and hyperpolarization of the recorded cell. To determine the effect of the selective 5-HT_{1A} receptor agonist R-8-OH-DPAT (1-300 nM) on membrane potential, continuous recordings were conducted in

current-clamp mode with no current injection (voltage follower). A similar protocol was used to measure the effect of α_1-adrenoceptor activation by phenylephrine (10 μM).

Voltage-clamp

In voltage clamp, voltage steps of -10 mV, 100 ms long, were imposed from a holding potential of -60 mV, this protocol was used to determine the access resistance, the cell membrane resistance and the membrane/leak current present at "rest". Measurements of access resistance were carried out by averaging five traces in which negative voltage step was delivered through the recording electrode. Access resistance was calculated as the ratio between the averaged capacitive current peak response and the applied voltage step (-10 mV).

To more directly determine the GIRK current evoked by perfusion of R-8-OH-DPAT, voltage-clamp recordings were conducted in the presence of TTX. Neurons were clamped at -60 or -65 mV and currents were elicited by applying repetitive voltage ramp commands (0.1 V/s) to -120 mV at varying intervals.

Acquisition parameters

In current-clamp recordings, signals were digitized at 25 kHz and low-pass filtered at 8 kHz, in voltage-clamp recordings, signals were digitized at 10 kHz and low-pass filtered at 3 kHz.

Loose-Seal Cell-Attached Recording

Rationale

To observe the "physiological" firing pattern(s) of individual neurons requires recording techniques that can monitor cell activity without producing any mechanical or intracellular fluid perturbations.

Common recording methods as whole-cell patch-clamp and cell-attached patch-clamp techniques have been widely used for this purpose, but they are far from

being the best suited choices. Their application in fact can result in the severe alterations of the cell, such as the deformation of the membrane to obtain a tight seal or the dialysis of the cell internal environment caused by the exchange with pipette solution in whole-cell configuration.

The loose-seal patch-clamp is a variant of the more popular cell-attached patch-clamp technique and permits to record the endogenous activity of a neuron for several hours without affecting the cell behaviour. At variance from cell-attached patch-clamp, this technique does not require special processing of the tissue to be achieved. The formation of a low resistance seal (<20 MΩ) on the surface of the target cell can avoid the process of "cleaning" necessary for the tight seal, and does not compromise the integrity of the cell membrane as a consequence of excessive suction. Furthermore, as the cell membrane remains integer, the physiological intracellular milieu also is maintained intact.

Finally, loose-seal cell-attached recording has a practical advantage over the tight-seal technique regarding the use of voltage-clamp mode to record firing rate activity. In fact, in tight-seal even few picoamperes injected by the amplifier can affect the patch membrane voltage, as the seal resistance is several magnitudes greater than the patch resistance, and therefore all the current generated by the amplifier would pass across the membrane patch. Thus in tight-seal the amplifier voltage command must imperatively be set at the correct value of membrane potential and continuously monitored, to ensure that no current is delivered to the patch-membrane. This is not always practical when drugs are applied, since they could change cell membrane potential and in general there is always the risk to affect the activity of the recorded neuron.

In contrast, loose-seal patch-clamp operates with seal resistances of few MΩ, i.e. lower than the patch resistance. Thus, even if amplifier generates some current due to incorrect estimate of the cell membrane potential or to a small drift of the reference, the current injected into the pipette by the amplifier in the attempt to counteract these erroneously estimated changes in cell membrane potential, would pass across R_{seal}, without affecting the cell activity.

Eventually, the extracellular recording of neuron firing rate using loose-seal cell-attached technique could seem similar to the single unit extracellular recordings with microelectrodes. However, we have observed under IR-DIC microscopy at high magnification that, to obtain a signal using sharp microelectrodes (~10 MΩ), the electrode should touch tightly cell membrane and this may elicit the formation of a large dimple on the surface of the cell. When observed this dimple expanded overtime, eventually changing the morphology of the neuron. As a severe deformation of the membrane may occur, in these cases the experimenter cannot be confident that the physiological cell activity was not altered by such a modification. Therefore we have chosen the loose-seal patch-clamp technique as it results one of the least invasive electrophysiological methods available and ensures very reliable and quite stable recordings in nearly every condition.

Experimental procedures

Visually-guided loose-seal cell-attached recordings were obtained using the same equipment and superfusion arrangement as for whole-cell recordings. Thus, slices were submerged in standard ACSF solution saturated with 95% O2, 5% CO2 gas at 33-35°C. When appropriate, ACSF was supplemented with a cocktail of receptor antagonists to block synaptic neurotransmission.

Loose-seal patch-clamp pipette solution

Loose-seal cell-attached recording electrodes were pulled from the same borosilicate glass capillaries used for whole-cell configuration, and had resistances of 2–3MΩ. The pipette contained 150 mM NaCl solution or a HEPES based ACSF-like solution comprised of (mM): 124 NaCl, 2,75 KCl, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1,3 and pH 7.4 with NaOH.

A sodium-based pipette solution with a composition nearly similar extracellular ACSF is used to avoid the perturbation of neuronal activity during recording. It allows the experimenter to clean the cell membrane by blowing solution out of the electrode tip before a seal is attempted, without depolarizing the cell and possibly weakening it. Pipettes were filled with NaCl saline solution in early studies. Because this is a non buffered solution, we became concerned that it could

influence the stability of recording over time. We thus switched to normal HEPES solution for later studies. No significant difference was observed in recording firing activity between these pipette solutions. We found pipettes with resistances of 2-3 MΩ when filled with normal HEPES pipette solution and held at potential of which amplifier read 0 pA current more suitable for these recordings. When pipette resistance was > 3 MΩ, we found the diameters to be too small, and GΩ seals often spontaneously formed. Conversely, pipettes < 1 MΩ were so large in diameter that cell morphology could be affected. In our experience, electrode resistance was the primary determinant of recording success.

Patching procedure

To approach the target cell, minimal positive pressure was applied to the recording pipette, which was then lowered into the bath and moved to a position above the surface of the slice. By focusing back and forth between the target cell and the pipette tip, the pipette was moved into position above the slice surface directly close to the target cell. Readings of pipette resistance and pipette offset were taken at this time. The pipette was then moved along an oblique trajectory and downward through the slice, to avoid the compression of the tissue over the cell. Positive pressure was then slowly released and usually a loose-seal between the recording pipette and the cell membrane was obtained.

To measure the seal resistance in voltage-clamp, voltage step of -10 mV, 40-100 ms long, were elicited from a holding potential that gives 0 pA current in current-clamp mode. Within 5-min, seal resistances typically ranged from 8-12 MΩ and either remained stable or increased slowly over time up to as high as 40 MΩ. These seals typically formed spontaneously and did not require application of negative pressure (suction) by mouth. Occasionally, when no spontaneous activity and no change in pipette resistance were observed in the first 5-min we applied negative pressure to improve seal resistance.

Recording quality checks

The typical firing pattern of serotonergic neurons was monitored under voltage-clamp using continuous recording. We checked periodically for seal resistance

during recording. Occasionally, seal resistance raised and reached the 100 MΩ range. To reveal if the recording would have been affected by the command voltage in these conditions, ramps of voltage steps were applied. As mentioned above, in the case of an appropriate loose-seal cell-attached configuration, injection of current through the pipette should not affect the spontaneous cell activity because the current injected should outflow through the loose patch (i.e. Rseal). Thus, if these current ramps produced a change in neuron firing rate, the recording was considered unreliable and it was aborted.

During the recording we also checked for stability of the baseline current. Typically, the trace would shift very little and very slowly once set to 0 pA. If any appreciable change in baseline occurs, we used the pipette offset command to shift the baseline to 0 pA as needed to negate current.

Acquisition parameters

In loose-seal cell-attached recordings, signals were acquired in continuous voltage-clamp mode with a gain of 10-20 mV/pA, digitized at 10 kHz and filtered at 3 kHz. To obtain more precise measures of action current parameters, signals were also acquired with a higher sample rate i.e. digitized at 100 kHz with a gain of 20 mV/pA and filtered at 8 KHz. The same acquisitions were done in current-clamp; no difference in kinetics was noticed between the signals recorded in these two modes.

CO2/pH challenge experiments in loose-seal cell-attached

To investigate the response of serotonergic neurons to changes in extracellular concentration of CO2 and pH, the experiments were performed using a modified oxygenating system.

Standard ACSF solution (i.e containing 26 mM $NaHCO_3$) was bubbled in three reservoirs, each one with a different mixture of O_2 and CO_2 which resulted in solutions having a different CO_2 content/pH (control solution: 95% O_2, 5% CO_2 pH ~7.4; hypercapnic, lower pH, solution contained 9% CO_2 and hypocapnic,

higher pH, contained 3% CO_2). The pH of oxygenated solutions was measured with a pH electrode before starting the experiment. In reporting the results, CO_2 values are given instead of pH, because CO_2 was experimentally controlled as the independent variable. When CO_2, was increased from 5% to 9%, there was typically a decrease in pH of 0.22-0.25 from the baseline pH of 7.4. Changes in CO_2/pH, were made by switching the feeding-perfusion tube between the three reservoirs. Changes in CO_2 were limited to the range of 3-9%, because levels beyond this range would be unlikely to occur under physiologic conditions.

Baseline firing pattern was recorded typically for 10-15 minutes before application of ACSF with different mixture of CO_2/O_2 (15 minutes). When the effects of 3% and 9% CO_2/O_2 on the same cell were tested, a recovery period of at least 15 min in normal CO_2/O_2 mixture (95% O_2, 5% CO_2 pH ~7.4) was left between applications.

Identification of serotonergic cells using electrophysiological parameters

As mentioned in the introduction and aims of this work, the principal objective of my studies was to characterize the functional modifications of serotonergic neuron activity using *in vitro* electrophysiological approaches in mutant mice in which overexpression of 5-HT_{1A} receptors was selectively induced in serotonergic neurons of the raphe. This involved the need to identify serotonergic cells during each electrophysiological experiment using their intrinsic characteristics and whenever possible using one distinctive parameter that could be recorded in whole-cell as well as in extracellular loose-seal cell-attached configuration.

Thus, in the initial phase of my thesis I studied the electrophysiological properties of serotonergic cells in non mutant, C57bl/6, mice. Serotonergic cells were identified with the use of whole-cell recordings combined with immunohistochemistry for L-tryptophan hydroxylase II (TpH 2) as a selective marker (Fig. 7). Several possible candidate parameters were investigated and in this section.

I will illustrate those that better characterize serotonergic cells in whole-cell recordings and that can be used to identify the recorded cell as a serotonergic neuron also in extracellular loose-seal cell-attached configuration.

Differences between serotonergic and non serotonergic neurons

Serotonergic neurons have several distinctive morphological, anatomical, and functional properties that, when considered together, allow to distinguish them from non serotonergic neurons. Serotonergic cells have a large soma (20-30 μm) of variable morphology and are localized predominantly along the midline of dorsal raphe region while non serotonergic neurons have smaller soma (10-15 μm), usually of bipolar shape, and are more dispersed throughout the raphe region.

When stimulated by α_1-adrenoceptor activation, serotonergic neurons display relatively slow and regular firing (0.1-4 Hz) with low variance in firing rate (typically of 5-20%), whereas non serotonergic neurons discharge with a wider range of firing

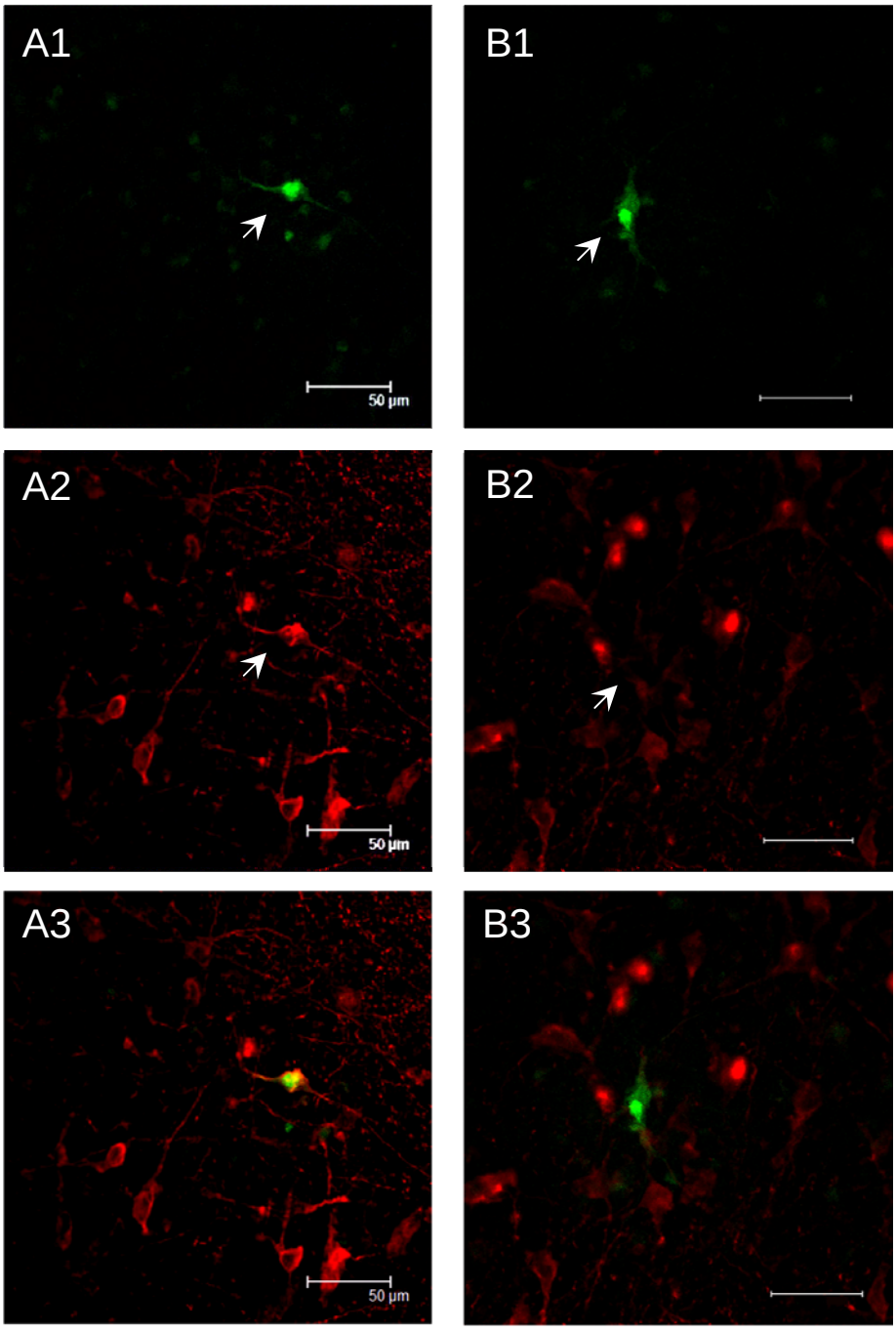

Fig. 7. Photomicrographs of TpH2-immunoreactive (5HT-neuron) and non TpH2-immunoreactive neurons (non 5HT-neuron), labelled with injection of a fluorescent dye. (A1, B1) recorded neurons labelled with Alexa fluor 488 (300 µM) are shown by arrows. Immunohistochemistry for tryptophan hydroxylase (TpH2) identified the label neuron as serotonergic (A2, TpH-ir) or not serotonergic (B2, non TpH-ir), see arrows. (A3,B3) merged.

rates (0.1-20 Hz) and often their firing is highly irregular with variance up to 100% (Fig. 8).

When directly stimulated by a series of steps current injection of increasing intensity, serotonergic neurons preserved their slow firing regularity reaching a constant firing rate level. Non serotonergic neurons increased their firing rate much more linearly with increase in the injected current and often did not show a plateau but cessation of firing (Fig. 9).

Furthermore, serotonergic cells possess basic properties (i.e. conductances) that underlie generation of action potential as well as its shape and duration, that are distinctive amongst cells of the dorsal raphe and that could be used to distinguish them from non serotonergic neurons. Thus, serotonergic cells have a broad action potential, with a shoulder during the repolarizing phase and a large slow-afterhyperpolarization (Fig. 7A, Fig. 10A1, see also Fig. 12 for details). In contrast, non serotonergic cells have shorter action potential and shorter AHP (Fig. 1B).

A further prominent difference is the fact that non serotonergic cells express HCN channels, which are not (or very weakly) expressed by serotonergic cells. Thus, non serotonergic cells have a substantial I_H current that is strongly activated at hyperpolarized cell membrane potentials and is revealed by a marked sag in the hyperpolarization produced by a step of negative current in whole-cell recordings (Compare Fig. 10B2 with 10B1 in a serotonergic cell).

Another distinctive property of serotonergic cells that is relevant to the present investigation is their high sensitivity to 5-HT_{1A} receptor activation. In fact, serotonergic cells respond with a marked hyperpolarization and decrease in firing rate to the application of relatively low concentration (10-30 nM) of selective 5-HT_{1A} receptor agonists (R-8-OH-DPAT or 5-CT), whereas non serotonergic cells show a weak, if any, response to these agonists, even when applied at very high concentration (100-300 nM) (Fig. 11).

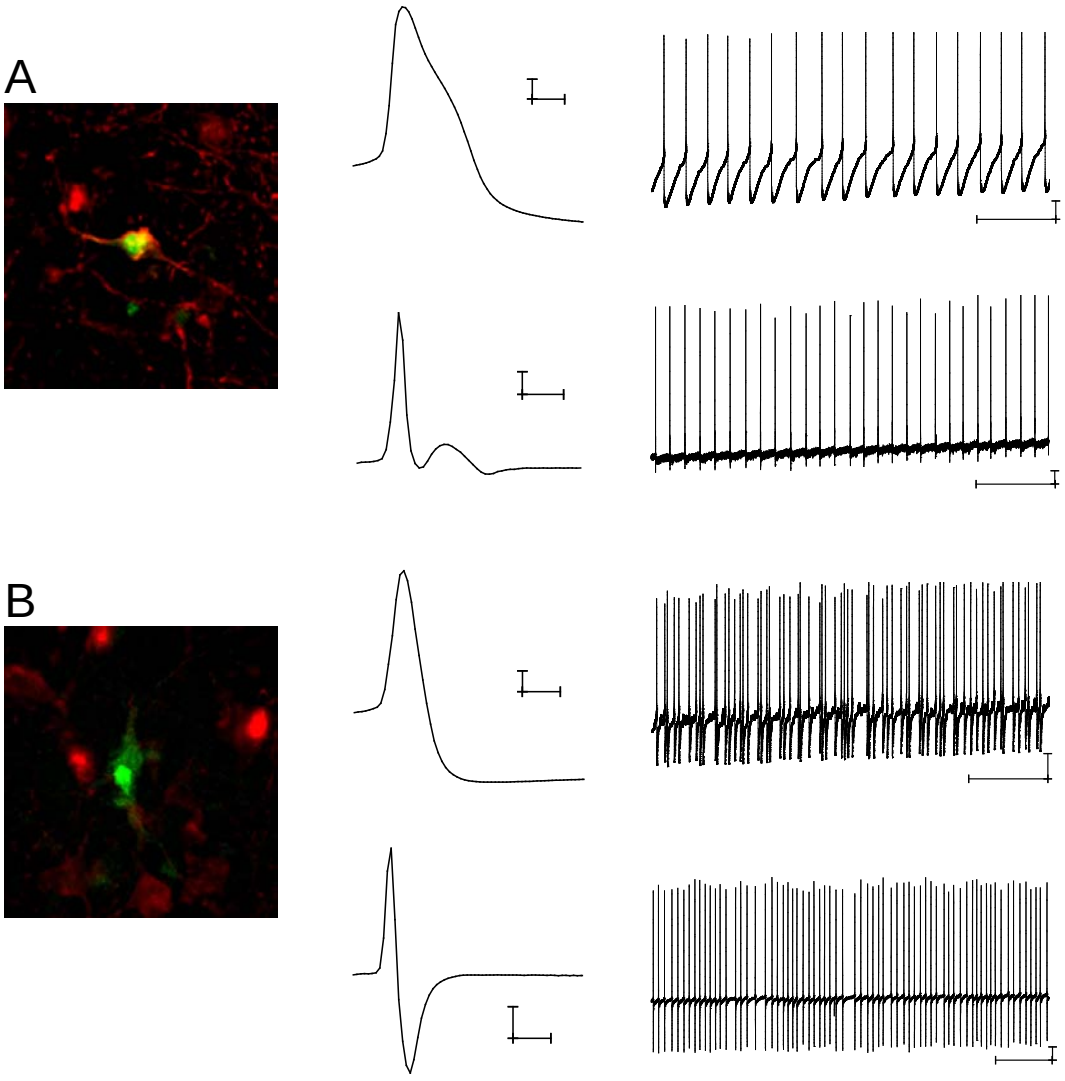

Fig. 8. Characteristical firing patterns of serotonergic and non serotonergic neurons. Panel (A) photomicrograph of the fluorescent dye labeled cell shown in Fig. 7A1-3, immunoreactive for TpH2 and traces of the respective action potential waveform and spontaneous firing rate recorded in whole cell (upper traces). Lower traces show the typical action current waveform and the pattern of firing recorded with loose-seal cell-attached recording from a serotonergic neuron. (B) the same set of information is shown as in (A) but for the non serotonergic cell in Fig. 7B1-3 (scales: action potentials 10 mV-1ms; firing in whole cell, 10 mV-2 s; action current, 50 pA-1ms; firing rate in loose seal cell-attached patch clamp, 50 pA/2 s).

