GABA signaling in the thalamus

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Table of contents

Summary

Inhibition of neuronal activity in networks of the mammalian central nervous system is essential for all fundamental brain functions, ranging from perception, to consciousness, to action. Both exacerbation and diminution of inhibition dramatically affect our behavioral capacities, indicating that, in the healthy brain, strength and dynamics of inhibition must be precisely balanced.

Inhibitory functions are primarily accomplished by neurons releasing the neurotransmitter GABA. According to their wide variety of functions, GABAergic neurons show a tremendous diversity in morphological, biochemical and functional characteristics. The combination of these diverse properties allows the brain to generate interneurons acting as, for examples, filters, co-incidence detectors or contrast enhancers. GABAergic signaling in thalamus plays an essential role in controlling sensory information flow from the periphery to the cortical processing centers, and in generating sleep-related neuronal rhythms. Surprisingly, however, the diversity of GABAergic neurons is remarkably limited in thalamic networks. Both functions mentioned have been tightly associated with two homogeneous groups of GABAergic neurons arising within thalamic nuclei or within the nucleus reticularis, a shell of inhibitory nuclei surrounding the dorsal thalamus.

The results arising from the present thesis challenge the view that the diversity of GABAergic signaling in thalamus is comparatively limited and proposes that, to fully understand GABAergic signaling in thalamus, at least two additional aspects have to be considered. First, it shows that GABAergic signaling arising from the nucleus reticularis can have a profound effect on the synthesis of second messenger compounds that are important in the control of neuronal rhythmicities and in the state-dependent control of gene expression. Second, it demonstrates the functional relevance of a previously undescribed extrathalamic and extrareticular inhibitory pathway that arises within the anterior pretectal nuclei, indicating that the architecture of GABAergic signaling in thalamus has to be complemented by a conceptually novel, powerful afferent pathway.

The first part investigates the modulation of cAMP synthesis by GABA in thalamocortical neurons through the activation of the G_i-coupled $GABA_B$ receptors. $GABA_B$ receptors can provide two different cAMP signals in the neurons. First, $GABA_B$ receptor activation depresses the level of cAMP inside thalamocortical

neurons. However, a large and long cAMP signal is observed when $GABA_B$ receptors are activated concomitantly with β-adrenergic receptors, which are G_s-coupled receptors. In the presence of $GABA_B$ receptor agonists, the moderate cAMP increase produced by β-adrenergic receptor activation is transformed into a large synthesis of cAMP. Remarkably, the activation of the $GABA_B$ receptors at the synapses between reticular neurons and thalamocortical neurons also potentiates the effects of β-adrenergic receptors. Thus, $GABA_B$ receptors modulate cAMP signals at synapses that are important for the regulation of the state of arousal.

The second part provides the first electrophysiological description of synaptic connections between the anterior pretectum group and the thalamic higher-order nuclei. Electric stimulation in the anterior pretectum group evoked inhibitory postsynaptic responses (IPS) in the thalamocortical neurons of the higher-order nuclei. We showed that the IPS responses were mediated via the $GABA_A$ receptors activated through monosynaptic connections between the APT and the higher-order nuclei. Functionally, the anterior pretectum modulated the discharge properties of the thalamocortical neurons, suggesting an important role of this nucleus in the dialogue between the thalamus and the cortex.

General introduction

The amino acid GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the central nervous system of mammals (CNS). Its primary inhibitory function is to control, dampen and coordinate the excitability of the principal excitatory neurons, which provide the main pathways of neuronal communication within and between neuronal networks of the brain (Freund and Buzsáki, 1996; McBain and Fisahn, 2001; Freund, 2003; Lawrence and McBain, 2003; Maccaferri and Lacaille, 2003; Whittington and Traub, 2003; Jonas et al., 2004). Although excitatory neurons often outnumber GABAergic neurons by up to 4-10-fold (Houser et al., 1983; Hendry et al., 1987), inhibitory neurons are highly divergent, strategically positioned and physiologically tuned to exert a functional control over excitatory communication (Miles et al., 1996; McBain and Fisahn, 2001; Freund, 2003) Accordingly, disturbances of GABAergic inhibition has been associated with fundamental behavioral disorders such as epilepsy, anxiety, sleep disturbances and schizophrenia (Pace-Schott and Hobson, 2002; Freund, 2003; Wong et al., 2003; Rogawski and Löscher, 2004). Moreover, important classes of medicines used since the beginning of the 20th century, such as barbiturates and benzodiazepines (BZs) (Möhler et al., 2002), were later recognized to potentiate GABAergic synaptic transmission. The action of these drugs ranges from sedative and anxiolytic to anti-convulsant, indicating that the potentiation of GABAergic transmission controls arousal, emotional states and prevents us from loosing consciousness. Conversely, drugs used in the generation of experimental epilepsy, such as penicillin and bicuculline, are now known to interfere with GABAergic neuronal communication, demonstrating that a reduction in inhibitory tone is one principal actor for human epilepsy (Kostopoulos, 2000; Wong et al., 2003; Rogawski and Löscher, 2004).

Besides controlling excitatory communication, neuronal networks containing exclusively GABAergic neurons have been recognized to act as generators and pacemakers for rhythmically patterned electrical activity that has been implicated in perception, associative learning, control of arousal (McCormick and Bal, 1997; Paulsen and Moser, 1998; Whittington and Traub, 2003). In many cases, this unique capability of such networks arises from a coupling of inhibitory cells via electrical synapses formed by gap junctions (Galarreta and Hestrin, 2001).

The multiple and fundamental roles of GABAergic transmission are paralleled by a bewildering diversity of GABAergic neuron types that is evident at the molecular, morphological, biochemical, synaptic and network level (Freund and Buzsáki, 1996; Gupta et al., 2000; Maccaferri and Lacaille, 2003).

This introduction gives a brief overview over the current state of knowledge on interneuronal diversity. It will then present an introduction into the current knowledge of the cellular basis of GABAergic signaling in thalamic networks, before highlighting the author's contributions that reveal two novel roles of GABAergic signaling in thalamic nuclei.

I Diversity of GABAergic neurons in the cental nervous system

The diversity of the interneurons manifests at multiple functional levels and is to date best understood in cortical networks. Therefore, the majority of the examples referred in the introduction arises from literature on neocortical and hippocampal circuits.

Principal or pyramidal cells (PCs) receive inhibitory inputs from different interneurons that have specific electric and architectural properties. The interneurons target precise somatodendritic locations of the PCs and of other interneurons. The synapses established by interneurons display particular pre- and postsynaptic properties. The response is then shaped by the different passive and active properties of the somatic and dendritic membranes. Finally, the interneurons are recruited in various conditions since they are innervated by different sources of excitatory and inhibitory inputs. All together, for distinct network functions, distinct interneuronal subpopulations are engaged to control the integrative properties of the neuronal circuitries.

1. Diversity of the morphological and biochemical properties of interneurons

Morphological studies, based on Golgi impregnations, first provided evidence for a diversity of non-pyramidal neurons, and these, therefore, originally provided names for morphologically different cell types (Cajal, 1911). Nowadays, the

localization of the soma and the distribution of the dendritic and axonal trees are still routinely used parameters to classify the interneurons in hippocampus and neocortex and are now known to have important functional correlates (Freund and Buzsáki, 1996; McBain and Fisahn, 2001; Thomson and Bannister, 2003). Thus, the localization of the dendritic tree defines the inputs that command the activity of the interneurons, whereas the axonal arborization establishes the target domains.

The interneurons were later subdivided based on the expression of different markers such as neuropeptides and calcium-binding proteins. The neuropeptides useful to class interneurons are the somatostatin (SST), the cholecystokinin (CCK), the Vasoactive Intestinal Polypeptide (VIP), the neuropeptide Y (NPY), the enkephalins and the substance P. The interneuronal neuropeptides are co-released with GABA and activate G-protein-coupled receptors (GPCRs) to modulate neuronal activity (Baraban and Tallent, 2004). Calcium-binding proteins that are commonly used to differentiate interneuron classes are parvalbumin (PV), calbindin (CB) and calretinin (CR) (DeFelipe, 1997; Kawaguchi and Kondo, 2002). The Ca^{2+}-binding proteins are Ca^{2+} buffers that trap free Ca^{2+} with variable kinetics (Clapham, 1995). Their role in interneuronal function is becoming increasingly clear and ranges from an involvement in short-term plasticity (Blatow et al., 2003a) to a determination of neuronal vulnerability during ischemia (Nitsch et al., 1989; Mattson et al., 1991; Sloviter et al., 1991; Freund and Magloczky, 1993; Dinocourt et al., 2003). Other biochemical differences, such as the expression of nitric oxide synthase and K^+/Cl^- transporter KCC2 are described. The nitric oxide synthase is generally co-localized in cortical neurons expressing the SST and NPY neuropeptides and CB (Smiley et al., 2000). The KCC2 is more abundant in all PV-positive interneurons of the CA3 and CA1 subfields of the hippocampus (Gulyás et al., 2001). The alliance of morphologic analysis with antibody staining for these markers is an appropriate way to classify interneurons.

Chandelier cells (or axo-axonic cells) and basket cells (BCs) are two types of interneurons that are distinguished based on morphological differences, but both innervate characteristically the perisomatic region of the PCs. Axons of the chandelier cells terminate exclusively on the initial segment of the axon. Two types of axo-axonic interneurons are described, one with a radial dendritic tree, the other with a horizontal dendritic tree (Freund and Buzsáki, 1996; Ganter et al., 2004). They contain the PV but no other markers (neuropeptides or Ca^{2+}-binding binding proteins)

known so far (Freund, 2003). The interneurons termed BCs display a vast range of subtypes. The most widely known BC type contains PV, whereas the other contains VIP and/or CCK (Freund, 2003). The BCs positive for PV or positive for CCK have in common that the axon terminates on the perisomatic region of the PCs, principally on proximal dendrites but also on the soma in the hippocampus and the neocortex (Freund, 2003). So two interneuronal types that target the perisomatic region of the PCs express PV, which is in agreement with the fact that no PV-positive cells are found to target the distal dendrites of the PCs in the hippocampus. Indeed, PV-positive neurons are primarily found in *stratum pyramidale* of the CA1 and CA3 and *stratum granulosum* of the dentate gyrus (Freund and Buzsáki, 1996; Nomura et al., 1997). However, in cortical layer II/III, the multipolar bursting neurons, a new type of PV-positive interneurons, have the majority of axonal branches terminating on dendrites and rarely in perisomatic regions (Blatow et al., 2003b).

In the hippocampus, the interneurons that innervate only the dendrites of the PCs express individually or in combination SST, NPY, enkephalin and/or CB. Different types are described and grouped in 8 classes, again based on morphological criteria (Cope et al., 2002). For example, in the hippocampal CA1 subfield, the SST-positive *oriens-lacunosum-moleculare* neurons (O-LM) have a horizontal dendritic tree that receives inputs from local PCs in the *stratum oriens* (Blasco-Ibanez and Freund, 1995). They project a dense axonal arbour into the *stratum lacunosum-moleculare* where they terminate on the distal dendritic shafts and spines of CA1 PCs (Freund and Buzsáki, 1996; Maccaferri et al., 2000). The CB-positive bistratified interneuron axons expand in the two layers where are located the apical and basal dendrites of the PCs, but they do not make terminals into the pyramidal cell layer. They make synapses on proximal and distal dendrites of PCs (Freund and Buzsáki, 1996).

In the neocortex, morphological criteria are additionally used to distinguish the bitufted or double bouquet cells, the Martinotti cells and the neurogliaform cells. The bitufted cells were described already by Cajal, have a bipolar dendritic tree and a preferentially descending axonal arborisation that innervates the dendrites of the PCs in a cortical column. They are CB- or CR-positive (del Rio and DeFelipe, 1997) or VIP-positive (Kawaguchi and Kubota, 1996). The Martinotti cells are SST- and CB-positive and have an ascending axonal arbor that reaches the cortical layer I (Kawaguchi and Shindou, 1998; Gupta et al., 2000). The neurogliaform interneurons

are multipolar cells with radial dendritic and axonal trees, the second is twice as wide as the first (Kawaguchi and Kubota, 1997; Tamás et al., 2003).

The interneurons described so far preferentially innervate PCs, but, with the exception of the chandelier cells, they also make synapses on other interneurons. A third type of interneurons specifically and uniquely targets other interneurons. In the hippocampus, they are named interneuron-specialized neurons (Freund and Buzsáki, 1996). The CR-positive interneurons are present in all layers of the hippocampus and they target other CR-positive and CR-negative interneurons such as CB- or VIP-positive interneurons but not the PV-positive BCs or chandelier cells (Gulyás et al., 1996).

2. Diversity in electrophysiological properties.

The diversity of the architecture and the expression of neurochemical markers described above is further combined with the expression of distinct ionic channel and neurotransmitter receptor subunits. The resulting variety of physiological properties, such as distinct spike-firing patterns or distinct postsynaptic responses, contributes essentially to the efficiency and the diversity of interneuronal functions.

a. Action potential discharge modes

Cortical interneurons were first recognized to be able to discharge at high-frequency without adaptation (200-600 Hz for hundreds of milliseconds), in contrast to PCs which discharge maximally at ~300 Hz with the frequency of the APs that declines within 50 ms to less than 100 Hz (Connors and Gutnick, 1990). In view of this property, these interneurons were termed fast-spiking neurons (FS). The action potential (AP) of the FS neurons has a short width (around 0.5 ms) and is cut short by a strong and fast afterhyperpolarization (McCormick et al., 1985). This discharge mode suggests that these cells were adapted particularly well for the maintenance of inhibitory drive in a wide range of frequencies (Connors and Gutnick, 1990). The fast-spiking neurons are usually the PV-positive cells (Cauli et al., 1997; Martina et al., 1998), such as the chandelier cells or the BCs (Kawaguchi, 1995). Interestingly, BCs in cortex, CA1 hippocampus as well as dentate gyrus, all share these properties.

In contrast, CCK-positive BCs and the neurons positive for other markers like SST, CR, CB and VIP discharge in a regular spiking mode, regular burst pattern or in

a delayed spiking pattern (Cauli et al., 1997; Kawaguchi and Kondo, 2002) indicating a large diversity of AP discharge modes.

In an effort to fully characterize cortical interneurons, Gupta et al. (2000) correlated morphological and biochemical characteristics with AP discharge patterns in a large sample of interneurons from cortical layers II/III (Gupta et al., 2000). They distinguished three main principal patterns of response to depolarizing current: discharges with or without accommodation, and a stuttering response characterized by irregular burst- and tonic firing. This study is the first to demonstrate that the combination of electrophysiological, morphological and synaptic properties (see below, chapter 3a) allows to fully subdivide cortical interneurons into 14 different subtypes.

b. Potassium channels

The characteristic AP pattern of interneurons is largely shaped by the expression of distinct ionic channels. The regular and fast discharge properties may be based on different properties of K^+ currents expressed at the membranes of the regular-spiking and FS neurons (Massengill et al., 1997). In FS cells and PCs, three different components of the K^+ current are distinguished depending on their sensitivity to 4-aminopyridine (4-AP) and tetraethylammonium (TEA), two well-known blockers of K^+ currents. These are a fast delayed-rectifier, a slow-delayed rectifier and an inactivating K^+ current. However, the proportion of each differs between PCs and FS neurons. The contribution of the fast delayed-rectifier is five-fold higher in FS than in PCs. In FS neurons, the afterhyperpolarization activated after each spike is mainly attributed to the fast delayed-rectifier current (Martina et al., 1998; Lien et al., 2002).

The highest proportion of fast delayed-rectifier K^+ current is associated with the strongest expression of Kv3.1 and Kv3.2 subunits in FS neurons. In contrast, PCs present a lower expression of Kv3 and instead a higher expression of Kv4 and Kv2 subunits (Martina et al., 1998; Lien et al., 2002). Kv3.1 immunostaining shows exclusive co-localization of the subunit with PV-positive neocortical interneurons and is not observed in PV-negative neurons. Kv3.2 subunit is preferentially observed in PV-positive neurons (Chow et al., 1999). Moreover, in heterologous systems, the expression of Kv3.1 or Kv3.2 subunits produces currents that have most of the biophysical and pharmacological properties similar to the fast delayed-rectifier current and that is not the case of the Kv2- and Kv4-mediated currents (Coetzee et al.,

1999). In mice lacking the Kv3.2 gene, PV-positive neurons, for which the Kv3.2 subunits are prominently expressed, show wider APs and do not exhibit FS discharges (Lau et al., 2000). Thus, the discharge property of the FS interneurons may be based primarily on the subunits Kv3.1 and Kv3.2, which form the fast delayed-rectifier channels.

The fast delayed-rectifier K^+ current is necessary for the FS property because the block of the channels composed of the Kv3.1 and Kv3.2 subunits, with low concentration of 4-AP, TEA leads to a broadening of a single AP and a reduced frequency of the train of spikes induced by injection of depolarizing current (Du et al., 1996; Martina et al., 1998). The specific properties of the currents mediated by the Kv3.1 and Kv3.2 subunits explain why the channels allow FS properties. In the following experiments, the fast delayed-rectifier currents are blocked pharmacologically and Kv3-mediated currents are added artificially with the fast dynamic-clamp technique. The dynamic-clamp technique permits to control the properties of the conductance applied in the neurons and thus, to investigate the impact of the alteration of one Kv3 current property on the discharge pattern of the neurons. The role of the activation threshold, the kinetic of deactivation and the inactivation of the current was investigated through this way. First, the currents mediated by the Kv3 subunits have to activate at high potentials (threshold potential is -20, -10 mV) to observe FS discharge. Thus, an artificially induced shift of the activation curve to more hyperpolarized potentials (~-40 mV, comparable to the Kv2- and Kv4-mediated currents) converts FS discharges to discharges with adaptation. Fast deactivation permits not to delay the generation of a new AP. An artificially deceleration of the kinetics of deactivation converts FS discharges to regular spiking discharges (Lien and Jonas, 2003). Moreover, the lack of inactivation of the fast delayed-rectifier current is primordial to maintain high-frequency discharges and a constant AP duration. Introduction of an artificial inactivation of the current led to a progressive broadening of the APs (Lien and Jonas, 2003). Conversely, the addition of artificial Kv3 conductance by the dynamic-clamp technique induces regularly-spiking PCs to produce FS discharges (Lien and Jonas, 2003). Thus, the time- and voltage-dependent properties of the fast delayed-rectifier current (likely composed of Kv3.1 and Kv3.2) are optimized for the FS property of hippocampal interneurons and thus interneurons devoid of this current display regular spiking.

c. Sodium channels

In addition to distinct K^+ currents, the properties of Na^+ channels further strengthen the propensity of interneurons to discharge at high frequencies. Thus, hippocampal BCs of the dentate gyrus display a Na^+ current with faster deactivation compared to PCs (the time constants are 0.13 and 0.20 ms, respectively, at -40 mV). Moreover, the voltage-dependence of the inactivation is shifted to more depolarized potentials (half potentials of inactivation is -58.3 and -62.9 mV, respectively), the kinetics of inactivation are slower (the inactivation time constants were 18.6 and 9.3 ms, at -55 mV) and the kinetics of de-inactivation are faster, thus promoting the rapid recruitment of Na^+ channels during ongoing AP discharges (Martina and Jonas, 1997).

d. Calcium channels

In cortical interneurons, the burst is composed of a calcium low-threshold spike, on which APs are superposed. The calcium low-threshold spike is based on the expression of a transient calcium current (the T-current, see chapter II, 2b), that is present in bursting neurons but not in regular spiking (Chen et al., 1996).

3. Diversity of synaptic connectivities

In addition to the diversity of intrinsic electrophysiological, morphological and neurochemical markers, interneurons display a marked, yet highly structured diversity in their synaptic connectivities. They are positioned in a particular network, receiving specific excitatory or inhibitory inputs and projecting to one or several specific targets.

a. Output diversity

Interneuronal - interneuronal connectivity

In the hippocampus, indications for synaptic connections between interneurons were provided by stimulation of afferent fibers, such as the Schaffer collateral pathway, that elicited di-synaptic inhibitory postsynaptic potentials (IPSPs) in several classes of interneurons (Buhl et al., 1996). Inhibitory connections between GABAergic neurons were demonstrated directly using recordings of synaptically connected BCs pairs in the CA1 area and dentate gyrus (Cobb et al., 1997; Bartos et

al., 2001) or corroborated by anatomical studies, showing, for example, the interconnectivity of hippocampal CCK- and PV-containing BCs (Nunzi et al., 1985; Katsumaru et al., 1988; Sík et al., 1995; Buhl et al., 1996). Interestingly, the degree of interneuronal connectivity is strongly dependent on their anatomical or neurochemical phenotype. While PV-positive chandelier cells exclusively target principal neurons (Martinez et al., 1996), several classes of interneurons are specialised to innervate other hippocampal interneurons such as a subset of VIP-positive neurons or CR-positive neurons (Acsády et al., 1996; Gulyás et al., 1996).