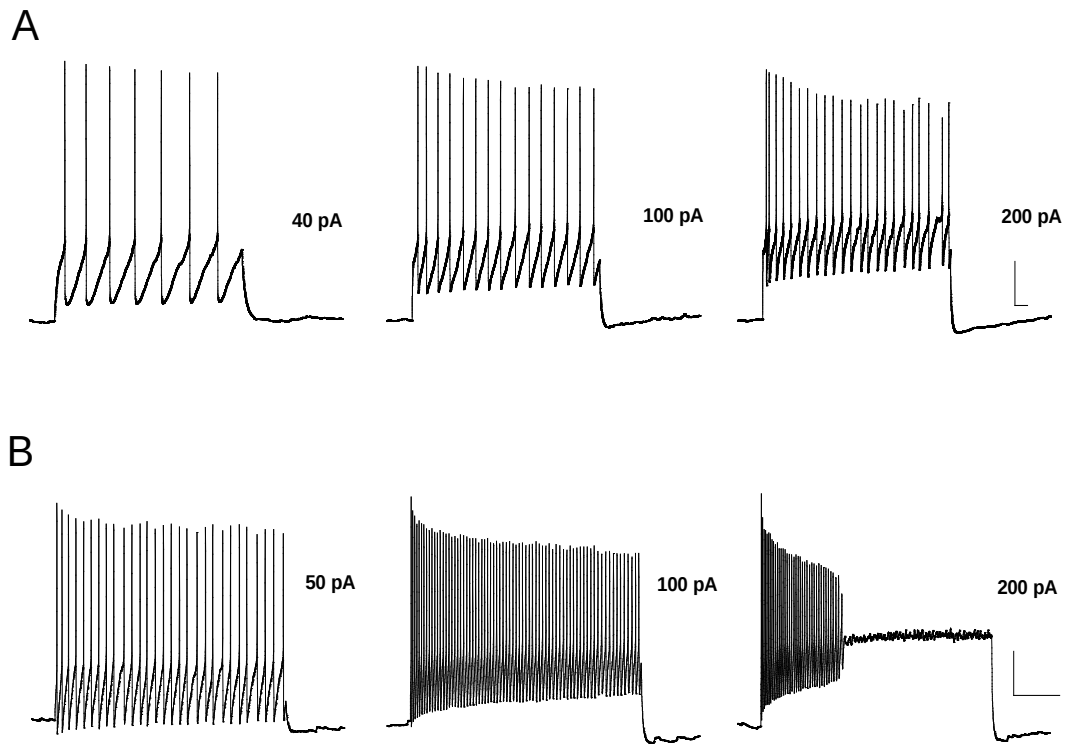

Fig. 9. Typical response of a serotonergic (A) and a non serotonergic (B) neuron to a series of depolarizing current steps injected through the recording electrode. (A) Slow and regular firing response of serotonergic neuron to injections of increasing current. Scales: 20 mV-1 s (B) response of a non serotonergic neuron. Scales: 20 mV-500 ms. Note that for injection of comparable current steps, the behaviour of the two cells were strikingly different. However, it should also be noted that the activity of the non serotonergic cell was regular and could be wrongly interpreted as serotonergic cell displaying a fast firing rate, but the two cells are discriminated by the action potential duration (see Fig. 13).

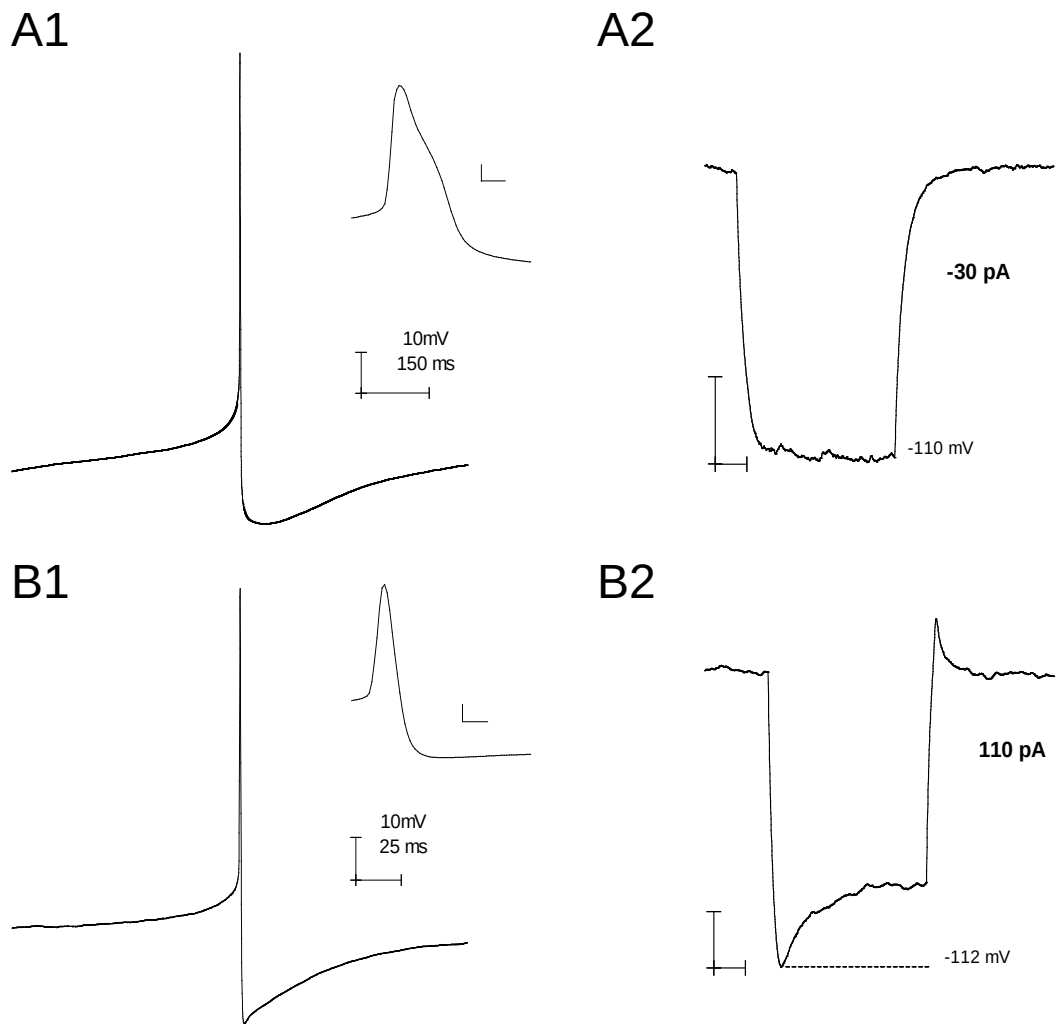

Fig. 10. Action potential waveforms and responses to hyperpolarizing current injections in a serotonergic (A) and a non serotonergic cells (B). (A1) typical broad action potential with shoulder (see *inset*) of a serotonergic cell, that shows a large afterhyperpolarization. (B1) typical action potential waveform of a non serotonergic cell; (A2, B2) response to negative current injection that revealed a prominent sag in a non serotonergic cell (B2) and the absence of sag in a serotonergic cell (A2). Scales: insets 10 mV-1ms; (A2, B2) 10 mV-200 ms.

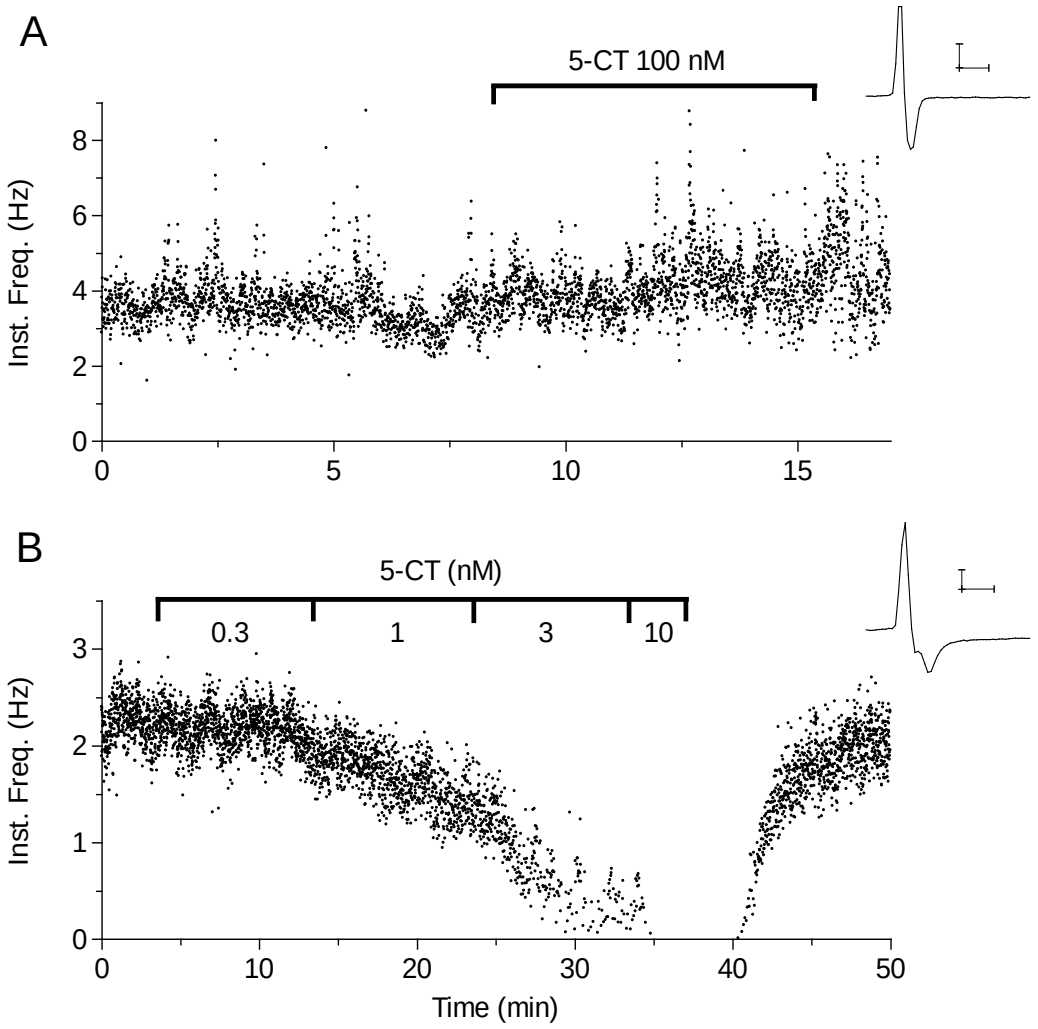

Fig. 11. Response of a serotonergic and a non serotonergic neuron to application of the 5-HT1$_A$ receptor agonist 5-CT in loose-seal cell-attached recording. (A) Time-course of instantaneous firing frequency showing the absence of response to 5-CT 100 nM in a non serotonergic neuron. (B) Time-course of the 5-CT concentration dependent inhibition of firing rate in a serotonergic neuron. Note that cessation of firing is reached with 5-CT 10 nM. *Insets*: action current waveforms, scale: 20 pA-1s.

Although any of these parameters is eventually useful for identification of serotonergic cells, the analysis of action potential waveform offers the most practical and immediate parameter for the identification of the recorded cell and, in most of cases, is sufficient for conclusive neuronal type identification. Therefore in the present study we used the action potential waveform to distinguish between serotonergic and non serotonergic cells. In addition, as we used an extracellular recording technique, the loose-seal cell-attached patch clamp, to monitor the firing behaviour of raphe neurons, I will describe the characteristics of the signal obtained with this technique and the meaning of its different phases in relation to the waveform of action potential recorded in whole-cell.

Action potential waveform analysis in whole-cell recording

Monoaminergic neurons display a long action potential with a plateau (shoulder) during the repolarizing phase that is absent in non serotonergic cells. The best suited parameter describing the duration of the action potential is its half height width of action potential (*HHW). HHW* can be defined as the time interval between the rising phase and descending phase of AP peak at the half point between peak amplitude and antipeak (negative peak) amplitude potentials (Fig. 12).

In C57bl/6 mice, HHW values of action potential in serotonergic cells are typically in the range of 1-3 ms while those of non serotonergic cells are comprised between 0.3 and 0.9 ms.

Considering that in loose-seal cell-attached patch-clamp the recorded waveform closely reproduces the kinetics of the neuronal action potential and can theoretically be approximated by the first derivative of the action potential recorded in whole-cell (see BOX 3), it becomes sensible to compare the action potential recorded in whole-cell with ***its*** first derivative, so to better understand the meaning of the different phases of the extracellularly recorded action current waveform.

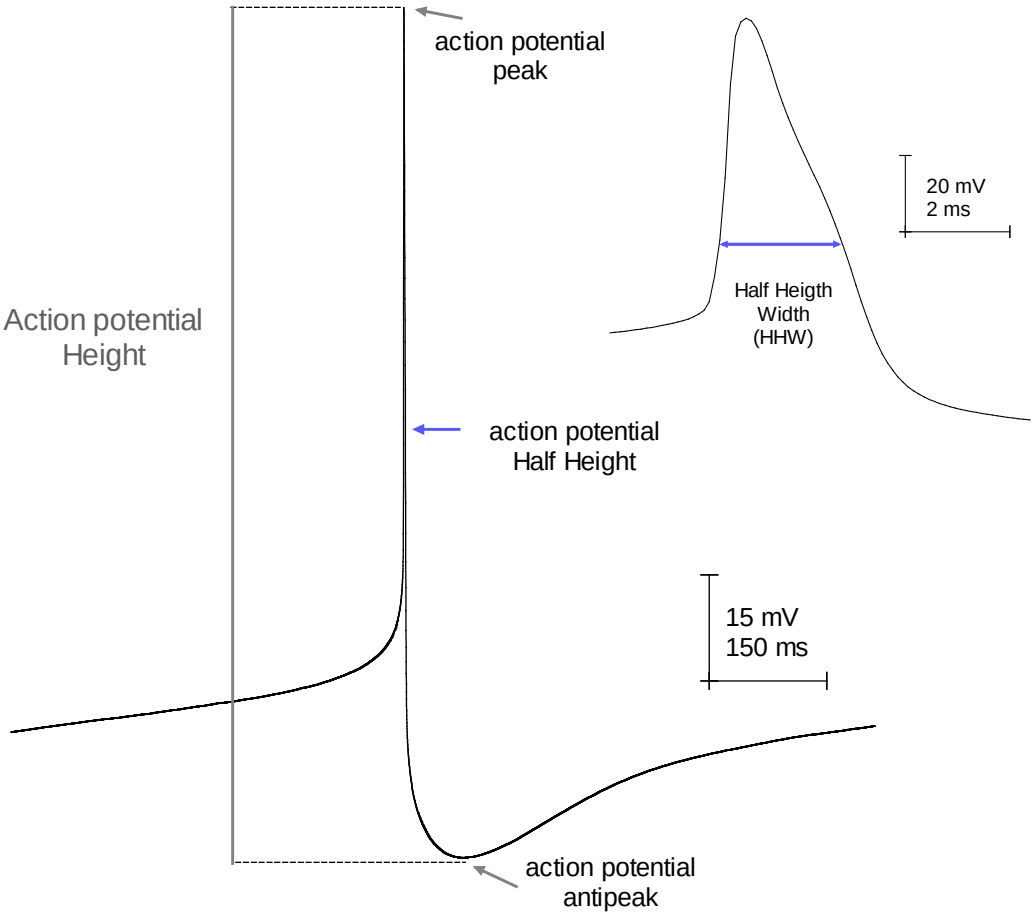

Fig. 12. Determination of the Half Height Width parameter (HHW). The measurement of action potential half-height is shown on the waveform recorded with a slow timebase to show the entire sequence of events preceeding and following action potential. *inset:* enlarged action potential. Double arrow shows the time interval taken as measure of HHW of the action potential.

As shown in Fig. 13, the first derivative in time (dV/dt) of the action potential presents peaks that correspond to points of maximal velocity of depolarization (*Upstroke*) and maximal velocity of repolarization (*Downstroke*). As action potentials have different shape in serotonergic and non serotonergic cells, their first derivative are also different. Non serotonergic cells display prevailingly a dV/dt with two peaks, one *Upstroke* and one *Downstroke*. Serotonergic neurons typically display three peaks, one *Upstroke* and two *Downstrokes*.

Thus, the measure of the interval between the upstroke and the downstroke(s) (*Upstroke* / *Downstroke* Interval: UDI) of the first derivative of the action potential recorded in whole-cell current-clamp, permits to quantify the duration of action potential. As illustrated in Fig 13B2,B3, in serotonergic neurones showing two downstrokes, the UDI parameter should be measured as the time interval between the *Upstroke* and the *second Downstroke* this is indicated by UDI_2 in Fig. 13B2 and 13B3, but for clarity I will refer to UDI throughout the following paragraphs. As shown in Fig. 14, UDI correlates in a very good manner (1:1 ratio) with the HHW of action potentials (UDI > 0.9 ms for serotonergic cells). Although the correlation between UDI and HHW is less strong in non serotonergic cells (UDI <0.9 ms; not shown) it appears clear that UDI >0.9 is distinctive of serotonergic cells and therefore can be safely used to identify these cells during recording.

In conclusion, since the signal recorded in loose-seal cell-attached corresponds to the first derivative of neuron action potential, the parameter UDI measured on action current recorded in loose-seal cell-attached patch-clamp configuration closely reports the duration of action potential, that is distinctive of cell type and can be used to distinguish between serotonergic and non serotonergic neurons (see Fig.13B3 vs A3).

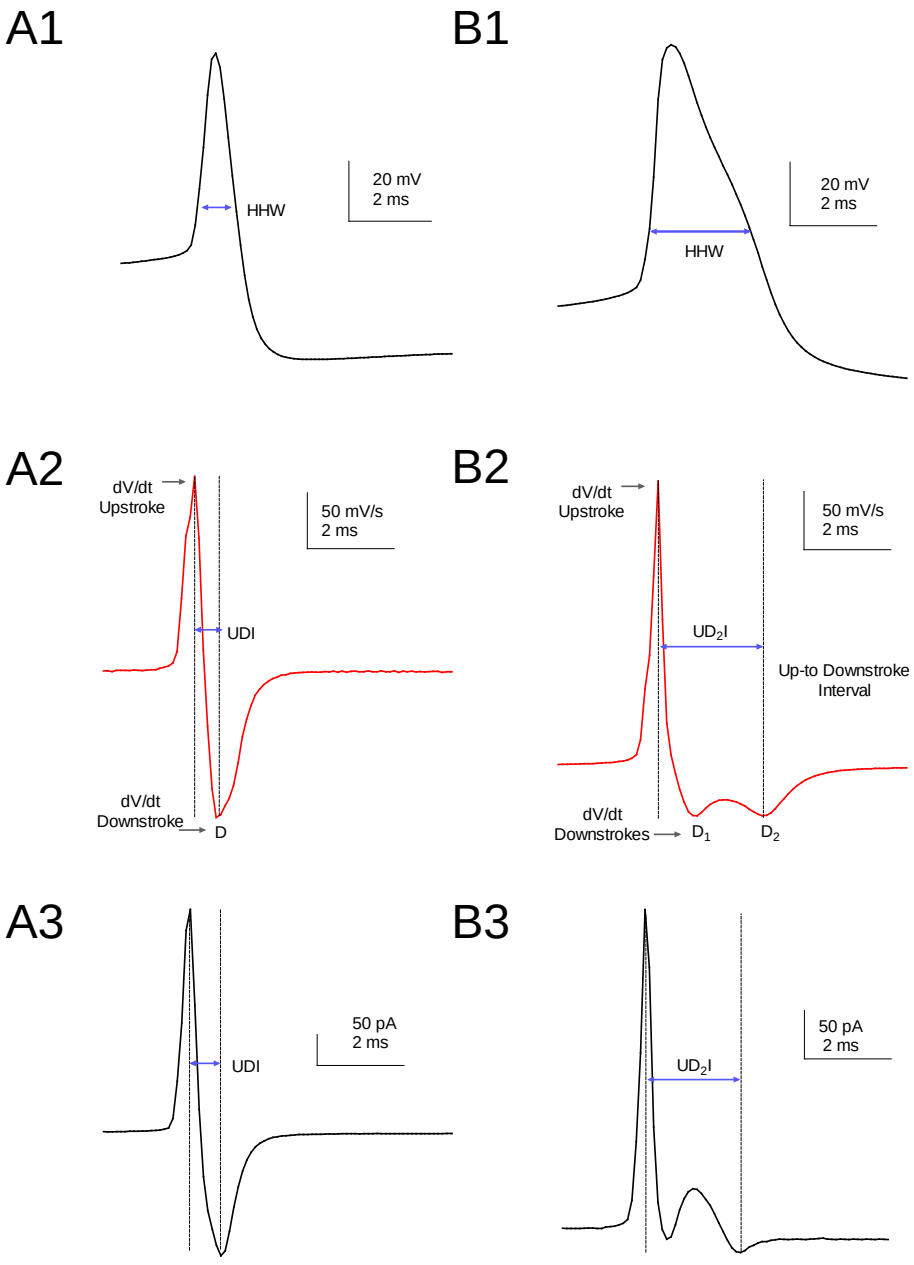

Fig. 13. Determination of Upstroke to Downstroke Interval UDI (UD_2I) parameter on the first derivative in time of action potential recorded in whole cell and on action current recorded in loose seal cell-attached. (A1-.3) is shown the half-height width (HHW) of the action potential recorded in whole-cell (A1) from a non serotonergic cell is compared with its first derivative (A2) and with the waveform recorded in loose-seal cell-attached mode. Note that the first derivative of the action potential closely resembles the action current recorded in loose-seal cell-attached. In (A2, A3) also shows how the UDI parameter on the first derivative in time of action potential and on action current is measured. (B1-3) show the corresponding traces and measures for a non serotonergic neuron. Note that, due to the presence of a shoulder during the repolarizing phase of action potential the first derivative in time of action potential and the extracellular recorded signal show two downstrokes and the measures are taken on the second (UD_2I).

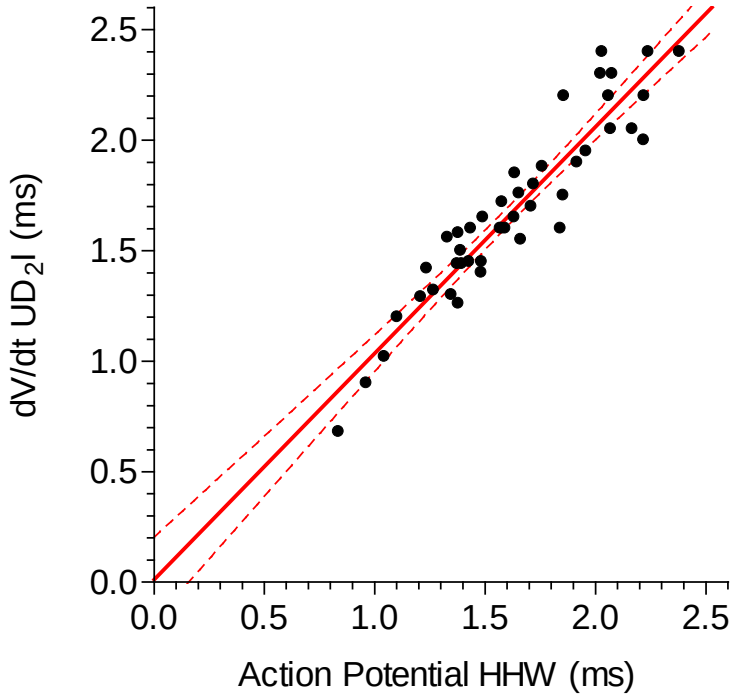

Fig. 14. Correlation between action potential Half Height Width parameter (HHW) and Upstroke to downstroke interval of the first derivative on time (UD_2I) of the action potential. The correlation of HHW and UD_2I (UDI) (UD_2I/ HHW= 1.02 ± 0.06, 95%CI 0.91-1.14, r^2=0.88; n=45).

BOX 3 ***THE SIGNAL RECORDED IN LOOSE-SEAL CELL-ATTACHED PATCH-CLAMP: THEORETICAL CONSIDERATIONS ON ITS ORIGIN AND SIGNIFICANCE***

This section reports a semiquantitative analysis that explains the origin of loose-seal recorded signal shape and the relationship between the extracellularly recorded signal (potentials/currents) and the transmembrane electrical events that originate extracellular signal.

In a simple spherical neuron the current generating an action potential (I_m) flows across an inactive region (where action potential is going to be generated), will consists of both capacitive and resistive components: $I_m = I_C + I_R = C_m (dV_m/dt) + G_m V_m$, where V_m is the transmembrane potential, C_m is the membrane capacitance, and G_m is the conductance of the inactive region of the membrane. Because the transmembrane current I_m draws current from the extracellular space, the time course of the inward current in the inactive region of the membrane will dictate the timecourse of extracellular potential (V_{ext}) across the extracellular space (i.e. across a resistance, R_{ext}). The time course of the extracellular recorded potential will be roughly proportional to I_m, or $V_{ext} \propto I_m$.

For electrical events that are relatively fast with respect to the membrane time constant (e.g. action potential $t >> \tau_m$), most of the current exiting in the extracellular space will be capacity current across the active region (where action potential has been generated). Thus for fast conductance change (ΔG_m fast respect to τ_m) the timecourse of the extracellular potential is roughly proportional to the first derivative of the transmembrane potential, or $I_m \propto dV_m/dt \propto V_{ext}$ (Humphrey *et al*., 1990; Johnston *et al*., 1995).

Therefore, loose-seal signal recorded in voltage- or current-clamp will have the same time-course but different polarity, the current recorded will be in the order of hundreds (to one thousand) of picoamperes and the voltage of few millivolts.

In conclusion, the extracellular potential/current recorded in loose-seal cell-attached patch-clamp will be closely proportional to the first derivative of transmembrane voltage.

Acquisition and analysis of electrophysiological parameters

Whole-cell recording

To distinguish between cells we preferably recorded spontaneous generated action potentials. If the cell did not fire spontaneously, we measured action potential parameters on action potentials evoked by the injection of steady current of minimal intensity (2-10 pA) or we used action potentials discharged on rebound depolarization that often follows an hyperpolarizing pulse. Action potentials were acquired at 25 kHz and detected with Clampfit proper procedure, setting the baseline at rest potential and the threshold at two third of AP amplitude. Individually detected events were peak-aligned and their average was used to measure HHW and UDI parameters.

Analysis of other parameters

From current-clamp and voltage-clamp recordings the following parameters were determined to characterize serotonergic neuron intrinsic properties:

- Membrane potential measured at break of patch in spontaneously firing cells. The following procedure was used to obtain an estimate of "resting" membrane potential (*RMP): RMP* was determined by interpolation from I_{HOLD} and R_{IN} measured on negative voltage step, 1mV/measured Rcell GΩ= unit pA; measured Ihold pA/unit pA= XmV, RMP=(-65 mV Vhold) + X mV),
- Membrane resistance R_{IN} was measured according to Ohm's law $R=\Delta V/\Delta I$, from the average of five negative voltage steps, as the mean value of last 40 ms of the elicited current pulse.
- Membrane capacitance C_M (determined online by a "macro" of the acquisition software fitting an exponential decay curve to the uncompensated membrane capacitive current transient, elicited by the voltage step),
- Time-dependent depolarization of hyperpolarizing current step, *depolarizing sag or* V_{sag} (determined from the largest hyperpolarizing step

as the difference between the average potential of the last 200 ms and the potential peak between the first 250 ms, $V_{sag}= V_{end}-V_{peak}$).

- Quantification of 5-HT_{1A} receptor-mediated activation of GIRK conductance by selective agonists (e.g. R-OH-DPAT). This was done as follows: ramps of negative potentials were imposed to the neuron membrane potential under voltage-clamp mode in the absence (control) and in the presence of the agonist. After digital subtraction of the control from the response in the presence of the drug, the *I-V* relationship between -85 and -95 mV was fitted by linear regression and the slope taken as the peak conductance.

Loose-seal cell-attached recording

Loose-seal recordings were high pass filtered with a bessel (8 pole) filter set at cut off frequency of 5 Hz. We used Clampfit (Axon Instruments) to detect and analyze the recorded action currents events. The instantaneous firing frequency obtained was collected to plot a time-course of each experiment.

Waveforms for identification of the cells were preferentially obtained from the earliest recording acquired with high gain and digitized at 100 kHz. When this recording was not available the average waveform of 1 minutes of early standard recording was taken. The waveforms of single events were collected at 40 ms before the upstroke and 100 ms after. The collected events were aligned at peak and the UDI parameter was measured on the average. The spontaneous firing rate *FR*, or the firing rate recorded after bath application of phenylephrine if the cell did not spontaneously discharge action potentials in normal ACSF, was measured as number of events over 1 or 3 seconds of stable recording.

Pharmacology

Analysis of responses to perfusion of drugs

All experiments in loose-seal cell attached recording in voltage clamp mode were carried out in ACSF supplemented with antagonists that block synaptic transmission ("cocktail") to isolate single cell activity from network.

The concentration-response curves for agonists (e.g. R-8-OH-DPAT, 5-CT) were obtained using cumulative concentration-response protocols. Drugs were applied until apparent steady state response was observed.

In whole-cell recording, the parameter used to construct the concentration-response curve was peak conductance, whereas in loose-seal cell-attached configuration we used the average firing rate over 1 or 3 minutes during steady state responses.

In both cases, steady state values of different drug concentrations were normalized to control (usually the firing rate in the presence of phenylephrine) and used to construct concentration-response curves.

The means of normalized measures were fitted with Prism 4 fitting facilities using a logistic equation: Y= bottom+ (top-bottom)/ 1+10ˆ((LogEC_{50}-Log [Agonist])*Hill Slope)), to obtain estimate for EC_{50} and slope of the curve (Hill slope).

Drugs

(+)8-Hydroxy-N,N-dipropyl-2-aminotetralin, R-8-OH-DPAT, was prepared as a 1 mM stock solution and diluted with distilled water to obtain intermediate stock solutions (a thousand times the experimental concentration), which were aliquoted and stored at -20 °C until use. All other drugs were prepared as stock solutions in distilled water, aliquoted and stored at -20 °C until use. All drugs were bath applied. When applied, organic receptor antagonists were perfused for 10 min before as well as during the agonist application. Agonists and inorganic salts were applied for ten or more minutes. N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-

N-(2-pyridyl)cyclohexanecarboxamide (WAY 100635, 50-100 nM), (+)8-Hydroxy-N,N-dipropyl-2-aminotetralin (R-8-OH-DPAT, 1-300 nM), 5-Carbossitryptamine (5-CT, 10-30 nM), were used as selective 5-$HT1_A$ receptor antagonist and agonists respectively. Phenylephrine was used as selective α1-adrenergic agonist. L-tryptophan (Sigma) was used as precursor of endogenous serotonin. To block neurotransmission, we used a mixture of antagonists that collectively block most excitatory (glutamatergic) and inhibitory (GABA and glycine) fast synaptic responses: $GABA_A$ receptor antagonist SR-95531 (10 µM) , $GABA_B$ receptor antagonist 3-N[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-P-benzylphosphinic acid (CGP 55845 A, 2 µM), NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (D-AP5, 20 µM), AMPA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM) or 6,7-Dinitroquinoxaline-2,3-dione (DNQX, 10 µM) and the glycine receptor antagonist strychnine (10 µM). This mixture of drugs will be indicated as ”cocktail” of synaptic blockers throughout the following sections.

Tetrodotoxin (TTX) was from Alomone (Jerusalem, Israel), all other drugs from Tocris Cockson (Bristol, UK) and Ascent scientific (Bristol, UK).

Statistical analysis

Data were analysed using, Clampfit (Molecular Devices) and Prism 4 software (GraphPad Software, San Diego, CA, USA). Unless otherwise stated, data were expressed as the mean ± SD and were analysed statistically with the use of two-tailed Wilcoxon or Mann–Whitney tests, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Fluorescent Dye intracellular labelling of the recorded cell and Immunohistochemistry

Dye filling

In a subset of experiments, recording from putative serotonergic neurons were made using a pipette solutions containing either 2 mM Lucifer yellow (Sigma-Aldrich) or Alexa Fluor 488 hydrazide, 300 µM (A10436 solid, Invitrogen). This allowed us to mark the recorded neuron with a high concentration of fluorescent dye, which facilitated subsequent *post hoc* identification of the recorded neuron. Following recording, the pipette was removed from the cell by pulling away and slowly lifting the pipette upward and, at the same time, trying to leave the soma intact. The formation of a giga-Ohm seal was observed.

After the electrode removal, cell coordinates in respect to acqueduct midline and lower rim were recorded and the slices were cut on their right upper edge with the tip of an electrode to recognize the side containing the cell during the mounting process.

Fixation

The slices were washed with ACSF in a well to eliminate dye residues from tissue surface. Then they were fixed with 4% paraformaldehyde in phosphate buffer solution (PBS; 0.1 M) for 1–24 h.

Immunohistochemical processing of slices was carried out in dim light and when the protocol did not require manipulation of the preparations, the well containing the slices was kept in a black box to avoid the exposure to light.

Epitope retrieval method

After fixation, the slices were rinsed three times in PBS 0.1 M. They were incubated, in eppendorf tubes, with sodium citrate buffer (10 mM sodium citrate, 0,05% Tween 20, pH 6) and heated 3 min at 90°C, to retrieve the antigen's epitope.

Aspecific antigens block

To limit the binding of antibodies to aspecific sites these were "blocked" by adding goat serum to the incubating solution together with a nonionic surfactant to facilitate penetration of antibodies in brain tissue. Thus, slices were washed in PBS 0.1 M for 3 times and then incubated from 4 to 6 hours in a solution containing: 10% goat serum, 0.5% Triton X-100, 0.05% NaN_3 in 0.1 M PBS.

Incubation with antibodies

To identify putative serotonergic neurons, fluorescent immunohistochemistry was performed by incubating slices overnight in the presence of a primary rabbit monoclonal antibody against L-tryptophan hydroxylase II (Gutknecht *et al*., 2008) diluted 1:900; in PBS 0.1 M with 0.05% NaN_3, at 4°C. After washing with PBS 0.1 M, slices were incubated overnight at 4°C with an antirabbit-Alexa Fluor 633-conjugated secondary antibody (Molecular probes) diluted 1:500 in PBS 0.1 M with 0.05% NaN_3.

Sections mounting

The sections were washed in PBS 0.1 M and in distilled water, then mounted on slides under microscopy to recognize the mark done for the correct orientation of the slice and recognition of recorded cell location. All excess of water was dried using a pipette and a narrow piece of paper tissue before a drop of gel-mounting medium for fluorescent specimens Vectashield (Vector Laboratories, Burlingame, CA) was put on the slide and the specimen coverslipped.

The slide was gently pressed with forceps and the coverslip was sealed with nail varnish. Mounted sections were stored at -20 °C.

Immunofluorescence visualization and image acquisition

Immunofluorescence labelling was visualized using a Leica confocal microscope. Images were captured using a digital camera and Leica Confocal software (Version 2.5, Leica). When using the confocal microscopy, 79 sequential photomicrographs (0.5 µm tissue thickness each) were obtained by lowering focus on z axis relative to the x-y slice surface plane (z-series). This resulted in a tridimensional scanning for a total length of 51 µm. 37 images were acquired at

the level of the cell body of the labelled neuron through a total depth of 22 µm. Alexa 633 was revealed separately from Alexa 488. The laser power and emission filters were adjusted for both the red and green fluorophor so that there was negligible possibility of false positive result. This was done by exciting at the optimal wavelength for the green fluorophor and detecting using the emission spectra for the red fluorophor and vice versa. An Image as "projection" of all photomicrographs acquired was adjusted for optimal colour balance and contrast using Adobe Photoshop software.

Mice genotyping

Tissue sampling and digestion

Mice at PND20-21 were divided from the mother and housed per gender. An ear tag was applied at each animal with the proper applicator. To obtain a sample of tissue for genotyping, the tip of the tail was excised (less than 0.5 mm) with a sterilized scalpel, on a surface cleaned with ethanol 70%, and inserted into a previously autoclave-sterilized eppendorf tube. For digestion, 200 µl of a buffer composed in mM: NaCl 20, pH 8-Tris HCl 50, pH8-EDTA 1, SDS 10% in distilled and sterilized water with 1 mg/ml proteinase K was added to each eppendorf tube and heated at 55°C for 4-5 h.

DNA extraction

After digestion, the samples were equilibrated adding to each 200 µl of phenolchloroform (4°C) and centrifuged 10 minutes at maximum rpm at 4°C to separate phases. Under a biological hood, 80 µl of the upper phase of each sample were removed and poured in a well of a flat-bottom 96-well plate, 160 µl of 100% ethanol (-20°C) was added in each well and the plate was leaved at RT at least 10 min without agitation. After this period the well was inverted gently but rapidly, onto a paper towel to remove the ethanol and wells were allowed to dry for 15 minutes under the hood. The DNA that would remain in the well was then resuspended in distilled (and sterilized) water.

PCR cycling

A PCR reaction was performed for each gene (Slc6a4^{tTA} or Htr1a^{tetO}). For each sample 2 µl of DNA solution were mixed with 23 µl of PCR buffer composed by: Primer 1 or common primer (Sert-F1 or Htr1a tetO-F1) 2 µM, Primer 2 (Sert-R1 o Htr1a tetO-R1) 1 µM, Primer 3 (TTA-R1 or Htr1a tetO-R2) 1 µM (Sigma), native Taq polymerase 0.04 U/µl, dATP 0.2 mM, dCTP 0.2 mM, dGTP 0.2 mM, dTTp 0.2 mM, KCl 50 mM, MgCl2 1.5 mM (Fermentas), bovine serum albumin 0.1 mg/ml (Sigma), in distilled (sterilized) water. PCR cycling (see Table 1) was started with less than 5-10 min delay from the buffer mixing.