In the sensory neocortex, different types of GABAergic interneurons show a high degree of mutual interconnectivity. The BCs are connected with other BCs but also with dendritic-targetting interneurons or double bouquet interneurons (Tamás et al., 1998). Therefore, it is apparent that interneurons not only differ in their postsynaptic target preference, with respect to both compartment and cell type-specific innervation, but also with respect to the differential degree of their efferent connectivity.

Finally, certain interneurons may also show a substantial degree of autaptic self-innervation (Cobb et al., 1997; Tamás et al., 1998), thus forming a monosynaptic inhibitory feedback loop.

Interneuronal – pyramidal cell connectivity

Interneurons control the dendritic, somatic and axonic compartments of PCs. The activation of perisomatic inhibiting neurons triggers a fast and large IPSP in the PCs that impairs or delays spike generation (Miles et al., 1996). Conversely, the activation of dendritically inhibiting neurons triggers a smaller and slower IPSP that reduces Ca^{2+} dendritic spikes (Miles et al., 1996). Somatically located synaptic terminals are larger and show broader active zones and more mitochondria suggesting a higher reliability of release at somatic synapses than at dendritic synapses (Miles et al., 1996).

In addition to compartmentalization, an important aspect of inhibitory inputs to PCs is the diversity of short-term plasticity, which includes short-term depression and short-term facilitation. The O-LM interneurons of the hippocampus target the distal dendrites and the small and slow inhibitory postsynaptic currents (IPSCs) elicited do not change for paired stimuli at short intervals. In contrast, the larger and faster IPSCs induced by chandelier cells display paired-pulse depression (Maccaferri et al., 2000).

The majority of the GABAergic synapses formed on neocortical PCs depresses during a train of discharges in interneurons but some classes of interneurons show facilitation or both facilitation and depression. A remarkable aspect of these diverse patterns of short-term plasticity is that they are associated with a tight target specificity (Gupta et al., 2000). Synaptic depression is also found during multiple stimuli (more than 500 stimulations) but is generally weaker than multiple stimuli depression observed in PCs (Galarreta and Hestrin, 1998), perhaps due to a more efficient recycling of the synaptic vesicles (Lüthi et al., 2001). Altogether, this suggests that strong activation of cortical networks eventually leads to a gradual dominance of inhibition.

The interneurons regulate PC activity via feedback or feedforward inhibition. If interneurons are innervated by collaterals of the afferent axons that target the PCs, the interneurons provide feed-forward (feedF) inhibition (Freund and Buzsáki, 1996), which manifests as a biphasic excitatory postsynaptic potential (EPSP) - IPSP sequence in PCs (Freund and Buzsáki, 1996). In opposite, if the source of excitation arises from local collaterals of PCs, the interneurons provide feedback (feedB) inhibition (Freund and Buzsáki, 1996). Some interneurons control the PCs by feedB or feedF inhibition only, some interneurons are innervated by both afferents and PCs, therefore they supply both feedB and feedF inhibition (Freund and Buzsáki, 1996).

The feedF inhibition controls the integrative properties of PCs by limiting temporal summation at the soma to a very narrow time window. This is most evident when applying two subthreshold inputs to Schaffer collaterals (Pouille and Scanziani, 2001). These two inputs induce an AP only when the two stimuli occurred almost concurrently (within a 1-5 ms time window, average 1.6 ms). In the absence of feedF inhibition, the production of an AP in the CA1 cell by the summation of the two EPSPs occurred for longer interstimulus intervals (~1-40 ms, average 14.8 ms). Therefore, feedF inhibition makes PCs behave as coincident detectors with a time window of 2 ms (Pouille and Scanziani, 2001). The FeedF inhibition reduces also AP backpropagation when it concerns distal dendrites (Tsubokawa and Ross, 1996).

In the feedB inhibition network, the interneurons are activated by the PCs they target providing a disynaptic loop. In cortical layer II/III, the PCs receive feedB inhibition on their proximal dendrites from VIP-positive bipolar neurons (Rozov et al., 2001). The EPSPs generated at the synapses between PCs and bipolar neurons are depressing due to the expression of S-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid (AMPA) receptors with a long-lasting desensitization. In

contrast, the IPSPs generated at the synapses between bipolar neurons and PCs are not altered during a train of pulses (Rozov et al., 2001). Consequently, the feedB inhibition will be operative only when PCs discharge low-frequency train of APs. For sustained PC discharges, inhibitory drive is reduced by the long desensitization of the AMPA receptors of the bipolar neurons (Rozov et al., 2001).

The bitufted neurons also provide feedB inhibition on the cortical PCs of layer II/III but the synapses are located at the distal dendrites. The EPSPs induced in bitufted neurons by a train of three discharges in PCs show multiple pulse facilitation and IPSPs induced by bitufted neurons in PCs show multiple pulse depression. Therefore, the inhibition of bitufted neurons will be more efficient for high-frequency discharges in PCs (Reyes et al., 1998). Thus, the subcellular target of feedB inhibition depend on the discharge frequency of PCs: high-frequency discharges lead to distal feedB inhibition via the bitufted neurons whereas low-frequency discharges lead to proximal feedB inhibition via the bipolar neurons.

Similarly, two types of feedback inhibition are also described in hippocampus. Low-frequency stimulations of PCs induced essentially perisomatic feedB inhibitions. Higher frequency stimulations results in perisomatic inhibition for the first stimulations followed by a dendritic feedB inhibition. The two types of inhibition are based on the specific conditions to recruit the perisoma-targetting and dendrite-targetting neurons (Pouille and Scanziani, 2004).

b. Input diversity

In addition to the diversity of the synapses they form on their target neurons, the interneurons also show differences in the distribution of the excitatory and inhibitory inputs they receive (Gulyás et al., 1999). Indeed, ~5% of the inputs on hippocampal PV-positive neurons are inhibitory whereas for the CB- and CR-positive cells, GABAergic inputs represent ~20-30% of all the synapses. Moreover, GABAergic synapses formed on PV- or CR-positive interneurons are in majority PV- or CR-immunoreactive, respectively and they both preferentially target the soma (Gulyás et al., 1999).

The properties of the synapses formed by PCs on interneurons are type specific. The types of synapses are distinct morphologically and molecularly, and therefore electrophysiologically but also with respect to synaptic plasticity. The case of the

synapses formed by hippocampal mossy fibers on three different targets will illustrate these points.

The mossy fibers are formed by the axons of the granular cells of the dentate gyrus. In dentate gyrus, recurrent collaterals of the mossy fibers (MF) target the proximal dendrites of BCs, which project back onto the granular cells for feedB inhibition (Freund and Buzsáki, 1996). The feedF target of the MF are the dendrites of the PCs and the local interneurons of the CA3 area (Acsády et al., 1998). The local CA3 interneurons provide feedF inhibition on CA3 PCs (Freund and Buzsáki, 1996). In the CA3 subfield, MF terminals have the structure of large boutons that form multiple release site synapses on PCs. Furthermore, from the large boutons extend small filopodia or small en passant terminals, which form single release site synapses preferentially on GABAergic neurons. Moreover, the convergence from one granular cell is higher to the CA3 interneurons compared to the CA3 PCs (Acsády et al., 1998).

At MF - CA3 interneuron synapses, the release probability and the quantal amplitude of the EPSPs are higher than at MF - CA3 PC synapses due to the expression in CA3 interneurons of AMPA receptors with higher single-channel conductance (Lawrence and McBain, 2003) and long postsynaptic densities (Acsády et al., 1998). Kinetic differences are also observed, as it was shown in other models, since the EPSPs generated in interneurons are shorter in duration than in the PCs (Gulyás et al., 1993; Thomson et al., 1993; Debanne et al., 1995; McBain and Fisahn, 2001). In the MF- dentate BC synapses, the EPSPs generated are very short. Several parameters explain these fast kinetics. The fast membrane time constants of the dentate gyrus BCs are partially responsible for the fast kinetics of the unitary EPSPs generated by an AP in granular cells (Geiger et al., 1997). Nevertheless, the principal parameter, which influences EPSP kinetics, is the properties of glutamate receptor as shown in BCs of the dentate gyrus. In these cells, the decay time constant of a quantal EPSP is similar to the time constant of the AMPA receptor deactivation. This is important for the temporal summation of the EPSPs in interneurons. Indeed, fast deactivating AMPA receptors are associated presynaptically to fast release of glutamate, therefore the window for temporal summation is very narrow (few milliseconds) and may allow the dentate gyrus BCs to operate as coincident detectors, meaning that suprathreshold depolarisation are reached only when several EPSPs arrive in this short time window (Geiger et al., 1997).

A low proportion of the slowly gating GluR2 (flip) and a high proportion of the rapidly gating GluR4 in the composition of the BC AMPA receptors explain the fast deactivation of the glutamatergic response in interneurons (Geiger et al., 1995) and the high single-channel conductance (Lawrence and McBain, 2003). The subunit compositions also dictate the permeability to Ca^{2+}. The GluR2 subunit expression limits the AMPA receptor conductivity to Ca^{2+}. Because of less GluR2 subunit expression, the interneurons have AMPA receptors with a higher permeability to Ca^{2+}. In contrast, the PCs express high levels of GluR2 subunits indicative of AMPA receptors with a low Ca^{2+} permeability (McBain and Fisahn, 2001).

The excitatory synapses between the granular cells and the CA3 interneurons are heterogeneous with respect to Ca^{2+}-permeability of the AMPA receptor (depending on the expression of GluR2 subunit) (Tóth et al., 2000). In the synapses with AMPA receptors having a low Ca^{2+}-permeability (CI synapses for Ca^{2+}-impermeable synapses), the *N*-methyl-D-aspartate (NMDA) component of excitatory postsynaptic currents (EPSCs) is higher. In opposite, at the synapses with highly Ca^{2+}-permeable AMPA receptors (CP synapses for Ca^{2+}-permeable synapses), the NMDA component is lower (Lei and McBain, 2002). The CI synapses display a postsynaptically induced and NMDA-dependent LTD, whereas the CP synapses have a presynaptically induced and NMDA-independent LTD (Lei and McBain, 2004). In the CA3 interneurons, LTD was possible but LTP was not induced. It was generally accepted that interneurons did not show LTP (McBain et al., 1999). However, it was recently found that LTP was induced at MF - dentate gyrus BC synapses indicating an input diversity at the level of long-term plasticity. Thus, at the excitatory synapses between dentate granule cells and BCs, high frequency stimulation paired with postsynaptic depolarization induced LTP with a presynaptic location (Alle et al., 2001).

c. The electric synapses.