Tab.1. PCR cycling protocol (GEN01)

step	time	temperature	action
1	1:00	95°C	
2	0:15	95°C	
3	0:15	64°C	
4	1:30	72°C	go to step 2, 2x
5	0:15	95°C	
6	0:15	61°C	
7	1:30	72°C	go to step 5, 2x
8	0:15	95°C	
9	0:15	58°C	
10	1:30	72°C	go to step 8, 20x
11	0:15	95°C	
12	0:15	55°C	
13	1:30	72°C	go to step 11, 10x
14	10:00	72°C	
15	forever	10°C	

Alleles identification

Once PCR was terminated 5 µl of running dye was added to each sample and 15 µl of this mix were loaded on 2% agarose gel with ethidium bromide, 10 µl of

100pb-1kb ladder was also loaded for reference. Identification bands for each allele were separated in gel electrophoresis after 1h at 110 V. Bands were visualized under an UV transilluminator. Slc6a4^{tTA} gene: wt allele (Sert-F1/Sert-R1) 306 pb, tta allele (Sert-F1/TTA-R1) 420 pb; Htr1a^{tetO} gene: wt allele (Htr1a tetO-F1/ Htr1a tetO-R2) 750 pb, tetO allele (Htr1a tetO-F1/ Htr1a tetO-R1) 338 pb

Tab.2. Primers sequences for both knock-in genes

Gene	Primer Name	N-pb	Primer sequence (5' to 3')
Slc6a4^{tTA}	1. Sert-F1	19	GTC AGG GTC CTT GGC AGA T
	2. Sert-R1	20	ATC TTC TTG CCC CAG GTC TC
	3. TTA-R1	20	TCC AGG GTC TCG TAC TGC TT
Htr1a^{tetO}	1. Htr1a tetO-F1	22	CAG TCT CTA GAT CCC CTC CCT T
	2. Htr1a tetO-R1	22	AAG GGC AAA AGT GAG TAT GGT G
	3. Htr1a tetO-R2	22	GGG CGT CCT CTT GTT CAC GTA G

RESULTS

The first main objective of our study was to evaluate the magnitude of the alteration in 5-HT_{1A} receptor functioning produced by the mutations introduced in $HTr1a^{RO}$ mice and resulting in a selective overexpression of 5-HT_{1A} receptors by serotonergic cells.

Our first goal was therefore to quantitatively compare the response of $HTr1a^{RO}$ serotonergic cells with that of serotonergic cell, in wild-type mice.

To afford this pharmacological investigation we decided to use loose-seal cell-attached recording to estimate the concentration-response relationship of selective 5-HT_{1A} receptor agonists on firing rate of serotonergic neurons. In fact, this technique is minimally invasive and permits long-lasting recordings that are required to investigate the effects of multiple applications of drugs.

Furthermore, the recorded parameters (action current/potential and neuron firing rate) reflect the activity of serotonergic neurons and would allow inference on the integrate effects of altered 5-HT_{1A} receptor stimulation on serotonergic cell functioning. However, this technique does not allow to apply a series of electrophysiological protocols/tests that, with other recording configurations (e.g. whole-cell patch-clamp), could help to identify the recorded neuron type.

On the other hand, to quantitatively compare the response of serotonergic cells in $HTr1a^{RO}$ and wild-type mice, unambiguous identification of the recorded cell type was required to avoid that results would be spoiled by the presence of non serotonergic cells in one of the two serotonergic neuron groups.

Identification of serotonergic neurons in wild-type and HTr1a^{RO} mice

Throughout the present work we used a "morpho-anatomical" criterion to select serotonergic cells *before* recording. Thus, we looked for neurons located in the midline of dorsal raphe region, where serotonergic cells are clustered and targeted cells that had larger diameter, because most of interneurons have a small soma. Therefore, non serotonergic neurons recorded in the present study were those that escaped the preliminary "morpho-anatomical selection", and resulted to be a small group. Nevertheless, their presence induced to apply further criteria to confirm the nature of the recorded cell, namely electrophysiological properties and pharmacological responses to phenylephrine and/or selective 5-HT$_{1A}$ receptor agonists (see also methods).

Thus, to identify serotonergic from non serotonergic neurons we routinarily used the electrophysiological criterion developed in previous experiments on C57bl/6 animals and based on the Up-to second Downstroke Interval (UD$_2$I or UDI) parameter of action potential currents recorded in loose-seal cell-attached configuration. As detailed in methods, this parameter is strictly correlated with the shape and duration of the neuron action potential.

As illustrated in Fig 15A, by ordering all neurons recorded in wild-type mice on the basis of their measured UDI, the resulting distribution of cells made apparent that the small number of non serotonergic neurones could be clearly discriminated from serotonergic neurons for their UDI < 0.9 ms.

Unexpectedly, when we applied the same electrophysiological criterion during recording from HTr1a^{RO} mouse neurons displaying typical serotonergic action current waveform and firing behaviour, we realised that the duration of their action currents was shorter than usual.

As illustrated in Fig 15B, the distribution analysis carried out on neurones recorded from dorsal raphe of HTr1a^{RO} mice made apparent that the histogram

was skewed towards the left (i.e. towards lower UDI values) for the whole population of recorded cells.

A more attentive analysis shows that twelve cells of HTr1a^{RO} mice displayed an UDI with a range of 0.9-1.1 ms, rarely seen in otherwise unequivocally recognizable serotonergic neurons of wild-type and C57bl/6 mice. For example, in our experiments on wild-type, only one serotonergic cell is found in this range (see Fig. 15A).

It seemed reasonable to assume that these cells were also serotonergic as their UDI was >0.9 ms, and had typical serotonergic waveform and firing behaviour. Nevertheless, when we became aware of this phenomenon, we tested a sample of the cells displaying a UDI comprised between 0.9 and 1.1 ms for their response to the application of selective 5-HT$_{1A}$ receptor agonists (R-8-OH-DPAT or 5-CT 3-10 nM), that is the second best choice between criteria to identify serotonergic cells.

All six cells tested were silenced by the application of R-8-OH-DPAT or 5-CT 10 nM, thus we concluded with a high degree of confidence that the others were also serotonergic. On the other hand, it should be noted that some of neurons displaying an UDI shorter than 0.9 ms could also have been serotonergic.

Investigating this issue resulted unpractical in terms of effectiveness regarding the main project and therefore we adopted the established criterion of an UDI shorter than 0.9 ms to discriminate non serotonergic neurons, although it remains possible that a small number of serotonergic neurons resulted excluded.

On the other hand, we became concerned of the possibility that serotonergic cells of HTr1a^{RO} mice underwent adaptive changes in their intrinsic cell membrane properties, which could affect the response to serotonin. Therefore, we compared several electrophysiological parameters of serotonergic cells recorded from HTr1a^{RO}

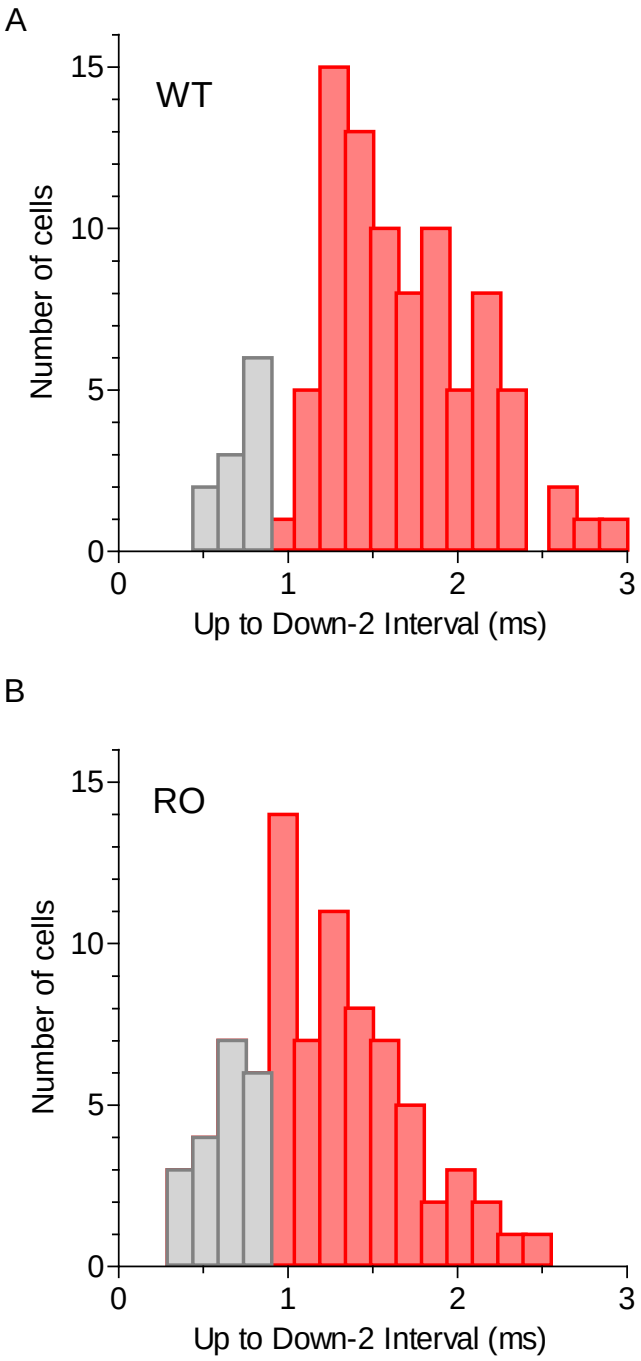

Fig. 15. Distribution histograms of the distinctive parameter Up-to Downstroke Interval (UD_2I) in wild-type (WT) and $HTr1a^{RO}$ mice (RO). Interneurons are clearly separated by this parameter from serotonergic cells in wild-type mice. Serotonergic cells (red) were found with UDI value > 1.1 ms, interneurons (grey) with UDI < 0.9 ms. Note that in wild-type mice (A) only one serotonergic cell out of 95 had an UDI comprised in the "boundary interval" between 0.9 and 1.1 ms. In $HTr1a^{RO}$ mice (B) the number of putative serotonergic cells found in the "boundary" range 0.9-1.1 ms increased to 12 out of 84, 6 of which were confirmed to be serotonergic using additional criteria (see text).

and wild-type mice to reveal possible alterations in basic intrinsic cell membrane properties of serotonergic cells in the mutant mice.

Electrophysiological properties of serotonergic neurons

First, we assessed whether the UDI parameter, that seemed systematically shorter in HTr1a^{RO} mice than in wild-type animals, was statistically different in the two mouse strains. As shown in Fig. 16, the Up-to second Downstroke Interval was indeed significantly shorter in HTr1a^{RO} mice (1.42 ± 0.40 ms, n=84) compared with that of wild-type animals (1.67 ± 0.43 ms, n=95, P value < 0.001, Mann Whitney test).

Since UDI of action currents corresponds to action potential half-height width (HHW, see methods), these results show that action potential duration of serotonergic cells in HTr1a^{RO}mice was also shorter than that of serotonergic cells in wild-type mice.

We examined for differences in serotonergic cells basic properties also whole cell experiments. However since they were performed to investigate 5-HT$_{1A}$ receptor activated GIRK-current, properties like resting membrane voltage, input resistance and action potential half-height width resulted modified by the special experimental conditions used. The only exception was total membrane capacitance that is independent by such manipulations (Fig. 16). Membrane capacitance was then found significantly smaller in serotonergic cells of HTr1a^{RO} mice (35.67 ± 6.73 pF, n=20) compared with that of wild-type (40.44 ± 7.67 pF, n=38, P value < 0.05, Mann Whitney test).

These results also strongly indicated that there were differences in electrophysiological or basic properties between serotonergic cells of wild-type and HTr1a^{RO} mice. We further investigated this issue using loose-seal cell-attached

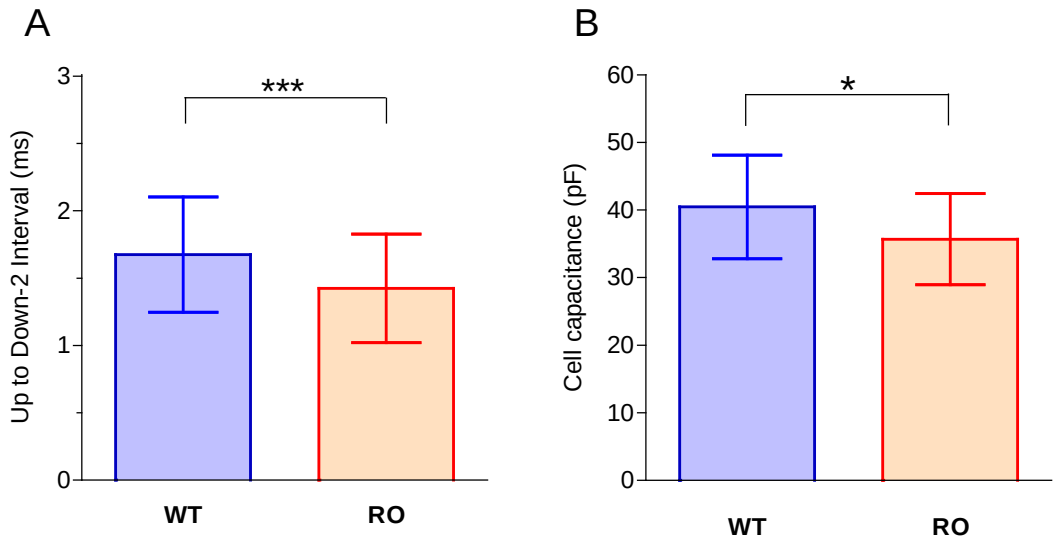

Fig. 16. The Up-to second Downstroke Interval and Cell capacitance are smaller in HTr1a^{RO} than in wild-type mice. (A) Bar graph shows the mean (± SD) values of UDI parameter for serotonergic neurons in wild-type (WT, n=95) and HTr1a^{RO} mice (RO, n=84) and. Asterisks indicate highly significant difference (P=0.0003, Mann Whitney test). (B) Bar graph shows the mean (± SD) values of cell membrane capacitance for serotonergic neurons in wild-type (WT, n=38) and HTr1a^{RO} mice (RO, n=20) and. Asterisks indicate a significant difference with P value < 0.05.

recording to obtain information on firing behaviour of neurons with recording conditions that produced minimal perturbation of neuron properties.

The first electrophysiological property we investigated was the firing rate of serotonergic neurons. Firing rate was measured i) as the spontaneous firing rate in conditions in which serotonergic neurons were pharmacologically isolated from major excitatory and inhibitory inputs, and ii) as the maximum firing rate of neurons during their maximal activation by stimulation of α1-adrenoceptors, that is in conditions similar to those of waking state *in vivo*.

The intrinsic spontaneous firing rate was measured as the number of action currents on 1-3 minutes of recording at steady-state superfusion of antagonists for fast and slow synaptic transmission, to isolate the cells from network activity.

The mean intrinsic firing rate was similar in serotonergic cells of wild-type (0.636 ± 0.856 Hz, n = 49) and HTr1a^{RO} (0.497 ± 0.792 Hz ,n = 27) mice, (Fig. 17, left).

As can be noted the intrinsic firing rate variability was very high. It was ranging from 0-4 Hz in both cells of the two mice strains, but most of them were silent in both groups, 59.2 % for wild-type, 66.7% of total cells for HTr1a^{RO} mice, (29/49 and of 18/27 respectively). The maximum serotonergic firing rate or "waking state"-related firing rate, was evoked by application of the α1-adrenoreceptor agonist phenylephrine and measured as the number of action currents in 1 minute of recording at maximum drug effect. The mean evoked firing rate was also similar, for serotonergic cells of wild-type (2.094 ± 1.058 Hz, n=69) and HTr1a^{RO} (1.906 ± 1.143 Hz, n=56) mice (Fig. 17, right.).

The magnitude of response to the α_1-adrenoceptor agonist phenylephrine was also evaluated. The change in firing frequency of serotonergic cells, relative to their basal firing rate, elicited by phenylephrine was found to be quite similar in serotonergic neurons of wild-type and HTr1a^{RO} mice, i.e. 1.615 ± 0.906 Hz (n = 40) and 1.409 ± 0.732 Hz (n=27) increase, respectively (Fig. 18).

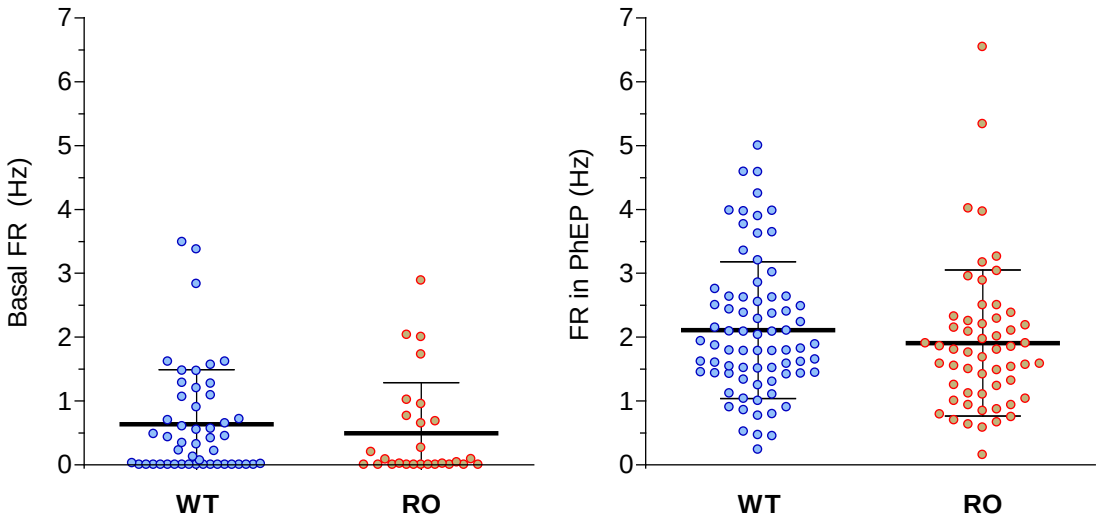

Fig. 17. Firing rate activity of serotonergic neurons in wild-type (WT) and HTr1a^{RO} mice (RO) is not significantly different. Left, the scatter plot shows the spontaneous firing rate in serotonergic cells isolated from network activity by the presence of glutamate AMPA and NMDA receptor antagonists and of GABAA, GABAB, and glycine receptor blockers (see methods for details on this “cocktail” of antagonists). Under these conditions, firing rate is not significantly different between serotonergic cells of the two strains of mice (n = 49 WT; n=27 RO; Mann Whitney test, P=0.285). It can be noted that the most of cells are silent in both groups with the same proportion. Right, scatter plot of firing rate in the presence of the α1-adrenoceptor agonist phenylephrine (PhEP, 10 µM, to reproduce the typical activation of serotonergic cells during waking state *in vivo*). In serotonergic cells of both mice strains the firing rate in phenylephrine is not significantly different (n=69 WT, n=56 RO; Mann Whitney test, P=0.133). Note that the two populations have quite the same variability.

From this initial set of experiments we concluded that, the decrease in duration of action potential made apart, serotonergic neurons in HTr1a^{RO} mice seemed to behave as "normal" serotonergic neurons in respect of their firing frequency and sensitivity to α1-adrenoceptor activation by phenylephrine. We therefore considered that the response to 5-HT$_{1A}$ receptor agonist could be investigated in HTr1a^{RO} mice serotonergic neurons without the risk of a systematic bias introduced by an alteration in firing properties of these cells.

Responses of serotonergic neurons to the selective 5-HT$_{1A}$ receptor agonist R-8-OH-DPAT

Serotonergic neurons of HTr1a^{RO} mice are known to express ~10 times higher density of 5-HT$_{1A}$ receptors compared to those of wild-type (Audero et al., 2008), but the direct proof that these newly (over)expressed receptors were able to respond to their direct activation by 5-HT$_{1A}$ receptor agonists was still missing.