In neocortex, different subclasses of interneurons are coupled by gap junctions such as the FS neurons and the low-threshold spiking (LTS) interneurons. The interneurons of one subclass were preferentially (3 out of 32 heterotypic electric synapses in Gibson's experiments) or exclusively (Galaretta's experiments) connected by electric synapses with interneurons of the same subclass, therefore electric coupling can give rise to a specific network of interneurons (Galarreta and Hestrin,

1999; Gibson et al., 1999). In the FS neurons that were connected by electric and chemical synapses, an AP in one cell elicited a biphasic response in the second cell, first a short depolarization via the electric coupling followed by a longer IPSP induced by chemical interconnections of interneurons (Gibson et al., 1999). Small depolarizating currents, which were subthreshold when injected in two cells at different time, were suprathreshold when injected simultaneously in two electrically connected neurons (Galarreta and Hestrin, 1999). Moreover, APs in one cell eased the generation of APs in the connected cells. Therefore, electric coupling allows connected neurons to fire synchronously (Gibson et al., 1999). The gap junctions that form the electrical synapses between the interneurons are composed of connexin 36. In connexin 36 knockout mice, the electric coupling between FS neurons and between LTS neurons is rare or absent (Deans et al., 2001; Hormuzdi et al., 2001). The absence of gap junctions reduces the capacity of interneurons network to generate rhythmic oscillations in the hippocampus and the neocortex (Deans et al., 2001; Hormuzdi et al., 2001), providing strong evidence of the role of the electric coupling in interneuronal networks to produce large-scale oscillations.

4. Diversity of the receptors

Three types of receptor are activated by the neurotransmitter GABA, the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptors.

$GABA_A$ and $GABA_C$ receptors possess a Cl^- channel that is integrated within the receptor protein. The binding of two GABA molecules on the receptor gates the Cl^- channels and mediates a fast inhibition lasting 10-100 ms. In contrast to $GABA_A$ receptors, $GABA_C$ receptors are defined by their insensitivity to bicuculline (Bormann, 2000).

The $GABA_C$ receptors will not be considered further, however, the $GABA_C$ receptors are composed of ρ_{1-3} subunits that are highly expressed in the retina. A weak expression of ρ subunits are described in the rat thalamus (Wegelius et al., 1998) and $GABA_C$ receptor currents are recorded in acutely isolated thalamic neurons (Schlicker et al., 2004). The $GABA_C$ receptors may have a role at the retinogeniculate synapses where GABA release by local interneurons produce, besides the $GABA_A$

and $GABA_B$ receptor-mediated currents, a Cl^- current that is bicuculline-insensitive (Zhu and Lo, 1999).

$GABA_B$ receptors are seven transmembrane-domains proteins that indirectly modulate ionic and cytoplasmic effectors via activation of G-proteins. The actions of $GABA_B$ receptors are thus slower in onset but more prolonged in duration (Greengard, 2001). $GABA_B$ receptors increase K^+ conductances (Newberry and Nicoll, 1984; Gähwiler and Brown, 1985; Thalmann, 1988), reduce Ca^{2+} currents (Mintz and Bean, 1993), inhibit adenylyl cyclases (Wojcik and Neff, 1984; Xu and Wojcik, 1986; Gerber and Gähwiler, 1994), activate phospholipase A_2 (Duman et al., 1986), control intracellular release of Ca^{2+} (Hirono et al., 2001) and vesicle recruitment at the presynaptic membranes (Sakaba and Neher, 2003).

a. Diversity of $GABA_A$ receptors

Molecular diversity

$GABA_A$ receptors are ubiquitously expressed in the CNS and form of a pentamer composed of various types of $GABA_A$ subunits. The 15 different subunits described are classed in 6 families: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε and θ. The GABA binding domain is at the interface between the α and the β subunits (Smith and Olsen, 1995). Most of the mature brain receptors are composed of the α, β, γ and δ subunits with the stoichiometry 2α, 2β and 1 γ/δ (Barnard et al., 1998). The properties of a $GABA_A$ receptor depend on their subunit composition (Costa, 1998; Hevers and Luddens, 1998). For example, the receptors composed of α_1 subunits have fast deactivation kinetics and display desensitization while the presence of the α_2 subunit allows faster activation kinetics and slower deactivation (Lavoie et al., 1997). The presence of the α_6 subunits or the δ subunits confers a 10- to 50-fold higher affinity for GABA than other $GABA_A$ receptors. This composition abolishes also receptor desensitization upon prolonged presence of the agonist (Saxena and Macdonald, 1994, 1996; Mody, 2001).

The diversity of $GABA_A$ receptor subunits seems to be important because they have a selective distribution in the brain (Wisden et al., 1992), which varies along the development (Laurie et al., 1992). The α_1 and α_2 subunits are widely expressed in the CNS but α_1 subunits are more strongly expressed in the cortex and the thalamus whereas α_2 subunits are preferentially expressed in the limbic sytem (Wisden et al.,

1992; Fritschy and Möhler, 1995). The α_6 subunit expression is restricted to granule cells of cerebellum and cochlear nuclei (Wisden et al., 1992). The δ subunit is strongly expressed in granule cells of the cerebellum and, to a less extent in the thalamus (Wisden et al., 1992; Nusser et al., 1998). The $GABA_A$ receptor subunits also display a highly organized cellular distribution. At the synapses formed by the PV-positive BCs on PCs, the postsynaptic membranes contain mostly the α_1 subunits (Klausberger et al., 2002). In contrast, at the synapses between the PV-negative BCs and PCs, the postsynaptic membranes contain mostly the α_2 subunit. The post-membrane of PCs apposed to chandelier cells have an intermediate ratio for α_1 and α_2 subunits compare to synapses formed by PV-positive and PV-negative BCs (Nyíri et al., 2001). The IPSCs generated by the PV-positive BCs display fast kinetics of deactivation and desensitisation. The IPSC generated by the PV-negative BCs display slower kinetics (Lavoie et al., 1997). Similarly, in the hippocampal CA1 PCs, two types of synaptic GABAergic IPSCs have been described. One is a fast $GABA_A$ response generated at the soma (presumably by the BCs and the chandelier cells) and the other is a slow $GABA_A$ response generated at the dendrites (presumably by the interneurons of *stratum lacunosum-moleculare*). Thus, they are generated by a different population of hippocampal interneurons and they are mediated by $GABA_A$ receptors composed of different subunits leading to a different pharmacological profile (Banks et al., 1998). The $GABA_A$ receptor subunits may also be confined in subcellular compartments such as in the cerebellum where the δ subunits are exclusively located extrasynaptically in the soma and dendrites of the granule cells (Nusser et al., 1998).

In the brain, endogenous ligands such as the neurosteroids, act as allosteric modulators of the $GABA_A$ receptors by facilitating the open state of the GABA-gated ion channels. The efficacy of neurosteroids depends on the subunit composition. Thus, the neurosteroids may selectively influence GABAergic signaling (Lambert et al., 2003).

Besides binding GABA and the neurosteroids, $GABA_A$ receptors also bind exogenous molecules such as the BZs, the barbiturates, the alcohol and some volatile anaesthetics (Mihic et al., 1997), which alter the properties of the receptors (Costa, 1998). The binding of the BZs, requires the $\alpha_{1,2,3}$ or α_5 subunits in combination with any of the β subunit and the $\gamma_{2,3}$ subunits (Costa, 1998; Möhler et al., 2002).

Benzodiazepines do not bind the receptor composed of the $\alpha_{4,6}$ or γ_1 or in absence of γ subunit. Benzodiazepines increase the response to GABA by allosterically enhancing the affinity of $GABA_A$ receptors to GABA, thereby increasing the frequency of the channel openings (MacDonald et al., 1989).

Generation of mutant mice for the different subunits allowed association of specific $GABA_A$ receptor subunits with particular pharmacological and behavioural characteristics. For example, mice expressing a genetically engineered BZ-insensitive α_1 subunit showed no diazepam-induced anterograde amnesia and a reduced anticonvulsant effect of BZ. In contrast, the myorelaxant, motor-impairing, ethanol-potentiating and anxiolytic-like properties of diazepam were not impaired (Rudolph et al., 1999). Conversely, the corresponding point mutation in the α_2 subunits reduced the anxiolytic effect of BZ (Löw et al., 2000). These two studies managed to link a complex behaviour to the expression of a specific $GABA_A$ receptor subunit, meaning α_1 subunit to memory and α_2 to stress emotion. This powerful strategy also permitted to demonstrate that the α_1 subunits temporally define the critical-period plasticity in the visual cortex (Fagiolini et al., 2004).

Physiological diversity

The Cl^- current induced by $GABA_A$ receptor opening depends on the driving force for Cl^- ions, namely the numerical difference between the reversal potential of Cl^- ions and the actual membrane potential of the postsynaptic membrane. Three situations are commonly found in native cells. A) If the reversal potential is more negative than the membrane potential, activation of the receptor induces an influx of Cl^- ions that hyperpolarizes the cell. B) If neurons have a resting membrane potential close to the reversal potential of Cl^- ions (around -65 mV), the net ionic flux is low and the effect of GABA is due to a decrease of membrane resistance that shunts other excitatory inputs C) If the membrane potential lies below the reversal potential, the flux of anions is outward and the effect is a depolarization of the membrane.

In cortical neurons of embryonic and newborn animals, GABA is excitatory because the intracellular concentration of Cl^- is high and the reversal potential is thus more positive than the resting membrane potential, such that GABA acts as a depolarizing neurotransmitter (Situation C). Remarkably, in immature neurons, it is the GABAergic excitation itself that induces the expression of KCC2, a K^+/ Cl^- co-transporter. KCC2 extrudes Cl^- and thus shifts the reversal potential of Cl^- to more

negative value (Ganguly et al., 2001). NKCC1 is a co-transporter driven by the Na^+ and K^+ gradients. Activation of NKCC1 leads to an increase in intracellular Cl^- concentration. NKCC1 is down-regulated along the development (Plotkin et al., 1997). The enhanced and reduced expressions of KCC2 and NKCC1, respectively, lower the cytoplasmic concentration of Cl^- that transforms the $GABA_A$ receptor responses from depolarizing in immature neurons to hyperpolarizing in mature neurons (Ganguly et al., 2001).