To quantify the level of functionality of these receptors in DRN serotonergic neurons of HTr1a^{RO} mice we measured the response of serotonergic cells to 5-HT$_{1A}$ receptor activation by the selective 5-HT$_{1A}$ receptor agonist R-8-OH-DPAT on a set of electrophysiological parameters.

R-8-OH-DPAT is a potent 5-HT$_{1A}$ agonists with high affinity and selectivity (>400-folds) for 5-HT$_{1A}$ receptors relative to other 5-HT binding sites (5-HT$_{1B,1C,1D,}$ 5-HT$_2$, or 5-HT$_3$) (Van Wijngaarden et al., 1990). In serotonergic cells, activation of 5-HT$_{1A}$ receptors by R-8-OH-DPAT is known to open G protein-coupled inwardly rectifying potassium channels (GIRK) and to hyperpolarize the neuron, thereby decreasing neuron firing rate.

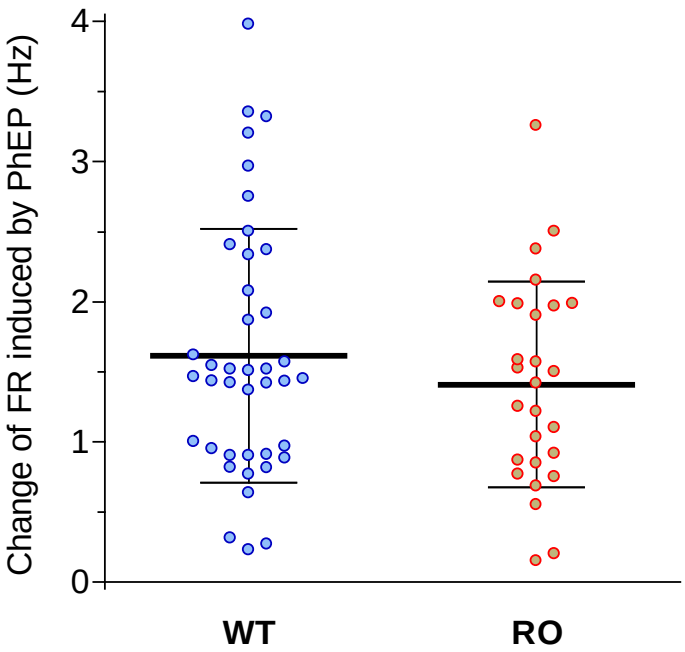

Fig. 18. Magnitude of the response of serotonergic cells to application of phenylephrine (PhEP, 10 µM) in wild-type (WT) and HTr1a^{RO} mice (RO). The change in firing rate induced by PhEP in both groups of serotonergic cells is not significantly different (P=0.516, Mann Whitney test), but the high variability of individual responses in both groups was very high.

Effect of R-8-OH DPAT on serotonergic neuron firing rate

In a first set of experiments, we quantified the response to R-8-OH-DPAT on firing rate using loose-seal cell-attached voltage-clamp recording.

All experiments were carried out in the presence of a "cocktail" of glutamate, GABA and glycine receptor antagonists to block fast and slow synaptic transmission. Under these conditions serotonergic cells were isolated from network activity intrinsic of raphe nuclei.

Thus, the effect of 5-HT_{1A} receptor stimulation on serotonergic neuronal firing rate could be studied in isolation from indirect mechanisms, induced by the action of the agonist on other raphe cells (e.g. GABAergic interneurons).

To reinstate *in vitro* the maximal noradrenergic drive of serotonergic neuron firing present during waking *in vivo*, phenylephrine (10 µM) was added to the superfusing fluid at least five minutes before application of R-8-OH-DPAT perfusion.

As shown in Fig. 19, the agonist R-8-OH-DPAT induced a concentration-dependent inhibition of serotonergic neuronal firing in both wild-type and $HTr1a^{RO}$ mice. This effect was reversible upon washout of the agonist (not shown for $HTr1a^{RO}$ mice). Using a similar protocol we collected data for assessment of concentration-response relationships in wild-type and $HTr1a^{RO}$ mice. This allowed a comparison of R-8-OH-DPAT effectiveness in decreasing the neuron firing rate in $HTr1a^{RO}$ compared to wild-type mice.

Firing rate values for the construction of concentration- response curves were measured as the number of action potential on 1-3 minutes of recording at the end of perfusion-time (10 min) for each R-8-OH-DPAT concentration. These firing rate values were normalized to control firing rate in phenylephrine for each recorded neuron. The averages of the normalized firing response elicited by different concentration of R-8-OH-DPAT in serotonergic neurons from both wild-type and $HTr1a^{RO}$ were then used and fitted by a logistic equation (see methods) to graph the

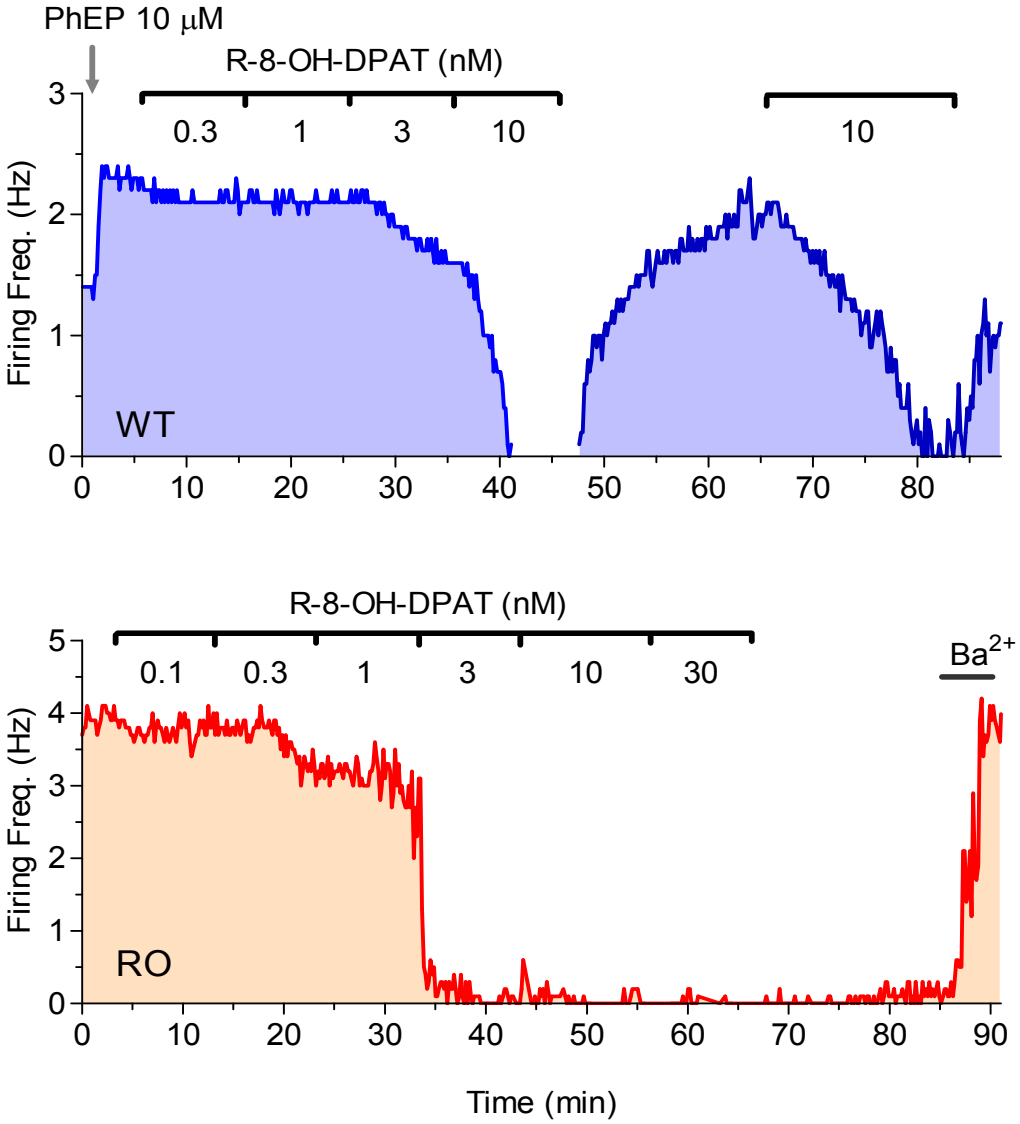

Fig. 19. Concentration-dependent inhibition of serotonergic firing rate induced by the selective 5-HT_{1A} receptor agonist R-8-OH-DPAT in wild-type and $HTr1a^{RO}$ mice. Graphs show time-course of firing frequency (expressed as number of action potential over 10 seconds) recorded from serotonergic cells with loose-seal cell-attached patch configuration in typical experiments. Upper panel: in a serotonergic cell from a wild-type (WT) mouse, phenylephrine (PhEP, 10 μM) increases the firing rate of the neuron. Addition of R-8-OH-DPAT concentration-dependently decreased neuron firing rate. The effect of R-8-OH-DPAT is completely reversible and pre-drug firing rate is recovered within ~ 15 min after agonist removal from superfusion ACSF. Lower panel shows a similar experiment performed on a serotonergic cell of a $HTr1a^{RO}$ (RO) mouse. Note that the experiment already started in the presence of phenylephrine. After perfusion of high concentration of R-8-OH-DPAT, barium was applied to avoid the need for long washout period. Barium at a concentration (150 μM) that preferentially blocks GIRK channels including these activated by 5-HT_{1A} receptors, produced full recovery of firing rate, indirectly confirming that the decrease of firing is mediated by this receptor.

concentration-response curves (Fig. 20) and obtain an estimate for their EC_{50} and slope.

As illustrated in Fig. 20, the concentration-response curve for R-8-OH-DPAT obtained from $HTr1a^{RO}$ mouse serotonergic cells was placed to the left of that from serotonergic neurones in wild-type mice, indicating greater effectiveness of R-8-OH-DPAT in $HTr1a^{RO}$ mice.

Thus, in wild-type serotonergic cells the EC_{50} value was 3.569 nM and the curve Hill-slope was equal to -1.7, (n=12). For $HTr1a^{RO}$ serotonergic neurones, the EC_{50} value was 1.585 nM and the curve Hill-slope was equal to -1.5, (n=11). The 95% confidence intervals of EC_{50} for the two fits were significantly different (3.13 to 4.06 nM for wild-type; 1.10 to 2.29 nM for $HTr1a^{RO}$ mice) as well as the Hill slopes (-2.036 to -1.338 for wild-type; -2.215 to -0.750 for $HTr1a^{RO}$ mice).

Fig. 20 also reports the comparison of the magnitude of responses elicited by 3 nM R-OH-DPAT in serotonergic cells from $HTr1a^{RO}$ mice and wild-type mice. The scatter plot shows that the 5-HT_{1A} receptor agonist elicited significantly greater responses in $HTr1a^{RO}$ compared to wild-type mice ($P<0.05$; Mann Whitney test).

Thus, our data show that the firing of serotonergic neurons of mutant mice was more sensitive to agonist-mediated 5-HT_{1A} receptor stimulation than that of wild-type mice.

Nevertheless, it must be noted that the sensitivity of $HTr1a^{RO}$ serotonergic neurons to 5-HT_{1A} receptor activation was less than what expected from a ten times 5-HT_{1A} receptor overexpression in these cells.

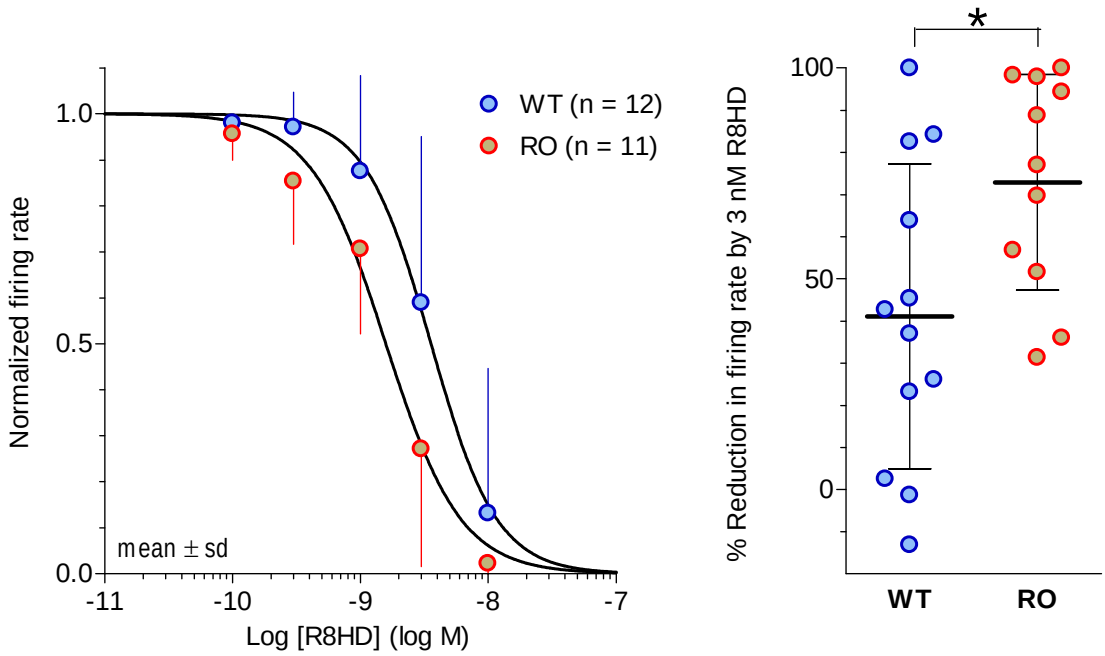

Fig. 20. Dorsal raphe serotonergic neurons of HTr1a^{RO} mice are more sensitive to 5-HT$_{1A}$ receptor activation than those of wild-type mice. Concentration-response relationship for R-8-OH-DPAT on firing rate of serotonergic neurons in wild-type (WT) and HTr1a^{RO} (RO) mice. The firing rate in R-8-OH-DPAT is normalized to firing rate in phenylephrine (see text). Right panel shows the effect of 3 nM R-8-OH-DPAT concentration on firing rate. Mean firing rates in 3 nM R-8-OH-DPAT are significantly different (asterisk: P=0.0423, Mann Whitney test) in the two mouse groups. Note the high variability in response of WT animals compared to RO mice.

Effect of R-8-OH DPAT on barium-sensitive inwardly-rectifying K^+ conductance

A precise measure of 5-HT_{1A} receptor-mediated effects cannot be performed in loose-seal cell-attached experiments as in this configuration only the firing rate is monitored, and no direct information can be obtained on the extent of membrane hyperpolarization that follows 5-HT_{1A} receptor stimulation in serotonergic neurons.

To further investigate 5-HT_{1A} receptor- mediated inhibition of serotonergic cells we extended our study using whole-cell voltage-clamp, which allowed to directly measure the extent of GIRK channel activation in the two strains of mice.

Indeed, whole-cell voltage-clamp recording permits to monitor the change of the 5-HT_{1A} sensitive GIRK current, thus provides a more precise evaluation of half-maximal effect (EC_{50}) and of the maximal response to the activation of 5-HT_{1A} receptors.

The GIRK channel current activation elicited by R-8-OH-DPAT is identified as an inward rectification of the total current measured in a voltage range between -60 and -120 mV.

Although R-8-OH-DPAT is generally effective in external solutions containing 2.75 mM K^+, higher potassium concentrations in the ACSF enhance the rectification induced by R-8-OH-DPAT (i.e. GIRK opening) allowing for more precise quantification of the effects produced by 5-HT_{1A} receptor activation. Therefore all voltage-clamp recordings were carried out in the presence of higher extracellular K^+ concentration (5.5 mM). In addition, we applied TTX (1 µM) to block sodium voltage-activated channels and synaptic transmission. Hyperpolarizing ramp voltage commands from a holding potential of -60 to -120 mV were used to characterize the *I-V* relationships of serotonergic raphe neurons before, during, and after exposures to R-8-OH-DPAT.

Figures 21 and 22 show traces and analyses of a typical experiment to illustrate how data were gathered and values for statistical comparison obtained.

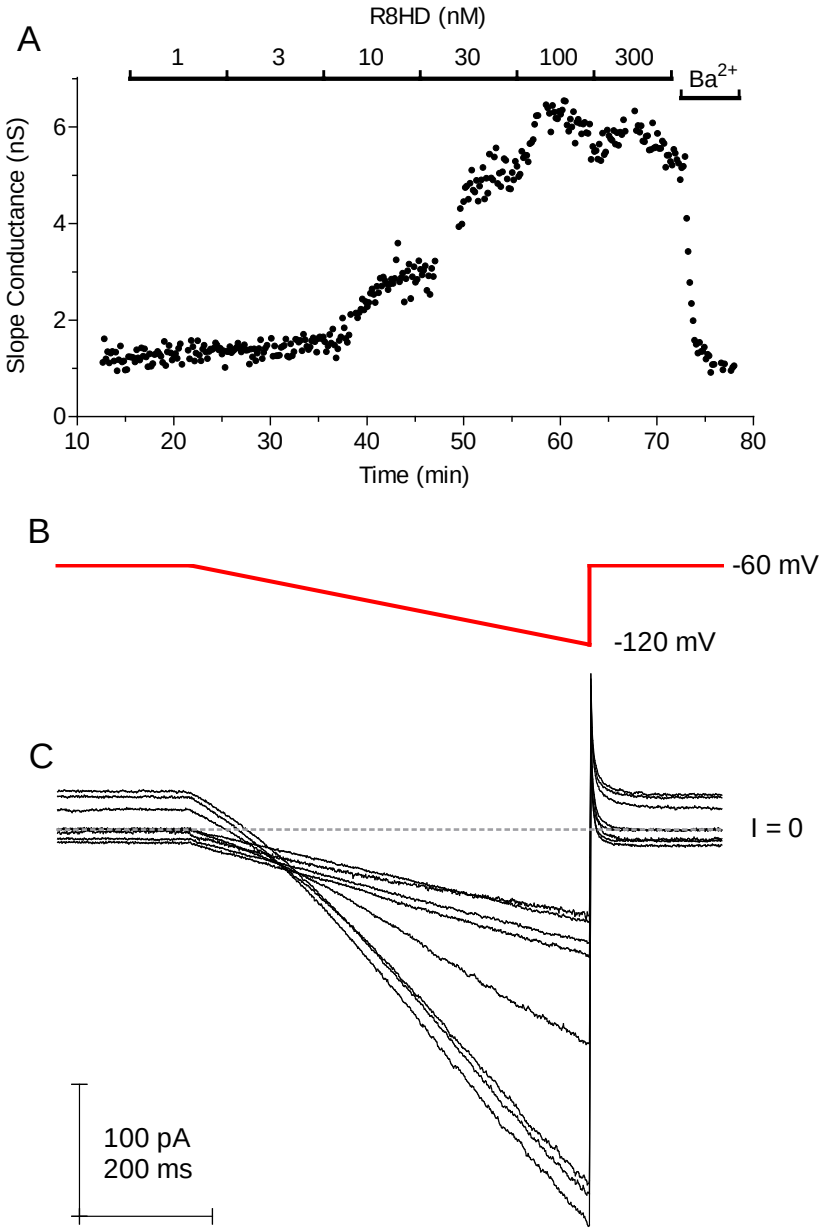

Fig. 21. Typical whole-cell voltage-clamp recording of conductance changes produced by R-8-OH-DPAT in a serotonergic neuron. (A) Time-course course of the slope conductance response to R-8-OH-DPAT application. Total cell membrane current elicited every 10 s by hyperpolarizing ramps through the recording pipette (B, C) to monitor the effects of increasing concentrations of R-8-OH-DPAT applied using a cumulative protocol. The effect was measured as slope conductance (see Fig. 22A). (B) illustrates the voltage ramp protocol applied to the neuron and (C) shows superimposed traces of the current evoked by the ramp in control and in the presence of increasing concentrations of R-8-OH-DPAT (1-300 nM), followed by application of barium (0.15 mM). In (A) note the slight reduction of conductance in 300 nM R-8-OH-DPAT (R8HD), probably due to receptor desensitization and the rapid recovery of conductance to control values in the presence of barium that blocks GIRK channels. Each trace in (C) is the average of five responses recorded in control and at steady-state drug effect for each drug application.

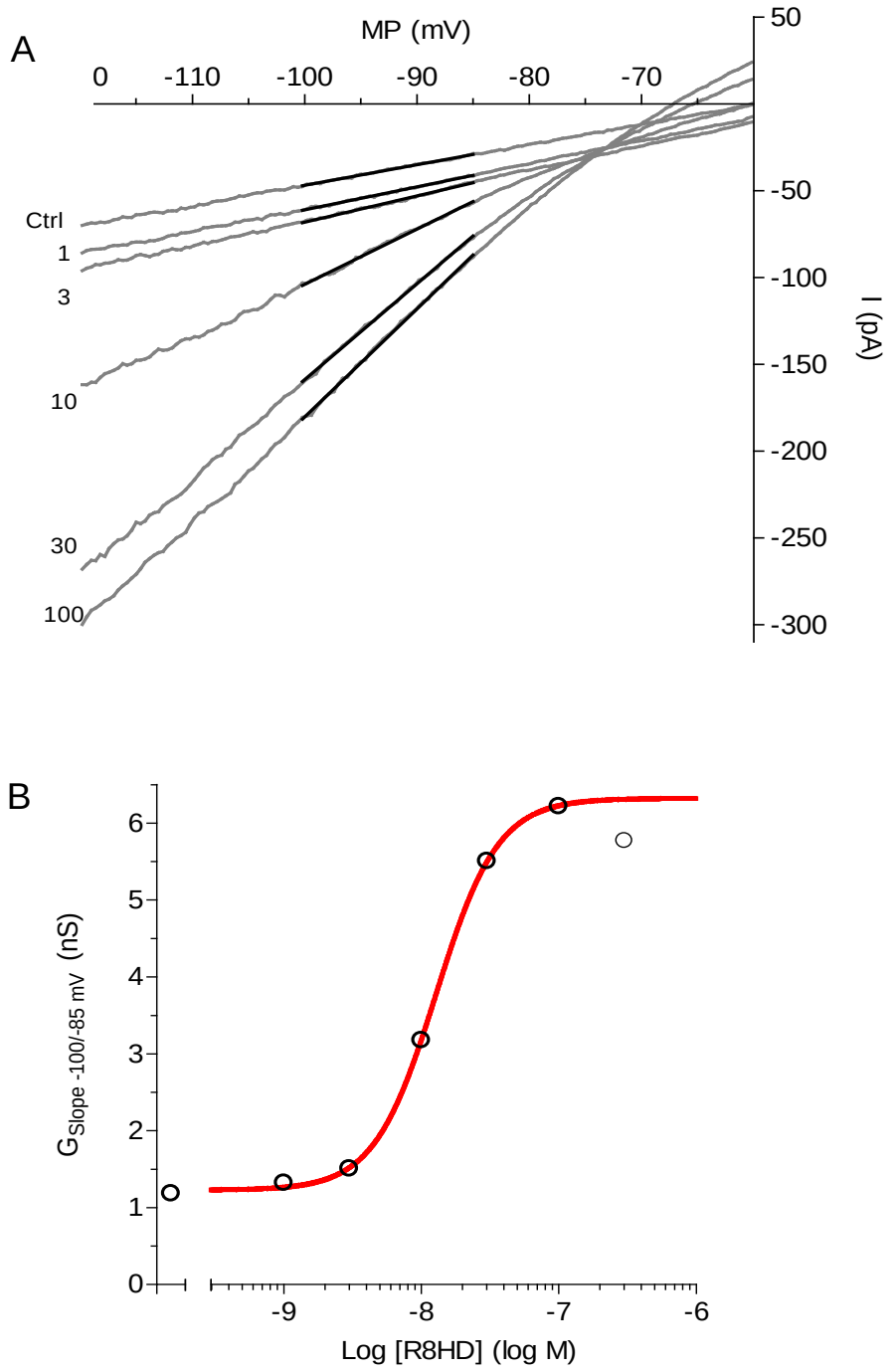

Fig. 22. Concentration-dependent activation of 5-HT_{1A} sensitive potassium conductance by R-8-OH-DPAT and the relative inwardly rectifier current in a typical whole-cell voltage-clamp experiment. Same cell as in figure 21). (A) panel, shows the current voltage relationship of 5-$HT1_A$ sensitive channels activated by R-8-OH-DPAT (concentrations in nM are indicated). Note the progressive increase in inward rectification with the increasing magnitude of current evoked by higher concentration of R-8-OH-DPAT. The linear regression fit to obtain the slope conductance is shown as a black line on each averaged traces. The (B) panel shows the concentration-response curve for slope conductance obtained as above. Note that the point at 300 nM was excluded for fit as desensitization occurred.

The current induced by R-8-OH-DPAT (Fig. 21B, 22A) displayed the characteristics of inwardly rectifying GIRK current activated by serotonin as described also for other preparations (Pan et al. 1990,1993; Penington et al. 1993a; Williams et al. 1988, Bayliss et al. 1997).

A distinctive feature of the 5-HT_{1A} receptor-activated inwardly rectifying K^+ current is the sensitivity to *low* concentrations of barium (Pan et al. 1990, 1993; Penington et al. 1993a; Williams et al. 1988). Thus, at the end of experiments we applied barium (0.15 mM) to further confirm the nature of current evoked by R-8-OH-DPAT.

To quantitate the effects of R-8-OH-DPAT on conductance, the *I-V* relationship between – 85 and -100 mV (Fig. 22A) was fitted by linear regression and the slope taken as the equivalent of conductance (slope conductance).

In Fig. 21A the slope conductance is plotted over time to show the time-course and the magnitude of the changes in conductance produced by application of increasing concentrations of R-8-OH-DPAT, using a "cumulative protocol".

Superfusion of R-8-OH-DPAT (1-300 nM) induced a concentration-dependent increase in slope conductance that fully recovered with the perfusion of barium 0.15 mM.

For the construction of concentration response curves, the slope conductance was calculated on I/V relationship obtained from the average of 5 traces taken at the end of R-8-OH-DPAT application for each concentration (Fig. 22A). The values collected were fitted by a logistic equation (see methods) to graph the concentration-response curves and obtain an estimate for their EC_{50} and slope (Fig. 22B).

The mean of EC_{50} values for wild-type was 15.61 nM with a 95% confidence interval from 3.65 to 27.56 nM, (n=9). The mean of EC_{50} values for $HTr1a^{RO}$ was 7.79 nM with a 95% confidence interval from 3.69 to 11.88 nM (n=7). When compared, the EC_{50} values for conductance, obtained for serotonergic cells, were not significantly different in $HTr1a^{RO}$ and wild-type mice (Fig. 23).

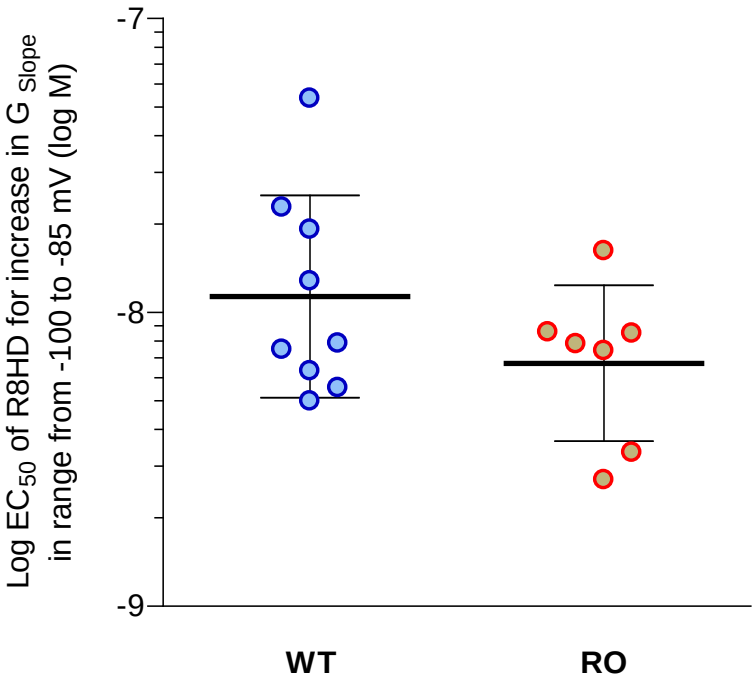

Fig. 23. Comparison between Log EC_{50} values of R-8-OH-DPAT (R8HD) for increase in 5-HT_{1A} sensitive potassium conductance in serotonergic cells of wild-type (WT) and $Htr1a^{RO}$(RO). Groups are not significantly different (P=0.407, Mann Whitney test).

We used the same experiments to evaluate the maximal 5-HT_{1A} sensitive potassium conductance activated by R-8-OH-DPAT. Total 5-HT_{1A} receptor sensitive potassium conductance was evaluated subtracting the residual conductance in the presence of barium, which in our condition blocked mostly the activated GIRK channel, from maximal conductance evoked by R-8-OH-DPAT (Fig. 24 A and B).

We found that the total 5-HT_{1A} sensitive potassium conductance in serotonergic cells was 8.654 ± 1.899 nS in wild-type (n=9), and 8.034 ± 1.350 nS in $HTr1a^{RO}$ mice (n=7), that were not significantly different (P=0.536, Mann Whitney test). The fraction of 5-HT_{1A} sensitive potassium conductance active in basal conditions in serotonergic cells of $HTr1a^{RO}$ and wild-type mice was also evaluated. 5-HT_{1A} sensitive potassium conductance active in basal conditions was defined as basal conductance minus the residual conductance in barium and its fraction is calculated on total 5-HT_{1A} sensitive potassium conductance, defined above.

A fraction of 5-HT_{1A} sensitive potassium conductance is active in basal condition both in wild-type, 4.861 ± 6.562 % of total (significantly different from residual in barium, P=0.0117, Wilcoxon test), and in $HTr1a^{RO}$ mice 21.39 ± 6.973 % of total (P=0.0156, Wilcoxon test). As shown in Fig. 24D, the fraction of 5-HT_{1A} sensitive potassium conductance active in basal conditions in $HTr1a^{RO}$ mice was significantly different from that in wild-type (P=0.0021, Mann-Whitney test).

This last finding indicates that a fraction of the total 5-HT_{1A} sensitive GIRK channels is already open in basal conditions and that this fraction is greater in $HTr1a^{RO}$ in comparison to wild-type serotonergic cells.

To confirm if a greater fraction of “GIRK like” basal conductance is present in $HTr1a^{RO}$ serotonergic cells also in conditions which *in vitro* mimic the activation of these neurons *in vivo* and/or it can affect the firing rate of $HTr1a^{RO}$ serotonergic neurons at rest, we performed additional tests in loose-seal cell-attached recording.

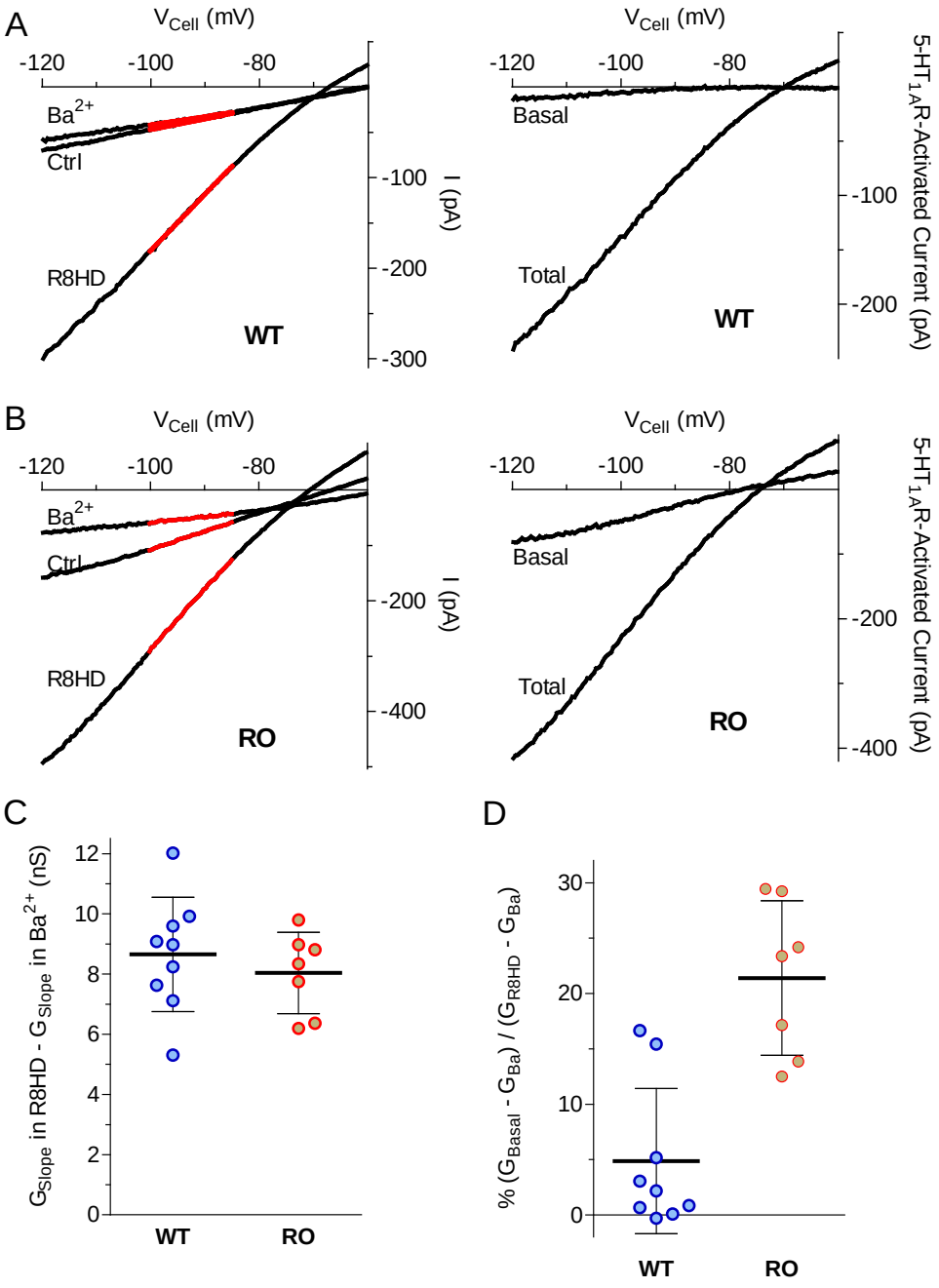

Fig. 24. Basal 5-HT$_{1A}$ sensitive potassium conductance and total evoked by R-8-OH-DPAT in serotonergic cells of wild-type and HTr1a^{RO} mice. In (A) wild-type single cell, the current in control (Ctrl), in maximal concentration of R-8-OH-DPAT (R8HD) and the residual current in barium; on each trace the linear regression interval used to obtain the respective slope conductance value is shown in red. Right, the net currents obtained from subtraction of current in barium from that in control (**basal** 5-HT$_{1A}$ sensitive) and from current in the presence of maximal concentration of R8HD (**total** 5-HT$_{1A}$ sensitive). In (B) the same for HTr1a^{RO} mice; the greater basal activated current can be noted. (C) comparation in wild-type and HTr1a^{RO} mice between total 5-HT$_{1A}$ sensitive potassium conductance (see text) and (D) the comparation of the fractional 5-HT$_{1A}$ sensitive potassium conductance active in basal condition (significantly different (P=0.0021, Mann-Whitney test,).

Effects of barium on serotonergic neuronal firing rate

We tested few cells in both mice for the presence of a "GIRK like" basal conductance in basal conditions. Barium was chosen to evaluate the level of GIRK channel activation, presumably mediated by 5-HT_{1A} receptors, because it directly and rapidly blocks the channels, its effect is easily reversible upon ion washout and low concentrations are needed to block all GIRK channels. To mimic *in vivo* condition in brain slices, phenylephrine was added to ensure the maximal activation of serotonergic cells, characteristic of waking state.

In a typical test, control baseline firing rate was monitored for 10 minutes; barium was applied for 5 minutes and then washed out for 10 minutes. The firing rate in control, in barium and washout was measured as number of action potential on 1-3 minutes at steady state. In some experiments R-8-OH-DPAT (30 nM) and/or the selective 5-HT_{1A} receptor antagonist WAY 100635 (50 nM) were applied to confirm the nature of recorded cells and the presence of 5-HT_{1A} receptor basal activation (data not shown).

Barium increased the firing rate of all cell tested (n=3) in $HTr1a^{RO}$ mice as shown for example in the Fig. 25. and in wild type animals (n=3).

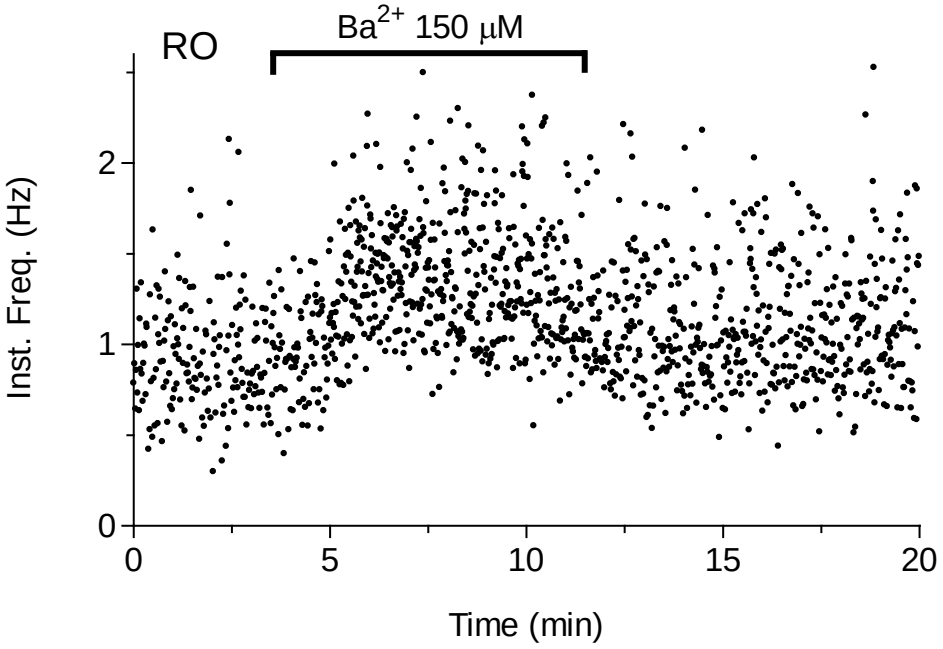

Fig. 25. Effect of barium on serotonergic firing rate in HTr1a^{RO} mice (RO). Timecourse of instantaneous frequency of a HTr1a^{RO} serotonergic cell shows the rapid and revesible increase in firing rate induced by barium (Ba^{2+}) 150 μM.

Regulation of serotonergic neuron activity in mice overexpressing 5-HT_{1A} autoreceptors ($Htr1a^{RO}$ mice).

5-HT_{1A} receptor-mediated autoinhibition of serotonergic neurone firing: response to the serotonin precursor L-tryptophan

As the physiological function of 5-HT_{1A} receptors expressed on serotonergic neurons is to mediate the autoinhibition of their firing activity, we quantified the magnitude of this process in wild-type and $HTr1a^{RO}$ mice.

In this context, it is worth mentioning that serotonin content in slices results lowered by damage caused by the slicing procedure and by the poor endogenous availability of L-tryptophan, which is the aminoacidic precursor of serotonin and that cannot be produced by animal cells. Previous studies demonstrated that supplementation of ACSF with L-tryptophan, restored the physiological content of endogenous 5-HT in slices (Mlinar et al., 2005; Liu et al., 2005). Therefore, addition of L-tryptophan to ACSF would reconduct synthesis of serotonin to normal level, would avoid further depletion of the neurotransmitter following pharmacological activation of serotonergic cells (Mlinar et al. 2005) and would re-establish a "physiological" level of serotonin in the extracellular space surrounding serotonergic cells.