GABA exerts excitatory effects in mature neocortical neurons as well. Thus, the resting potential of cortical neurons recorded in acute slices is around -79 mV, more negative than the reversal potential of Cl^- ions at the soma and the dendrites (~-70mV). Nevertheless, $GABA_A$-mediated response is not only excitatory but also inhibitory depending on the subcellular location where it is induced. First a dendritic GABAergic depolarization turns a subthreshold somatic EPSP elicited simultaneously into a suprathreshold EPSP. In contrast, a somatic GABAergic depolarization inhibits a suprathreshold EPSP elicited simultaneously because the effect of shunting inhibition is stronger than the depolarization induced by the flux of Cl^-. When the glutamatergic EPSP is delayed by at least 5 ms, the somatic GABAergic depolarization is now excitatory and facilitated the glutamatergic EPSP to reach AP threshold. Therefore, when the GABAergic depolarization is spatially and temporally distant from the glutamatergic EPSP, GABAergic response facilitates the EPSP (situation C). On the contrary, when the GABAergic depolarization and the glutamatergic EPSP are evoked simultaneously and nearby, GABAergic response inhibits the EPSP (situation B) (Gulledge and Stuart, 2003).

b. Tonic activation of $GABA_A$ receptors

The synaptic activation of $GABA_A$ receptors responsible for fast inhibition of the targeted neurons is also termed as phasic response, in contrast to tonic activation of $GABA_A$ receptors. The tonic activation consists in a constant activation of extrasynaptic receptors by ambient GABA present in the extracellular milieu. Tonic activation of $GABA_A$ receptors was described in granule cells of the cerebellum (Brickley et al., 1996), the granular cells of the dentate gyrus (Stell and Mody, 2002) and the interneurons of the hippocampal CA1 subfield (Semyanov et al., 2003).

In the cerebellum, the association with the α_6 subunit produces a receptor showing three properties require for tonic inhibition: high affinity for GABA, slow desensitization and a long open time (Brickley et al., 1996; Nusser et al., 1998; Brickley et al., 2001). The ambient GABA levels are controlled by GABA uptake mechanisms and residing extrasynaptic GABA is estimated to be superior to 0.4 μM (Attwell et al., 1993). This minimal concentration is sufficient to activate high-affinity receptors containing the α_6 subunits and the δ subunits (EC_{50} for GABA is 0.2-0.5 μM) (Saxena and Macdonald, 1996). The tonic inhibition reduces the excitability of cerebellar granule cells and alters the sensitivity of the neurons to excitatory inputs (Semyanov et al., 2004). In hippocampal interneurons, a functional role for tonic inhibition is also described (Semyanov et al., 2003). In these neurons, $GABA_A$ receptor-dependent tonic activation is mediated by high affinity receptors that are pharmacologically different from the $GABA_A$ receptors responsible for phasic inhibition at the interneuronal-interneuronal synapses and at the interneuronal-PC synapses. Thus, similarly to cerebellum, a particular subunit composition of the $GABA_A$ receptors underlies tonic inhibition, but, in contrast to the cerebellum, the δ subunits seem not to be required. The tonic inhibition of interneurons reduces their excitability and thus modulates the inhibitory drive to the PCs. In addition, in this preparation, the blockade of GABA uptake unmasks tonic inhibition of PCs, which shares similar pharmacological properties with the tonic inhibition of interneurons. Thus, tonic inhibition of interneurons may arise from spillover of GABA coming from interneuronal-interneuronal synapses and not from the interneuronal-PC synapses where GABA uptake may prevent GABA spillover (Semyanov et al., 2003).

c. $GABA_B$ receptors

$GABA_B$ receptors were originally identified in pharmacological experiments showing that 1) GABA could act through a bicuculline-insensitive receptor 2) These effects were mimicked by application of baclofen, a compound that is still used against spasticity (Bowery et al., 1980), 3) These receptors were GPCRs (Asano et al., 1985). A first subunit was cloned in 1997 and was named $GABA_{B(1)}$ which partially showed the properties of native receptor (Kaupmann et al., 1997). A second subunit, $GABA_{B(2)}$, was cloned in 1998 (Kaupmann et al., 1998) and it was demonstrated that co-expression of the $GABA_{B(1)}$ and the $GABA_{B(2)}$ proteins was absolutely required for

a functional receptor. The $GABA_B$ receptor is the first example of a heterodimeric metabotropic receptor (Filippov et al., 2000), a concept now widely established for GPCRs (Bouvier, 2001; Milligan et al., 2003). The genetic disruption of the $GABA_{B(1)}$ subunit or the $GABA_{B(2)}$ subunit in mice prevents the major functional effects normally observed after activation of $GABA_B$ receptors (Schuler et al., 2001; Gassmann et al., 2004).

d. Activation of $GABA_B$ receptors

In contrast to activation of $GABA_A$ receptors, $GABA_B$ receptors need stronger stimulations of the GABAergic axons to be activated synaptically (Dutar and Nicoll, 1988). Thus, at the majority of GABAergic synapses, $GABA_B$ receptor activation is generally not observed following activation of a single presynaptic interneuron, even when this neuron produces a train of APs at high-frequency (Scanziani, 2000). Conversely, extracellular stimulation routinely produced a $GABA_B$ response, indicating that a coordinated release of GABA may be required for $GABA_B$ receptor activation (Scanziani, 2000). In the CA3 area of the hippocampus, $GABA_B$ receptor-mediated currents have been studied in detail (Scanziani, 2000). Thus, blocking GABA uptake allows $GABA_B$ receptors to be activated even by a single neuron suggesting that extrasynaptic $GABA_B$ receptors are activated by spillover of GABA out of the synaptic cleft. It is estimated that, in the presence of functional GABA uptake, the cooperation of 2 to 20 stimulated interneurons is required to generate a $GABA_B$ response (Scanziani, 2000).

The search for the mechanism underlying the requirement of increased stimulation intensity to activate $GABA_B$ receptors has led to an intense debate over the subcellular location and the properties of $GABA_B$ receptors. The location of $GABA_B$ extrasynaptically is one reason that helps to explain the necessity of higher stimulation to activate $GABA_B$ as the GABA released has to spillover the synaptic cleft (Isaacson et al., 1993; Scanziani, 2000). An additional explanation is that activation of the K^+ channels demands the cooperative binding of several (four) G-proteins to the G-protein inward rectifier channels as suggested by computational modelling. In this hypothesis, a threshold level of G-proteins has to be activated by $GABA_B$ receptors to produce detectable K^+ currents (Destexhe and Sejnowski, 1995).

In two exceptional cases, the synaptic release of GABA by a single cell was sufficient to allow the activation of a unitary $GABA_B$ response. At synapses between the neurogliaform interneurons and the PCs, a single presynaptic AP elicited a biphasic postsynaptic response composed of fast $GABA_A$ and slow $GABA_B$ components (Tamás et al., 2003). At the GABAergic synapses between the neurons of the reticular nucleus and the thalamocortical (TC) neurons, unitary $GABA_B$-mediated hyperpolarizations are possible. A unique AP in neurons of the nucleus reticularis (nRt) resulted in a pure $GABA_A$ response in TC neurons but a prolonged burst of APs in nRt neurons was sufficient to produce a response with a $GABA_B$ component (Kim et al., 1997).

II GABA signaling in the thalamus.

1. Basic cellular and synaptic organization of the thalamus

a. Organization of the thalamic nuclei

The thalamus is an aggregate of nuclei located within the diencephalon, which is composed of the epithalamus, the dorsal thalamus, the ventral thalamus and the hypothalamus. The dorsal thalamus directly sends projections to the cortex and is principally responsible for the gating functions traditionally ascribed to the thalamus (Jones, 1991; Sherman and Guillery, 1996).

The nuclear subdivision of the dorsal thalamus, from now on referred to as 'the thalamus', arises predominantly from the topographically organized projections arriving either from 1) the sensory periphery or 2) from cortical layer V. These driving inputs are defined as the determinants of the receptive field properties of thalamic neurons, but they constitute a minor portion (5-10%) of the total number of synapses (Jones, 2002; Guillery, 2003). The remaining synapses arise from modulatory inputs via brainstem and cortical layer VI afferents. Modulatory inputs shape the synaptic properties of driving afferents and the intrinsic electrophysiological characteristics of thalamic cells, but do not primarily determine the receptive field (Sherman and Guillery, 1996; Sherman, 2001c; Jones, 2002; Guillery, 2003).

Based on the nature of driving inputs, thalamic nuclei are subdivided into first-order and higher-order nuclei (HOn). The first-order nuclei or relay nuclei are driven

by afferents from the sensory periphery and serve as an obligatory relay for all sensory information except olfaction. For example, the dorsal lateral geniculate nucleus (dLGN) and the ventrobasal nucleus (VB) are two nuclei specialized to relay the external visual and somatosensory information to the primary visual and somatosensory cortex, respectively. The HOn receive input from cortical layer V and from sensory afferents but the first are driving inputs and the second are modulatory inputs (Diamond et al., 1992b; Sherman and Guillery, 2002). Frequently, activation within HOn is associated with more complex functions related to sensory processing, such as multisensory analysis, selective attention and expectation of reward (Kinomura et al., 1996; Ahissar et al., 2000; Komura et al., 2001b).

Compared to first-order nuclei, little is known about the connectivity of HOn and about their function in TC and cortico-cortical communication. Presumably, the representations of each sensory modality are contained in at least one relay nucleus and one HOn. For example, the latero-posterior, the latero-dorsal and the posterior nuclei are HOn specialized to treat the visual and the somatosensory information, respectively (Bourassa and Deschênes, 1995; Bourassa et al., 1995; Sherman and Guillery, 1996). Moreover, first-order and HOn are interconnected via inhibitory nRt neurons, further indicating a tight communication between the 'simple' first-order and the more complex HOn (Crabtree et al., 1998; Crabtree and Isaac, 2002).

b. Cellular and synaptic structure

Compared to cortical and hippocampal circuits, the cellular architecture of thalamic nuclei appears remarkably simple, containing essentially only three types of neurons, the TC neurons, the thalamic interneurons, and the nRt neurons. Furthermore, compared to hippocampus and cortex, GABAergic signaling in thalamus appears strikingly uniform, with only two sources of inhibition from two seemingly homogeneous cell groups with spatially restricted distributions. The cellular structure, the principal synaptic (driving) inputs and the axonal projections of these three cell groups are briefly presented here. For a detailed account of neuronal subgroups within the nRt, the reader is referred to Pinault (2004).