Under these conditions one would also expect that the newly synthesised serotonin, released by serotonergic cells, would activate 5-HT_{1A} receptors expressed on the somatodendritic region of serotonergic neurons and thereby (auto) inhibit their firing activity.

In fact, superfusion of L-tryptophan rapidly (~10 min to reach steady-state response) reduces serotonergic neuron activity and this effect is completely reversed by the 5-HT_{1A} receptor antagonist WAY100635 (50 nM), demonstrating that the L-tryptophan-induced inhibitory response is mediated by 5-HT_{1A} receptor activation (Mlinar et al., 2005; Liu et al., 2005) see Fig. 26.

Collectively, these findings show that the autoinhibition mediated by 5-HT_{1A} receptors can be restored in slices by the perfusion of L-tryptophan.

On the other hand, assuming that the synthesis of serotonin, hence its release and extracellular level, would directly depend on the extracellular concentration of the precursor L-tryptophan, it can expected that the degree of autoinhibition produced by a given concentration of L-tryptophan in ACSF would strictly depend on the number of 5-HT_{1A} receptors expressed by serotonergic cells.

Thus, to evaluate the level of autoinhibition in serotonergic cells overexpressing 5-HT_{1A} receptors compared to that present in serotonergic neurons with normal expression of these receptors, we measured the change in firing rate of serotonergic neurons induced by different concentrations of L-tryptophan, using loose-seal cell-attached voltage-clamp recording.

To study the effect of L-tryptophan on dorsal raphe serotonergic cells isolated from network we added a “cocktail” of antagonists to ACSF, so to block fast and slow excitatory and inhibitory synaptic transmission (see methods). Phenylephrine (10 µM) was added to the bath previously to the L-tryptophan perfusion, (i.e. 5 minutes) to reproduce the maximal noradrenergic drive present during waking *in vivo*.

In a typical experiment, L-tryptophan was applied after collection of a stable neuron firing rate in phenylephrine for 10-20 minutes, to ensure a stable baseline reference for calculating the autoinhibition of neuron activity produced by L-tryptophan.

L-tryptophan was applied for 10-15 minutes for each concentration, and then washed out for 10 min. In some experiments, barium was applied to confirm that L-tryptophan-mediated response was induced by the activation of GIRK channels (Fig. 26). In all $HTr1a^{RO}$ serotonergic cells tested (n=4), L-tryptophan produced a concentration-dependent decrease of neuron firing and a complete inhibition with 30 µM, while in wild-type neurons little, if any, decrease of firing was observed in all cases (n=5). Typical examples are illustrated in Fig. 26.

The firing rate values for the construction of concentration response curve were measured at the end of perfusion-time (10-15 min) for each L-tryptophan concentration as the number of action potential on 1-3 minutes of recording. These firing rate values were normalized to control firing rate in phenylephrine. The averages of the normalized firing response elicited by different concentration of L-tryptophan for both wild-type and $HTr1a^{RO}$ serotonergic neurons were fitted by a logistic equation, to obtain estimate for the maximal effect on firing. The maximal effect for wild-type is 0.88 ± 0.05 respect to control, in other words there is only 12 % decrease in firing rate from that in phenylephrine, with the maximal concentration of L-tryptophan (30 µM). In $HTr1a^{RO}$ mice instead, the maximal effect is 0.07 ± 0.16 or a 93 % reduction in firing rate from that of control in phenyleprine. To exclude that the effect of L-tryptophan on $HTr1a^{RO}$ cells was due to lack of functionality of serotonin transporter (see in methods, $HTr1a^{RO}$ mice production and characteristics), we performed the same experiment on $HTr1a^{RO}$ mice fed with doxycycline (DOX, n=3) (Fig. 27). This drug prevented the overexpression of 5-HT_{1A} receptors and let the possible effects due to the Sert null-allele be unmasked. No differences between concentration response curve of L-tryptophan in DOX-treated $HTr1a^{RO}$ mice and wild-type were seen, confirming that the different effect of L-tryptophan in $HTr1a^{RO}$ mice is linked to the 5-HT_{1A} overexpression.

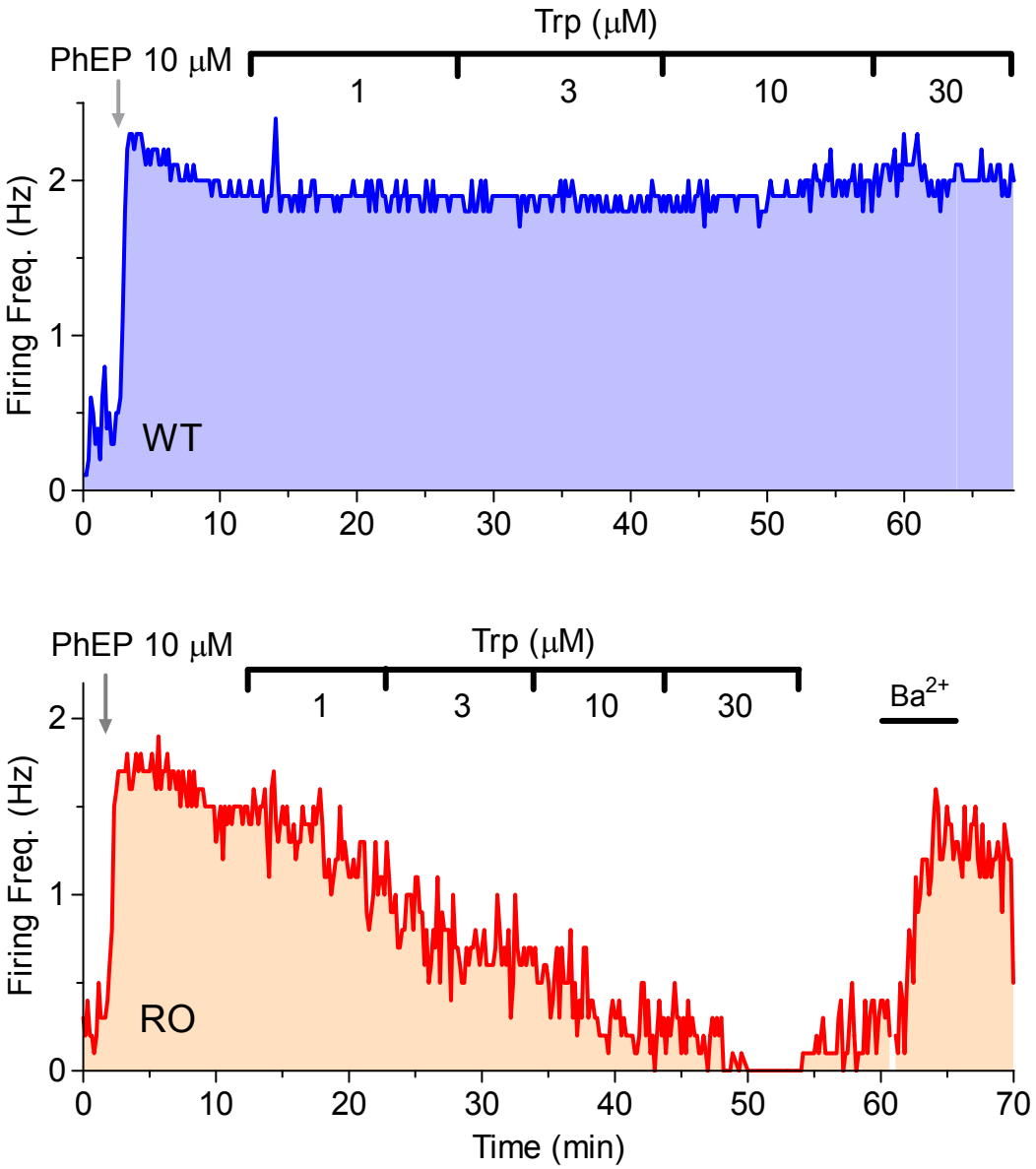

Fig. 26. Concentration-dependent (auto)inhibition of firing rate induced by serotonin precursor L-tryptophan in wild-type (WT, upper panel) and HTr1a^{RO} mice (RO, lower panel). Time-course of the effect produced by increasing concentrations of tryptophan added to ACSF to produce concentration response curves with a cumulative protocol. Arrows indicate the beginning of phenylephrine (PhEP) application (10 μM, kept constant thereafter). Note that phenylephrine-induced increase in firing rate slightly fades after an initial peak and stabilizes within 5 min from beginning of application. Baseline for calculating the effect of L-tryptophan applications is taken in the following 5-10 minutes of steady-state response.

Upper panel: typical experiment in a serotonergic neuron from a wild-type mouse showing that in normal mice L-tryptophan evokes little, if any, inhibition of firing rate. Lower panel: in a serotonergic cell of a HTr1a^{RO} mouse firing is silenced by 30 μM L-tryptophan. In this cell, the full recovery of firing rate during barium application (150 μM) confirmed that the decrease in firing induced by L-tryptophan, was mediated by GIRK channels.

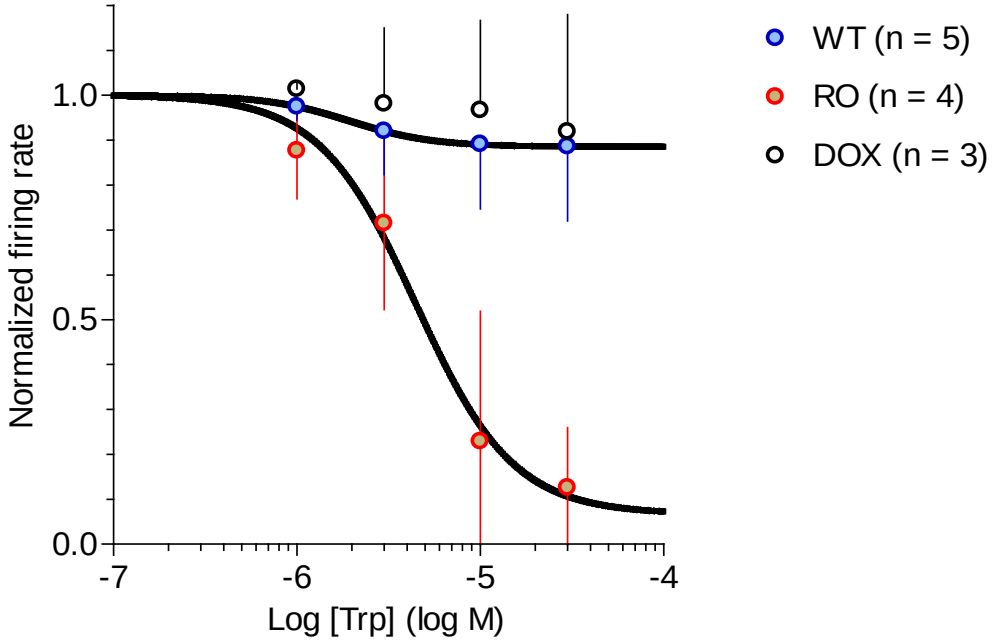

Fig. 27. Concentration-response relationship of L-tryptophan on firing rate of serotonergic neurons in wild-type (WT), HTr1a^{RO} (RO), and Doxycycline fed-HTr1a^{RO} mice (DOX). The firing rate in L-tryptophan is normalized to firing rate in phenylephrine, (see text).

CO_2/pH chemosensivity of serotonergic neurons in 5-HT_{1A} raphe overexpressing ($Htr1a^{RO}$) mice.

Responses of serotonergic neurons to changes in extracellular CO_2/H+ concentration.

Serotonergic neurons have been shown to be sensors of carbon dioxide and pH. They respond to hypercapnic acidosis with an increase in activity *in vitro* (Richerson, 1995; Wang et al., 2001), and *in vivo* (Larnicol et al., 1994; Veasey et al., 1995; Haxhiu et al., 2001; Johnson et al., 2005), and this causes an increase in 5-HT release *in vivo* (Kanamaru and Homma, 2007).

There is also evidence that medullary serotonergic neurons can act as chemoreceptors and stimulate breathing during hypercapnic ventilator response (HCVR) either by increasing the gain of respiratory neurons or by enhancing the activity of nonserotonergic chemoreceptors (Putnam et al., 2004; Li et al., 2006; Mulkey et al., 2007).

Chemosensitive serotonergic neurons in the midbrain are believed to mediate non-respiratory responses to hypercapnic acidosis that might be just as important for survival as increased ventilation, such as arousal from sleep (Berry et al., 1997), an intense feeling of anxiety (Klein et al., 1993) and changes in cerebrovascular tone (Madden et al., 1993).

A developmental defect in carbon dioxide chemoreception has been proposed to have a role in the etiology of SIDS (Richerson et al., 2004), syndrome that is known to be linked to serotonergic system deficits.

In the present work we were studying the serotonergic cell activity of a mouse that, in addition to higher level of autoinhibition, shows severe and often mortal autonomic dysregulation that resemble the events of SIDS. Therefore we asked whether 5-HT_{1A} receptor overexpression could cause a change in serotonergic

neuron homeostatic properties, like CO_2/pH sensitivity, suggesting a possible explanation for the SIDS-like autonomic deficits in these mice.

To inquire this issue, we devised to assess if HTr1a^{RO} and wild-type mice display different responses to CO_2/pH challenge.

The experiments were carried out in loose seal voltage-clamp recording to minimally perturb the spontaneous activity of the cells. Slices were initially perfused with a "cocktail" of antagonists to block synaptic neurotransmission such to isolate the single cells activity from network. Phenylephrine (10 µM) was routinarily added to ACSF to reproduce the maximal noradrenergic drive present during waking *in vivo*.

In a typical experiment 10-20 minutes of firing rate in phenylephrine were recorded before perfusion of ACSF with 9 % or 3 % CO_2 to have a stable baseline (Fig. 28). Serotonergic neurons were exposed to an increase in CO_2 to 9 % or a decrease to 3 % at constant $NaHCO_3$ concentration for 15-20 min, resulting in a change in pH from 7.4 to 7.18 or 7.63 respectively. Then, slices were left to recovery normal activity for at least 15 min with an ACSF with CO_2 5 % (normal) following each application with ACSF at different fraction of CO_2.

In some cells two successive and alternate application of ACSF with 9 % and 3% CO_2 were done to further confirm serotonergic neuron chemosensitivity (Fig. 28)

The firing rate in control, in the presence of ACSF with 9-3 % CO_2 and washout was measured as number of action potential on 3 minutes at steady state. The effect of CO_2 change was expressed as percent change of firing from baseline, taking as "baseline" firing rate the mean between firing rate in control and the relative washout for each different application.

In wild-type, serotonergic cells respond to increase of CO_2 to 9 % with an average increase in firing rate of 15.35 ± 8.37 % (n=11), significantly different from baseline

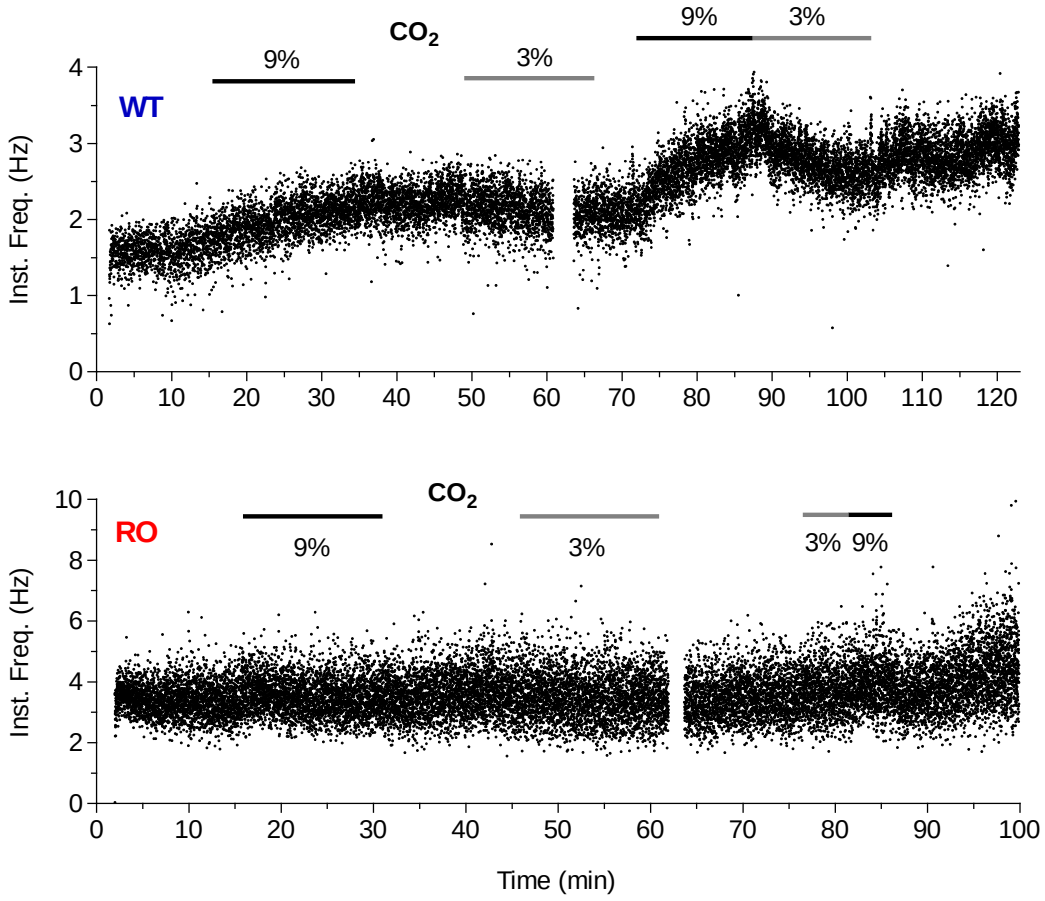

Fig. 28. Effect of change in concentration of CO_2 in bath ACSF on firing of serotonergic neurons of wild-type (WT) and HTr1a^{RO} mice (RO). The two panels show the time-course of experiments were 3-9% CO_2 were repeatedly applied. Upper panel shows the degree in changes of firing during 3 or 9% CO_2-ACSF perfusion in wild-type animals. Lower panel shows that in HTr1a^{RO} mice no response is evoked by 3 or 9% CO_2-ACSF perfusion.

firing rate (P=0.001, Wilcoxon test). Conversely, following a decrease in CO_2 to 3 % they respond with an average decrease in firing rate of 13.01 ± 8.19 % (n=10), also significantly different from baseline (P=0.002, Wilcoxon test).

In $HTr1a^{RO}$ mice, we found on average a 3.80 ± 10.61 % increase of firing following an increase of CO_2 to 9 % in the bath (n=9) and an average decrease in firing rate of 3.99 ± 13.94 % following a decrease in CO_2 to 3 % (n=5), each not different from baseline (P=0.300 and P=1.00, respectively, Wilcoxon test). Serotonergic cell responses to 9% application and 3% application were found to be significantly different, (P= 0.015 and P= 0.004 respectively, Mann-Whitney test) in $HTr1a^{RO}$ compared with wild-type mice values (Fig. 29).

Therefore we can conclude that the sensitivity to CO_2 change in extracellular space was absent in serotonergic neurons of $HTr1a^{RO}$ mice.