Thalamocortical neurons

Somatodendritic morphology: In dorsal thalamic nuclei, the principal neurons are the glutamatergic TC neurons (Kaneko and Mizuno, 1988). In cats and rodents,

these are multipolar neurons with large somata (20x30 μm diameter) and radially arranged dendrites (Yamamoto et al., 1985; Yen et al., 1985), although more polarized dendritic trees are also described (Brecht and Sakmann, 2002). *In vivo* studies in the visual system have provided a functional subdivision of TC neurons into X-, Y-, and W-cells which is based on the linearity of responses to visual inputs, the receptive field structure, and the axonal conduction velocity (Sherman, 1985). This differential responsiveness of TC neuron subtypes correlates with distinct morphological (Friedländer et al., 1981; Crunelli et al., 1987) and electrophysiological (Bloomfield et al., 1987; Crunelli et al., 1987) characteristics. However, in other thalamic nuclei, TC cells appear as a more homogeneous group with no clear relationship between morphological and functional properties (Brecht and Sakmann, 2002).

Synaptic driver inputs: The driving inputs established by sensory afferents are glutamatergic (Montero and Wenthold, 1989; Turner and Salt, 1998) and terminate on the proximal dendrites of the TC neurons (McAllister and Wells, 1981; Wilson, 1989). The sensory ascending tracts, such as the optic tract and the medial lemniscus, are composed of large-diameter axons that form large terminals on the TC neurons of the dLGN (Bowling and Michael, 1980) and of the VB (McAllister and Wells, 1981; Liu et al., 1995b), respectively.

Axonal projections: The TC neurons project axons to the cortex. The TC axon terminals are principally located in the layer IV of the cortex (Herkenham, 1980) and form asymmetric glutamatergic synapses on the spiny dendrites of the neurons of this layer (Kharazia and Weinberg, 1994; Sherman and Guillery, 1996). On their way to the cortex, TC axons project collaterals to the neurons of the nRt (Scheibel and Scheibel, 1966; Yamamoto et al., 1985; Yen et al., 1985; Harris, 1987). Recently, it was also proposed that intralaminar axonal collaterals of TC neurons observed previously (Ferster and LeVay, 1978; Friedländer et al., 1981) innervate local interneurons to provide feedB inhibition (Cox et al., 2003).

Local interneurons

The other types of neurons found in the dorsal thalamic nuclei are the local interneurons. The proportion of interneurons varies as a function of the nuclei and the species. They can represent up to 30% of the cell bodies as revealed by the glutamic

acid decarboxylase (GAD) staining. This enzyme transforms glutamate into GABA. The primates have a higher proportion of interneurons than the rodents. For example, in rodent somatosensory thalamus, no interneuron is present (Arcelli et al., 1997), while in the dLGN, the interneurons represent 20% of the neurons (Gabbott et al., 1986). In the primates, the local GABAergic neurons represent around 35-40% of the neurons in the dLGN and the VB (Arcelli et al., 1997).

Somatodendritic morphology: The interneurons are small (8-14 μm diameter) GABAergic neurons (Ohara et al., 1983). They have a polar appearance with two highly branched dendrites arising from the opposite side. Most of the interneurons have a short ramified axon arising from the soma or the proximal dendrites (Rafols and Valverde, 1973; Montero, 1987; Gabbott et al., 1988).

Interneurons in thalamic nuclei are unique, because besides the classic synapses formed at the axonal terminals (F1 synapses), the dendrites of interneurons form a triadic synaptic arrangement (named F2 synapses) with the retinogeniculate terminals and the postsynaptic dendrites of TC cells of X-type (Wilson et al., 1984; Sherman and Guillery, 1996, 2002).

Synaptic triad: The triad has the following structure: The retinal axon makes asymmetric glutamatergic synapses on a TC cell dendrite and a dendrite of an interneuron that contains elements of a presynaptic structure, such as a cluster of synaptic vesicles (Ralston, 1971; Hamos et al., 1985). The interneuron then establishes a dendro-dendritic synapse on the same TC cell dendrite and is induced to release the vesicle after activation by the retinal tract, independently of the activity of other cellular compartments (Ralston, 1971). In this manner, the interneuron provides a highly localized feedF inhibition that controls the transmission efficiency of the prethalamic pathways. This triadic arrangement is embedded in a glial sheet to form a glomerular structure (Williams and Faull, 1987), thus ensuring the focal regulation of GABA release onto postsynaptic GABA receptors.

Besides prethalamic connections, the interneurons are innervated by corticothalamic (CT) collaterals, as shown with morphological studies (Weber et al., 1989; Montero, 1991; Erisir et al., 1998). They also receive excitatory inputs coming from intrageniculate collaterals of TC neuron axons (Cox et al., 2003).

Axonal projections: Besides the dendrodendritic synapses found in triads, interneurons form axodendritic or axosomatic inhibitory synapses (F1 terminals) onto TC neurons of the X- and Y-type (Guillery, 1969; Famiglietti and Peters, 1972).

Nucleus reticularis neurons

Somatodendritic morphology: The nRt is composed of a homogeneous population of small neurons (10-20 μm diameter) that express the GAD (Houser et al., 1980; Yamamoto et al., 1985). Via Golgi impregnations of the nRt, Scheibel & Scheibel (1966) first provided a detailed description of the homogeneous group of oval or slightly elongated, multipolar cells with long, relatively unramified dendrites that were stretched out along the axis of the nRt, so perpendicularly to the direction of the TC and CT tracts.

Principal synaptic inputs: The nRt lies in the path of axonal projections between the thalamus and the cortex and receives glutamatergic synaptic inputs from both areas (Scheibel and Scheibel, 1966). The cortical synapses on nRt cells represent the majority (>60%) of synapses present on the dendrites of nRt cells (Carman et al., 1964; Ohara and Lieberman, 1981; Liu and Jones, 1999), whereas TC synapses amount to only 20%. This numerical dominance of cortical synapses at least partially explains the powerful control of nRt function, and thus indirectly of thalamic activity, by the cortex (Contreras et al., 1996; Bal et al., 2000; Blumenfeld and McCormick, 2000; Zhang and Jones, 2004).

Axonal projections: The nRt neurons project exclusively to the dorsal thalamus (Jones, 1975; Houser et al., 1980; Ohara and Lieberman, 1981; Steriade et al., 1984). Before, the reticulothalamic axons may give rise to intra-reticular collaterals (Cox et al., 1996). In dorsal thalamus, the nRt neurons terminals synapse on the soma, the proximal dendrites of rodent VB neurons (Peschanski et al., 1983) and dLGN neurons (Ohara et al., 1980) or on their intermediate dendrites (Kim et al., 1997).

2. Electrophysiological properties the thalamocortical neurons

Unique electrophysiological properties of TC neurons endow thalamic networks with the capacity to generate rhythmic neuronal activity in an autonomous manner. These rhythm-generating properties are essential for the generation of oscillatory

activity within TC networks that is observed during different phases of slow-wave sleep. The early stages of non-REM sleep is characterized by the recurrence of 6-15 Hz oscillations that last 1-3 s and named spindle oscillations. Then, during the late stages of sleep, 0.5-4 Hz oscillations named delta waves appear (Steriade et al., 1993; McCormick and Bal, 1997; Jones, 2002). For at least these two sleep-related rhythms that are sequently present in the electroencephalogram (EEG) of mammals, the cellular and the synaptic basis are remarkably well understood and is explained to a large extent by the activity of TC neurons and the GABAergic inhibition of these cells by the nRt

a. Action potential discharge modes

A principal property of TC neurons is the ability to generate rebound burst of APs upon transient membrane hyperpolarization. Jahnsen and Llinás (1982, 1984 a, b) were the first to describe these characteristic AP discharge patterns via intracellular recordings from guinea-pig TC cells (Llinás and Jahnsen, 1982). These authors found that when the membrane potential of TC neurons was below –60 mV, a step depolarization produced a burst of APs composed of a low-threshold spike (LTS), on which fast spikes were superimposed. They also showed that a hyperpolarizing potential induced a rebound burst when the membrane potential was set back to the resting potential. In contrast, when the membrane potential was above –60 mV, the same step depolarization evoked tonic firing of Na^+ spikes. These observations were surprising because they showed that TC neurons displayed a dual AP discharge mode dependent upon the recent history of membrane potential trajectory. Moreover, it indicated that, paradoxically, TC neurons could discharge APs as a rebound response to inhibitory synaptic input. Jahnsen and Llinás further characterized the ionic conductances underlying this anomalous electrophysiological behavior and determined that the conductance that generated the LTS was inactivated at potential above -55 mV and that the removal of this inactivation required membrane hyperpolarization and was time-dependent (Jahnsen and Llinás, 1984a). Using ion substitution and blocker experiments, the LTS was found to be carried by Ca^{2+} channels, whereas the superimposed fast spikes were carried by TTX-sensitive Na^+ channels (Jahnsen and Llinás, 1984b).

The functional correlate of the dual discharge mode was found when recording *in vivo* from animals during different states of arousal. During sleep, *in vivo*

intracellular recordings showed that TC neurons of dLGN had a hyperpolarized membrane potential and that the main discharge pattern observed consisted in a burst of APs. Conversely, during waking or rapid-eye-movement sleep (REM sleep), the membrane potential was depolarized and the firing pattern of the cell consisted mostly in tonic firing (Hirsch et al., 1983; McCarley et al., 1983; Weyand et al., 2001). Thus, the AP discharge pattern was correlated with the arousal state and suggested that the burst discharge mode prevalent during sleep phases could contribute to the failure of thalamic neurons to faithfully transmit sensory signals to the cortex. In contrast, during the tonic firing mode, cells respond to incoming activities with individual spikes at frequencies that are proportionnal to the intensity of the stimuli. Indeed, analysis of the input-output relationship of TC neurons revealed that the burst discharge mode did not faithfully transmit information because the frequency of AP discharge during a burst did not depend on the amplitude of the inputs (Sherman, 2001a).

Although not nearly as common, arrhythmic bursting can also occur during wakefulness in a minority of dLGN cells and a variety of studies have emphasized a critical role of burst discharges in sensory processing. Bursts were observed at the onset of a visual stimulus presented for the first time, whereas subsequent presentations of a similar stimulus induced a burst less often (Weyand et al., 2001). Moreover, decreased arousal of the cat enhanced the probability to observe a burst in response to a visual stimulus (Weyand et al., 2001). Like the single spikes, thalamic bursts are informative for the cortex but with a different content. Indeed, in the FS interneurons of the cortical layer IV, the first spike of the TC bursts, which occurred mainly in drowsy animals, was more efficient to induce a suprathreshold response than a spike during regular firing (Swadlow and Gusev, 2001). It is therefore proposed that in an inattentive animal, the appearance of a new stimulus entails a burst in the dLGN TC neurons. This first burst would lead to a reliable activation of the cortex. This is consistent with the detector role attributed to the burst (Sherman, 2001b). In awake state, the bursts may serve to detect fast changes in the sensory signals whereas the spikes decode a detailed representation of the signal (Nicolelis and Fanselow, 2002b).

b. The low-threshold calcium current

The ability of the TC neurons to produce a rebound burst of APs is the consequence of the expression of a particular type of voltage-gated Ca^{2+} channel, also called the T-channel (I_T) or Low-Voltage Activated (LVA) Ca^{2+} channel (Coulter et al., 1989; Huguenard, 1996), in the proximal dendrites and the soma (Suzuki and Rogawski, 1989; Destexhe et al., 1998; Williams and Stuart, 2000b).

A transient Ca^{2+} current, different from two other Ca^{2+} current types, was originally identified in sensory neurones of the chick dorsal root ganglion and termed T-current, with the 'T' highlighting the transient appearance of the current (Nowycky et al., 1985). In TC neurons, the T-current manifests as a rapid inward current that has a low threshold of activation ~-65 mV compared to the high-voltage activated (HVA) Ca^{2+} channels which activates at potentials above ~-30 mV. The current inactivates within tens of milliseconds and the voltage-dependence of the steady-state inactivation is ~-83 mV. Both kinetics of activation (τ=2-8 ms) and inactivation (τ=20-50 ms) are dependent on voltage, being faster at more depolarized potentials (Coulter et al., 1989). At hyperpolarized potentials below -90 mV, I_T recovers from inactivation within less than 500 ms (time constant of recovery = 250 ms at -92 mV) (Coulter et al., 1989). Upon transient membrane hyperpolarization, such as it occurs during inhibitory synaptic input arising from GABAergic neurons, neurons endowed with I_T can thus, at the end of the hyperpolarization, generate a rapid, all-or-none depolarization that can reach AP threshold and promote a burst-like discharge of APs at frequencies up to 250-400 Hz (Jahnsen and Llinás, 1984b).

The calcium channels are tetramers composed by the α1 subunit that is thought to form the pore of the channel and is associated to β, α2/δ and γ subunits (Catterall et al., 2003). At least ten α1 subunit transcripts have been cloned and three transcripts, the α1G (Cav3.1), the α1H (Cav3.2) and the α1I (Cav3.3) are related to T-channels (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999) . The α1G was the first cloned and showed strongest expression in the TC neurons (Perez-Reyes et al., 1998; Talley et al., 1999). In mice lacking the α1G subunits, the TC neurons lacked I_T and failed to generate rebound burst responses following transient membrane hyperpolarization. The transgenic mice were resistant to the generation of spike-and-wave discharges typically observed in the relay nuclei of the thalamus after injection of drugs that promoted these epileptiform oscillations in wild-type mice (Kim et al.,

2001b). These results indicate a crucial role of I_T in the generation of synchronized oscillations in TC networks. The consequence of the lack of α1G subunits on the sleep of these animals has so far remained unexplored.

c.The hyperpolarization-activated non-selective cationic current

In addition to showing a strong propensity to generate rebound low-threshold calcium bursts, TC neurons express a hyperpolarization-activated cation current (I_h), classically referred to as the pacemaker current. The properties of I_h are detailed here in the review 'Regulation of recombinant and native hyperpolarization-activated cation channels' (part III). The pacemaker current activates at potentials below –60 mV and is carried by both Na^+ and K^+ ions, with a higher permeability to K^+ ions (P_{Na}/P_K ~ 0.2-0.4). Recently, a small permeability for calcium was identified via imaging techniques for the current produced by recombinant expression of human HCN4 transcript in HEK293 cells or for the native current in dorsal root ganglia neurons (Yu et al., 2004a). Within the hyperpolarized activation range, the current is mainly carried by an influx of Na^+ ions across the membrane, as evident from Na^+ imaging studies in dopamine cells of the substantia nigra pars compacta (Knöpfel et al., 1998). The kinetics of activation are usually best described by a biexponential time dependence (McCormick and Pape, 1990a; Santoro et al., 2000).

A remarkable characteristic of I_h is its modulation through cyclic nucleotides. Cyclic AMP (cAMP) increases the amplitude of the current and accelerates the kinetics of activation (DiFrancesco and Tortora, 1991; Robinson and Siegelbaum, 2003).

Four subunits of the HCN (Hyperpolarization-activated cationic non-selective) channels, HCN1-4, were recently cloned and they were found to generate currents that showed properties strongly reminiscent of those found in intact cells. Immunocytochemistry and in situ hybridization essays has shown that the TC neurons express mainly HCN2 and HCN4, the two subunits most sensitive to cAMP (Moosmang et al., 1999; Santoro et al., 2000).

The combination of I_T and I_h is the basis for the delta oscillations, a type of slow rhythmic discharges at 0.5-4 Hz that are found both *in vitro* (McCormick and Pape, 1990a; Leresche et al., 1991; Soltesz et al., 1991; Destexhe et al., 1993; Steriade et al., 1993; Hughes et al., 1998) and *in vivo* (Steriade et al., 1991) and that contribute to the

emergence of the slow EEG waves during deep sleep phases. These oscillations are cell-autonomous as they are produced in the absence of synaptic activity and of AP generation (McCormick and Pape, 1990a; Leresche et al., 1991; Soltesz et al., 1991; Destexhe et al., 1993). Briefly, when the cell is hyperpolarized around –75 mV, the h-current is activated and it drives the potential back to depolarized state. The slow kinetics of activation of I_h permit a sufficient de-inactivation of the T-current. The opening of T-channels elicits a fast depolarization that triggers the burst of APs. The depolarization of the cell inactivates I_T, de-activates I_h and results in the repolarization of the potential, which re-activates I_h and triggers a new burst.

3. GABAergic signaling in thalamus I: the nucleus reticularis

The principal inhibition of thalamic nuclei is exerted by the nRt that forms a shell of inhibitory cells surrounding the thalamus. The nRt exerts both a global inhibition of thalamic nuclei and is involved in the genesis of neuronal synchronization and rhythmicity in TC networks (McCormick and Bal, 1997; Steriade, 2001; Jones, 2002), and a local inhibition that acts to control the gain and precision of information transfer during sensory processing (Norton and Godwin, 1992; Nicolelis and Fanselow, 2002b; Pinault, 2004). In parallel to the first chapter of this thesis, we focus here on the cellular and synaptic basis of nRt function and the diversity of its actions in thalamic networks.

a. Electrophysiological properties of the nucleus reticularis neurons

Similar to the TC neurons, the nRt cells have two different AP discharge modes, a tonic mode and a burst mode as shown *in vitro* (Spreafico et al., 1988; Bal and McCormick, 1993) and *in vivo* (Domich et al., 1986; Mulle et al., 1986). The capacity to burst is also associated with the expression of low-threshold calcium currents. Nevertheless, the burst discharge differs from that generated by TC neurons due to the fact that the nRt T-channels have molecular and physiological properties distinct from those expressed in TC neurons. The current activates at more depolarized potentials (~-50 mV), permitting nRt cells to generate bursts at more positive potentials (Huguenard and Prince, 1992). Due to decelerated inactivation, the burst lasts longer so that 6 to 8 APs are generated instead of only 1 to 3 APs in TC neurons (Bal and

McCormick, 1993; Contreras et al., 1993). The time and voltage-dependent properties of the activation and inactivation observed in nRt I_T are thought to result from the strong expression of the α1I subunit (Talley et al., 1999). Indeed, recombinant expression of α1I subunits leads to a current with a more depolarized voltage-dependence of the activation and a slower activation (the time constant is 4.7 ms) and inactivation (the time constant is 55 ms) compare to the α1G and α1H subunits (the activation and inactivation time constants are, 1.7-1.8 and 14-15 ms) (Perez-Reyes et al., 1998; Lee et al., 1999). Finally, in contrast to TC neurons, nRt neurons are capable to produce burst discharges in a repetitive manner. This is mostly due to the fact that calcium influx during low-threshold calcium bursts is tightly coupled to calcium-dependent afterhyperpolarizing currents (Avanzini et al., 1989; Bal and McCormick, 1993) which repolarize sufficiently the membrane potential to allow for fast recovery of inactivation of I_T (Bal and McCormick, 1993).

In comparison to TC neurons, nRt cells show a qualitatively similar, yet biophysically distinct intrinsic propensity to discharge in bursts. Due to the equipment with a peculiar set of intrinsic conductances, this intrinsic bursting is facilitated to occur at fairly depolarized potentials, and usually entrains the generation of at least two or three more rebound bursts. These pronounced discharge properties of nRt cells make them uniquely suited to act as pacemaker cells for the initiation of rhythmic activity driven by GABAergic synaptic inhibition.

b. Synaptic connectivities within the nucleus reticularis

Connection by chemical synapses

The main axon of 65% of reconstructed nRt neurons gives rise to intra-reticular collaterals before running into the dorsal thalamus of young rats (Cox et al., 1996), but in adult rats no intra-reticular axon collaterals has been found so far in other studies (Pinault et al., 1995a, b). If they exist, the intra-reticular collaterals may form GABAergic synapses on the proximal and distal dendrites of the nRt neurons (Liu and Jones, 1999). Moreover, intra-reticular inhibition could also be mediated through dendro-dendritic synaptic contacts that have been described on nRt neurons (Deschênes et al., 1985; Pinault et al., 1997). Accordingly, local stimulation of nRt neurons results in intra-reticular inhibition mediated by both $GABA_A$ receptors and

$GABA_B$ receptors (Bal et al., 1995b; Ulrich and Huguenard, 1996; Sanchez-Vives et al., 1997; Shu and McCormick, 2002).

Intra-reticular inhibition prevents the occurrence of hypersynchrony in network activities of the TC system. Disinhibition of nRt cells via local application of bicuculline *in vitro* (Bal et al., 1995b; Jacobsen et al., 2001) resulted in a transformation of sleep-related, weakly synchronized oscillations into a hypersynchronous activity typical for generalized epilepsies (McCormick and Contreras, 2001). The $GABA_A$ receptors that mediate intra-reticular inhibition contain the β_3 subunits (Wisden et al., 1992). Mice in which the β_3 subunit was genetically removed were epileptic and showed 3 Hz oscillations in the EEG that were abolished by administration of ethosuximide (DeLorey et al., 1998). Moreover, *in vitro*, hypersynchronous multi-unit activities were observed in the majority of the thalamus of knock-out mice. Therefore, both pharmacological and genetic evidence strongly supports a crucial role of nRt neurons and their reciprocal inhibition in both the generation and the limitation of synchronized oscillatory activity (Huntsman et al., 1999).