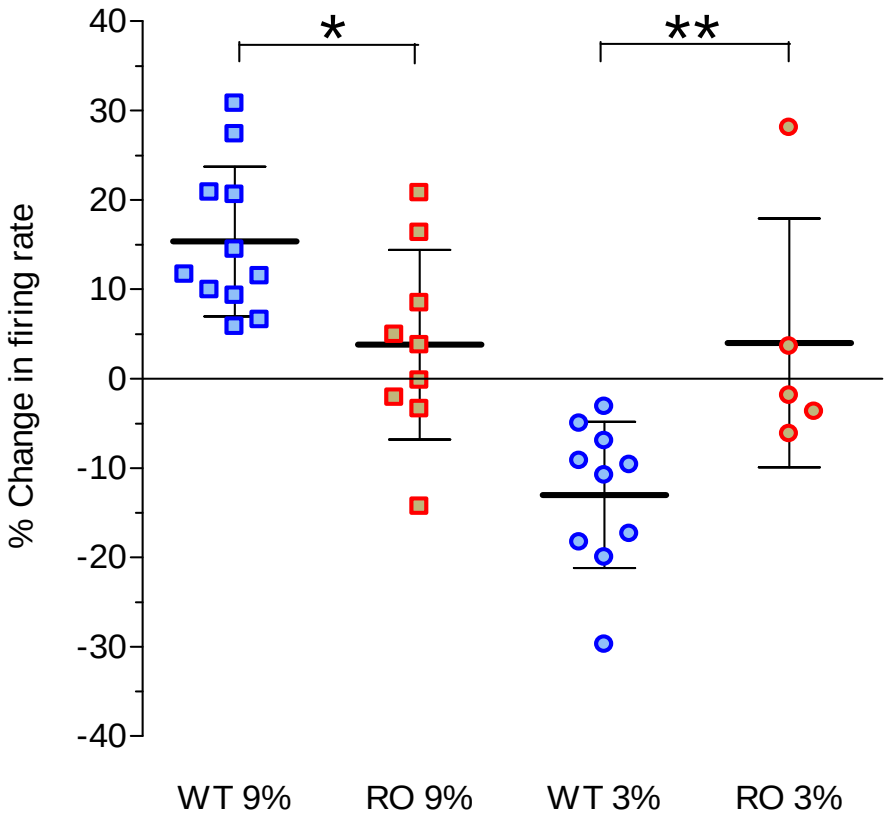

Fig. 29. Comparison between the fractional firing rate response to change in bath CO_2 concentration in wild-type (WT) and $HTr1a^{RO}$ mice (RO) serotonergic neurons. Note the lack of response in $HTr1a^{RO}$ mice. Serotonergic cell responses to 9% application and 3% application were found to be significantly different in $HTr1a^{RO}$ compared with wild-type mice values (P= 0.015 and P= 0.004 respectively, Mann-Whitney test).

DISCUSSION

The principal objective of the present work was the functional characterization of serotonergic neuron activity in the dorsal raphe of mutant mice bearing selective overexpression of 5-HT_{1A} receptors in these cells.

The interest for an in-depth knowledge of functional characteristics of serotonergic neurons in this mutant mouse resides in the fact that at early postnatal age these mice present severe autonomic disregulation that often produces life-threatening cardiovascular crises. These, eventually result in newborn mice death, which presents striking similarities with sudden infant death syndrome (Audero et al., 2008). Equally interesting is the fact that in the surviving animals is present an altered behaviour of increased aggression (Gross C., personal communications).

The research developed on three major intermingled lines, namely: i) assessment of distinctive parameters allowing the electrophysiological identification of serotonergic neurons when recording with advanced extracellular techniques (loose-seal cell-attached patch); ii) comparison of electrophysiological cell characteristics, firing pattern(s) and response to 5-HT_{1A} receptor activation of serotonergic cells, in $HTr1a^{RO}$ compared to wild-type mice; iii) study of chemosensitivity of serotonergic cells in wild-type and $HTr1a^{RO}$ mutant mice.

Our results show that autoinhibition is dramatically increased in serotonergic cells of dorsal raphe, despite the limited (two folds) increase in their 5-HT_{1A} receptor-mediated response, and that serotonergic neuron chemosensitivity, that is physiologically relevant to the respiratory alteration found in SIDS victims, is virtually absent in these mice.

Serotonergic neurons of 5-HT1A^{RO} mice

The major obstacle that hampers understanding of serotonergic cell functioning resides in the difficulties to electrophysiologically recognise serotonergic neurons which has often led to ambiguous results and contrasting interpretation.

Namely, as serotonergic cells represent only a fraction of neurones localised in raphe nuclei (Steinbusch, 1981), their identification is required for investigating their response to various stimuli during electrophysiological experiments. Currently, electrophysiological identification of serotonergic neurons is based on their "characteristic" slow and regular pattern of firing and sensitivity to 5-HT$_{1A}$ receptor agonists (Vandermaelen & Aghajanian 1983).

However, these criteria are not compelling, as demonstrated by the variety of firing patterns and electrophysiological properties revealed in studies where *post-hoc* immunohistochemical identification of the recorded cell was applied.

In addition, as immunohistochemical identification produces uncertain results (up to 40% false negative or doubtful labelling) it is not surprising that contrasting findings on electrophysiological, pharmacological and morphological characteristics of serotonergic cells have been published (Allers and Sharp, 2003; Kirby et al., 2003; Beck et al., 2004; Marinelli et al., 2004).

In the present study, we identified serotonergic neurons in wild type and HTr1a^{RO} mice combining a "visually guided selection" under IR microscopy (large neurons located in raphe midline) with the presence of an electrophysiological criterion (i.e. action potential duration and shape: HHW or action current UDI) that we developed and validated in C57bl/6 mice.

The electrophysiological criterion defined neurons as serotonergic ones when the HHW of their action potential (or the UDI of their action current in loose-seal cell-attached patch) is greater 0.9 ms.

In rare cases in which a further confirmation of neuron identity was considered useful, the addition of a pharmacological criterion (high responsiveness to 5-HT_{1A} receptor activation, i.e. neuron responds to low concentrations of R-8-OH-DPAT: 3-10 nM) allowed unambiguous identification of neurons. The combination of these criteria resulted well suited for discriminating serotonergic cells from other neurones in dorsal raphe, with a confidence greater than 99%, both in whole-cell and loose-seal cell-attached recordings. In addition, since one of the objectives of our research was to investigate the responsiveness of cells to 5-HT_{1A} receptor activation, pharmacological additional criteria were almost always present in the protocol (e.g. responsiveness to α1 adrenoreceptor and/or 5-HT_{1A} receptor agonists).

The primary conclusion of this study is that serotonergic neurons of $HTr1a^{RO}$ mice differ from those of wild-type mice for a shorter action potential/current and their smaller size as assessed by measuring cell membrane total capacitance.

Thus, the action current of $HTr1a^{RO}$ serotonergic cells resulted shorter than that of wild-type. This was apparently not due to an “acute” response to experimental conditions, as the waveform of action potential current was not modified e.g. by the effect of drugs applied. Rather, this modification is more likely due to developmental difference between the two mice, possibly linked to 5-HT_{1A} receptor overexpression (see below).

This was the only firing characteristic we found different. The intrinsic firing rate or the firing in the presence of phenylephrine were found similar as well as the response to phenylephrine activation, suggesting that apart from narrower action potential $HTr1a^{RO}$ serotonergic cells have an activity behaviour closely similar to that of wild type.

Unfortunately, in most of whole-cell patch-clamp experiments it was impossible to reliably measure cell membrane potential at rest as well as cell input resistance and/or action potential shape because of spontaneous cell firing and/or modifications of these basic cell parameters introduced by recording conditions/protocols designed for the main investigation needs.

In whole-cell patch-clamp, however, we found that cell capacitance of HTr1a^{RO} serotonergic neurons was lower than that of wild type suggesting that the cell size and/or the dendritic arborization are decreased.

We did not further investigate this issue. However, shorter action current and smaller neuron size could indicate a developmental modification in HTr1a^{RO} serotonergic cells that is consistent with the alteration in raphe serotonergic neurons found SIDS victims. In fact, Paterson and Colleagues (2006) observed an increase in the population of serotonergic cells showing immature granular aspect and a decrease in serotonergic neurons with multipolar cell body.

5-HT1$_A$ receptor expression levels and functional response to its activation

We applied loose seal cell attached recording and whole-cell patch-clamp recording techniques to provide the first direct evidence of 5HT$_{1A}$ receptor functionality in identified serotonergic cells of HTr1a^{RO} mice.

We showed that the serotonergic raphe neurons of HTr1a^{RO} mice, apart from the duration of action potential, have firing properties that are similar to those serotonergic neurons in wild type mice, and that the response to noradrenergic receptors activation, typical of waking condition, were also similar.

Thus, we considered that functional behaviour of serotonergic cells was not significantly affected by the mutation and that we could compare the response to 5-HT$_{1A}$ receptor activation on firing rate of serotonergic neurons in wild type and HTr1a^{RO} mice.

In this attempt we blocked the raphe network activity using a “cocktail” of antagonists for fast and slow synaptic transmission to study the effect of 5-HT$_{1A}$ receptor activation in isolation from the influence of principal external inputs (see in Adell et al., 2002).

We have chosen to monitor serotonergic firing rate because it is an integrative parameter, which is informative about the behavioural activity of the neuron and, using the loose-seal cell-attached voltage clamp recording technique, it can be studied on intact cells with minimal perturbations.

In HTr1a^{RO} mice, we found that the 5-HT$_{1A}$ receptor-mediated inhibition of firing rate was roughly doubled compared to wild-type mice, which was consistent with an overexpression of functionally activable 5-HT$_{1A}$ receptors. However, taking into consideration that the estimated overexpression of 5-HT$_{1A}$ receptors approached ten times the normal density of these receptors in the raphe (Audero et al., 2008), this limited increase in functional response was unexpected.

This inconsistency suggested the possibility that an adaptative mechanism prevented the firing rate to be changed over a given extent, masking the real response to 5-HT$_{1A}$ receptor activation. Therefore, we performed whole-cell experiments to compare the extent of activation of GIRK channels by 5-HT$_{1A}$ receptor stimulation in Htr1a^{RO} and wild type mice.

GIRK channels are directly activated by the βγ subunit of G-protein associated with 5-HT$_{1A}$ receptors and they are the principal mediators of serotonergic activity inhibition (Bayliss et al., 1997; Penington et al., 1993a, 1993b).

Our results show that the maximal GIRK conductance activated by R-8-OH-DPAT was similar in serotonergic cells of both mouse strains. Similar to firing rate inhibition, the concentration-response relationship of 5-HT$_{1A}$ receptor-dependent activation of GIRK current in Htr1a^{RO} mice was placed leftward to the curve in wild-type mice and EC$_{50}$ values differed by a factor of two.

These findings confirm that the functional response to 5-HT$_{1A}$ receptor activation does not linearly follow the number of receptor expressed, and suggest that the pool of 5-HT$_{1A}$ receptor-coupled G proteins and/or the availability of G protein coupled potassium channels are primary players in determining the extent of response.

5-HT1$_A$ functionality and autoinhibition in 5Htr1a^{RO} mice

From the same set of whole cell experiments we gain important information on the functionality of 5-HT$_{1A}$ receptors in these two mouse strains. We found that in basal conditions a greater GIRK-like conductance is activated in serotonergic neurons of HTr1a^{RO} mice compared to that present in wild-type serotonergic cells.

This was verified also in more physiological conditions, i.e. using loose-seal cell-attached patch recording from deeply-seated cells in the slice to avoid the excessive loss of endogenous serotonin surrounding perikarya and the regulatory mechanism associated with serotonin neurotransmission, normally present *in vivo*.

Under these conditions the application of barium, a GIRK channel blocker, increased the firing rate "at rest" more in HTr1a^{RO} mice (30 % circa) respect to wild type. In addition it was observed that the 5-HT$_{1A}$ receptor antagonist WAY 100635 was able to increase firing rate to the same extent of barium (data not shown) suggesting that the basal GIRK channel conductance is activated by a pool of 5-HT$_{1A}$ receptors stimulated "at rest". All together, these findings indicated that the basal 5HT$_{1A}$ receptor-mediated inhibition of serotonergic activity is more pronounced in HTr1a^{RO} mice respect to wild type. However, this seems in contrast with the fact that in loose seal cell attached experiments we were unable to detect a significantly lower "intrinsic" firing rate, or a lower firing rate in phenylephrine, in HTr1a^{RO} serotonergic cells compared to those present in wild type mice.

We interpreted this failure with the higher variability in the extent of autoinhibition present in the *in vitro* condition, where the content of serotonin in slices is greatly reduced compared to physiological level and, in addition, depends on the depth of serotonergic cell location within the preparation (Mlinar et al., 2005, Liu et al., 2005). Indeed, when endogenous synthesis of serotonin was restored by supplementation of L-tryptophan in ACSF, the 5-HT$_{1A}$ receptor-mediated inhibition of serotonergic firing rate was about nine times greater than

the one in wild type, thus correlating well with the difference in 5-HT_{1A} receptor density in raphe serotonergic cells of these two mice.

On the other hand, the discrepancy between the effect of synthetic 5-HT_{1A} receptor agonists (R-8-OH-DPAT, 5-CT) and endogenous serotonin was striking and difficult to explain.

The effect of L-tryptophan can be influenced by several factors regulating the synthesis and turnover of serotonin or the processes of release and uptake (Adell et al., 2002); therefore we hypothesised that the mutants could have other factors influencing the firing response to L-tryptophan/newly synthesised serotonin. For instance, HTr1a^{RO} mice are heterozygous for the gene coding for the membrane transporter (*see* Experimental procedures) and therefore the increase in response could have been caused by a partial decrease in serotonin uptake. Should re-uptake mechanisms be altered this would not affect the response to exogenous, synthetic, agonists for 5-HT_{1A} receptors, but would possibly affect the response to endogenously released serotonin. Our experiments in which we tested the sensitivity to L-tryptophan in doxycycline-treated HTr1a^{RO} mice (in which the 5-HT_{1A} receptor overexpression was blocked) demonstrate that the increase in response to L-tryptophan is not linked to a reduced expression of membrane serotonin transporter.

We are therefore confident that L-tryptophan-induced firing rate inhibition was strictly dependent on 5-HT_{1A} receptor density on serotonergic neurons. Moreover, at the end of some experiments the perfusion of barium recovered the firing rate to control values, confirming the involvement of GIRK channels in mediating L-tryptophan response. Finally, the selective 5-HT_{1A} receptor antagonist WAY100635 (Corradetti et al., 1996) was applied with the same result, as previously shown (Audero et al., 2008), confirming that inhibition of neuron firing rate was *via* activation of 5-HT_{1A} receptors during L-tryptophan superfusion.

In conclusion, although we do not have a direct explanation for the discrepancy between the effects of synthetic 5-HT_{1A} receptor agonists and endogenous

serotonin, it appears evident that in conditions approaching the physiological state *in vivo*, where the supply of L-tryptophan is not restricted and serotonin is freely accessing the overexpressed 5-HT_{1A} receptors, autoinhibition of serotonergic cells is extremely enhanced in $HTr1a^{RO}$ mice, compared to controls.

CO_2/pH chemosensivity of serotonergic neurons in $Htr1a^{RO}$ mice

Once we had defined electrophysiological parameters distinctive of serotonergic cells with an unprecedented accuracy we used this knowledge for the identification of serotonergic neurones during recording from $Htr1a^{RO}$ mice, which has been proposed as animal model of Sudden Infant Death Syndrome, SIDS (Audero et al., 2008). This allowed us to begin our study on how an altered autoinhibitory feedback could affect autonomic functions to a life-threatening level as in SIDS.

SIDS is a combination of genetic, metabolic and environmental factors that culminate in the death of an infant. In Italy, SIDS is still one major cause of infant deaths (0.5% of live births). Several studies suggested that autonomic control and arousal responsiveness are altered in these infants and implicated in SIDS causation (Moon et al., 2007). It has been proposed that the mechanisms leading to SIDS represent a failure in the neural integration of the cardiovascular and respiratory systems, with a concomitant failure to arouse from sleep (Harper et al., 2000; Horne et al., 2004). An increasing number of studies indicates that genetic, developmental, or functional alterations of the serotonergic system may contribute to SIDS (Okado et al., 2002; Sawaguchi et al., 2003; Paterson et al., 2007; Rand et al., 2007; Hodges et al., 2008; Morley et al., 2008; Thach, 2008; Duncan et al., 2010). This is not surprising given the modulatory actions of serotonin on many physiological functions (e.g. arousal, sleep, cardiorespiratory, and thermoregulatory functions) that resulted anomalous or impaired in newborns that succumbed to SIDS. Notably, serotonergic cells respond to modification in extracellular CO_2/pH with changes in firing frequency, thereby modulating

cardiorespiratory centres and participating to homeostatic reflexes (Washburn et al., 2002; Severson et al., 2003; Richerson, 2004; Dias el al., 2007; Mulkey et al., 2007; Guyenet et al., 2008; Li et al., 2008). In this context, our hypothesis is that increased autoinhibition dampens/blocks serotonin system response to chemical stimuli such as hypoxia, hypercapnia or acidosis, which occur during sleep in infants and normally elicit respiratory responses, arousal and avoidance reflexes.

We tested this hypothesis in $Htr1a^{RO}$ mice in which excessive serotonergic autoinhibition had been already associated with spontaneous bradycardic and hypothermic crises that occurred during a limited developmental period and frequently progressed to death.

In the present thesis I have shown that CO_2 chemosensitivity was absent in $HTr1a^{RO}$ mice providing a further analogy with human pathology and strengthening the notion that the autonomic disregulation present in these mice could be used for elucidating functional mechanisms of SIDS-like autonomic deficits.

We found significant differences between the response to CO2 of wild type and $HTr1a^{RO}$ mice. However, the chemosensitive response in wild-type mice was smaller than that described by other Authors both in mice and rats (Richerson 1995; Wang et al., 2002; Severson et al., 2003).

A reduced chemosensitivity is unlikely to be caused by application of CO_2 concentrations not sufficient to change pH in our conditions, as we always controlled the pH of our solutions.

Furthermore, experiments were performed in the presence of synaptic blockers to isolate the neuronal intrinsic properties, and therefore we do not expect that local feed-back regulation of serotonergic cell firing by surrounding terminals and neurons would dampen the response to CO_2. We made additional tests in the absence of phenylephrine to exclude that α_1-adrenoceptor activation could mask the changes in neuron firing rate produced by CO_2, but we did not find a greater increase in responses to CO_2 challenge in this condition. It must be noted that we recorded from a selected population of dorsal raphe serotonergic cells that may

have smaller responses to CO_2 compared to those observed in medullary raphe nuclei that are the most studied as chemoreceptors.

Although we observed a small increase of firing rate in dorsal raphe, it may still be significant for respiratory chemoreceptors, since there are many ways for a small response of a neuron to be amplified downstream and cause a large increase in breathing (Richerson et al, 2005).

Finally, we cannot discard the possibility that the wild-type animals, we used for their genetic background common with that of $HTr1a^{RO}$ mutants, have smaller CO_2 sensitivity than non transgenic mice like C57bl/6. Although we did not directly test this possibility, yet, we think that great differences based on genetic background are unlikely to be present in wild-type mice, since they retain a response to CO_2 and grow perfectly healthy.

In conclusion, the suppression of this homeostatic property seems linked to increased serotonergic autoinhibition in $HTr1a^{RO}$ mice and the importance of this regulatory mechanism may be relevant to the SIDS phenotype of these animals.

CONCLUSIONS AND PERSPECTIVES

The present study shows that 5-HT_{1A} receptor overexpression in serotonergic cells of dorsal raphe nuclei produces limited changes in the functional response to direct 5-HT_{1A} receptor activation, but greatly enhances the response to activation of the same receptors mediated by endogenous serotonin. Thus, autoinhibition is enormously increased in these cells. Further studies are needed to fully elucidate the mechanisms by which overexpression of 5-HT_{1A} receptors leads to excessive autoinhibition in $HTr1a^{RO}$ mice.

The second important finding of the present work is that serotonergic cells with 5-HT_{1A} receptors overexpression lost their sensitivity to changes in extracellular CO_2 concentration. This provides a possible explanation for the SIDS-like events in these mice.

It would be of interest for future studies to investigate if this deficit extends to other raphe nuclei and if it is important *in vivo* in experimental conditions of hypercapnia.

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