Accordingly, an increase of intra-reticular inhibition prevents synchronous oscillations. The benzodiazepines stopped oscillations elicited by stimulation in the internal capsule in wild-type mice (Sohal et al., 2003). In mice expressing a genetically engineered BZ-insensitive α_1 subunits, application of clonazepam (a BZ) suppressed evoked oscillations, whereas in mice expressing the BZ-insensitive α_3 subunit, application of clonazepam had no effect. Subunits of the α_1-type are expressed in TC neurons while nRt neurons express α_3 subunits (Wisden et al., 1992). Therefore, the BZ effect was mediated by the potentiation of $GABA_A$ inhibition within the nRt (Sohal et al., 2003). The intra-reticular inhibition may reduce the number of bursts in an oscillation by reducing the excitability of the cells. Through this way, it limits the synchronization of the discharges in nRt cells and TC cells (Sohal and Huguenard, 2003).

Connection by electrical synapses

Based on the detection of tight bundling of dendritic trees (Scheibel and Scheibel, 1966; Ohara and Lieberman, 1985; Liu and Jones, 1999) and the presence of dendritic spikes (Contreras et al., 1993), it has long been suspected that nRt neurons

may communicate via both axodendritic and dendrodendritic electrical contacts and thus form a densely connected electrical network with the capacity for strong synchronization. Moreover, connexin 36 is expressed in nRt (Deans et al., 2001; Liu and Jones, 2003), indicating the probable formation of gap junctions between nRt neurons. Indeed, dual recordings from closely apposed nRt cells reveal that the majority of nRt neurons are electrically coupled (Landisman et al., 2002). The electric synapses in nRt have the characteristics of a low-pass filter: the high-frequency AP discharges in one cell are more attenuated by electric coupling than the lower frequency discharges. There is almost no attenuation for spike discharge frequencies inferior to 10 Hz while 100 Hz spike discharges are attenuated by ~75% (Landisman et al., 2002). The electric coupling between two nRt cells is also particularly efficient to transfer charge movement induced by injection of negative current or by induction of rebound bursts in one cell. The gap junctions between nRt neurons are almost entirely dependent on connexin 36 since in animals lacking this protein, apposed nRt neurons do not display any electric coupling (Landisman et al., 2002). The nRt is composed of relatively small clusters of electrically coupled cells that can synchronize rhythmic activity over relatively short distances (40 μm) (Landisman et al., 2002) whereas synaptic GABA inhibition occurs via long-range connections (until 300 μm) (Sohal et al., 2000). Electric coupling may thus serve to locally synchronize small clusters of neighbouring neurons during thalamic synchronized oscillations, such as during spindle waves in slow-wave sleep (Long et al., 2004).

c. Thalamocortical and corticothalamic inputs

The neurons of the nRt are driven by two types of excitatory inputs, one arising from TC collaterals, the other from CT collaterals. Thalamocortical inputs into nRt cells have been well characterized *in vitro* studies on slice preparations maintaining the connectivity between TC and nRt neurons. The activation of the TC neurons by local application of glutamate or by electrical stimulation induces EPSPs in the nRt (Bal et al., 1995b). In paired recordings, the unitary EPSPs elicited by APs in TC neurons are found to summate sublinearly during both tonic and burst firing, suggesting the absence of a boosting effect of high-frequency discharge on the functional connection between TC and nRt neurons (Kim and McCormick, 1998; Gentet and Ulrich, 2003). Nevertheless, the summated EPSPs generated by one

rebound burst in a TC neuron induce bursts in a nRt neuron (Kim and McCormick, 1998), although the convergence of TC inputs during synchronized TC rhythms, such as spindle waves, greatly increases the reliability of burst generation in nRt cells (Kim and McCormick, 1998). The TC-nRt EPSPs are mediated predominantly by AMPA receptors, but also show a NMDA receptor-mediated component at resting membrane potentials (Bal et al., 1995b; Jacobsen et al., 2001; Gentet and Ulrich, 2003), consistent with the expression of weakly Mg^{2+}-sensitive NMDA receptor subunits in nRt cells (Wenzel et al., 1995; Wenzel et al., 1997).

The number of CT synapses far exceeds that attained by TC projections (Liu and Jones, 1999). Moreover, CT projections innervate both nRt and TC neurons, yet CT synapses show a four-fold higher expression of AMPA receptor subunits in the postsynaptic membrane of reticular neurons, suggesting both a numerical and physiological dominance of cortical effects on nRt neurons (Golshani et al., 2001). Accordingly minimal CT fiber stimulation elicits EPSCs with amplitudes up to three-fold greater than the EPSCs amplitude evoked at CT-TC synapses. Like the TC-nRt connections, activation of CT fibers produces not only EPSCs but also di/poly-synaptic IPSCs due to activation of neighbouring nRt neurons (Zhang and Jones, 2004). Via this powerful influence on nRt neurons, it is perhaps not surprising that cortical inputs to the thalamus is essentially involved in the synchronization of thalamic oscillations (Contreras et al., 1996; Bal et al., 2000; Blumenfeld and McCormick, 2000) and in the sharpening of receptive fields (Alitto and Usrey, 2003).

d. Inhibition of thalamocortical neurons by the nucleus reticularis neurons

The output projections from the nRt are limited to the thalamic nuclei, including both first-order and HOn. Therefore, nRt and TC neurons form reciprocally connected loops in which consist the basic cellular network able to self-sustain synchronized network activity that contributes to both sleep and epileptic rhythmic waves characteristically observed in EEG recordings (Steriade et al., 1993; Steriade et al., 1994; McCormick and Bal, 1997; Siegel, 2004). An *in vitro* ferret preparation, which preserves the ability of thalamic circuit to produce the spindle waves, has been used to detailed the biophysical mechanisms for their generation in the thalamus (von Krosigk et al., 1993). Activation of excitatory inputs from TC or CT tracts induce bursts in nRt cells that lead to a barrage of IPSPs in thalamic neurons. The resulting

hyperpolarization of the membrane of TC cells permits the removal of inactivation of I_T and the repolarization of membrane potential can be followed by a rebound calcium spike associated with bursts of APs. The thalamic bursts induce a barrage of EPSPs in reticular neurons that de-inactivates I_T, allowing the generation of bursts and then a new cycle can start again (von Krosigk et al., 1993; Bal et al., 1995a,b; McCormick and Bal, 1997). The frequency of the spindle waves (6-15 Hz) is chiefly determined by the duration of the IPSPs in TC neurons (Bal et al., 1995a,b). Moreover, nRt-mediated inhibition of TC neurons during states of waking acts in a feedB manner to control the gain of information transfer from the periphery to the cortex (Le Masson et al., 2002).

The extracellular stimulation of the nRt in horizontal rodent thalamic slices generally evokes biphasic inhibitory responses in the TC neurons (Huguenard and Prince, 1994a; Warren et al., 1994; Warren et al., 1997) with both $GABA_A$ and $GABA_B$ receptor-mediated components. At reticulothalamic synapses, activation of the $GABA_B$ receptor component could be observed at similar stimulation intensity as the $GABA_A$ component (Huguenard and Prince, 1994a). Paired recordings of connected nRt neurons and TC neurons allowed studying the properties of unitary nRt-TC connections (Cox et al., 1997; Kim and McCormick, 1998). Unitary IPSCs evoked in TC neurons by an AP in nRt neurons showed small amplitude (20 pA in average) and high failure rate (~60%) in few neurons (3 out of eight). Bursts of APs in nRt neurons reduced the failure rates (a three folds decrease) but did not alter the response properties. The strong pairs (5 out of 8 pairs) showed large amplitudes (190 pA in average) and no failure. Bursts in presynaptic nRt neurons strongly amplified the response (increase by 270% of the control charge current). The difference between the two synapse subtypes may arise from morphological differences as the weak and the strong synapses were estimated to present 1-3 release sites against 5-70 release sites, respectively (Cox et al., 1997). The $GABA_B$ receptor-mediated responses are observed whether the nRt cell produced prolonged burst discharges (Kim and McCormick, 1998), indicating that nRt neurons can give rise to unitary $GABA_B$-mediated responses in response to prolonged bursts. In contrast to TC-nRt connections, IPSPs generated by nRt cells show pronounced facilitation at higher frequencies (Kim and McCormick, 1998), indicating that the impact of nRt burst discharges is further strengthened by the presynaptic properties of nRt synapses.

Modelling of inhibitory feedB has shown that the strength of feedB inhibition decreases the reliability between sensory input and the TC neurons discharge (Le Masson et al., 2002). When TC neurons were hyperpolarized as during sleep, the correlation between the arrival of sensory inputs and discharges was negatively correlated with the strength of feedback inhibition. During arousal, or *in vitro* after application of noradrenaline, the TC neurons were more depolarized and the efficiency of sensory inputs to trigger spikes was increased even in the presence of strong feedB (Le Masson et al., 2002), indicating that nRt neurons can control the amount of information that can be transmitted by TC neurons to the cortex.

4. GABAergic signaling in thalamus II: Local interneurons

a. Electrophysiological properties of local interneurons

A large variety of data on the electric properties of the interneurons have been reported. Some studies showed a higher input resistance and a longer membrane conductance of the interneurons compared to TC neurons (McCormick and Pape, 1988; Zhu et al., 1999b). Indeed, the input resistance was ~500 MΩ and the membrane time constant was ~100 ms for rat interneurons and the values are 100 MΩ and 25 ms for the TC neurons in the data presented by Zhu and colleagues (Zhu et al., 1999a). Some studies showed a shorter time constant (36.8 for the interneurons and 58.2 ms for TC) (Williams et al., 1996) and a similar cell resistance (Pape and McCormick, 1995; Williams et al., 1996), 80 and 160 MΩ, respectively. The discrepancies of the data were not commented but they were not due to species differences, as Williams and Zhu used both rats.

The presence of I_T (Pape et al., 1994) allows the interneurons to generate LTS with a duration of 100-300 ms (Pape and McCormick, 1995). Depolarization of the cell membrane induces several bursts of spikes at a frequency between 25 and 150 Hz (Zhu et al., 1999b). In a previous paper, the interneurons produced high-frequency tonic firing upon depolarization of the cell (Pape and McCormick, 1995) and no burst activity. A high resistance (500 MΩ in Zhu's study against 80 MΩ in Pape's study) of the membrane of the recorded interneurons seemed to be required to observe oscillatory discharges (Zhu et al., 1999b).

A passive model of interneurons, considering the passive and morphological properties of the soma and dendrites of interneurons, has concluded that the dendritic tree and the soma with the proximal dendrites form two isolated compartments, and only the two last may influence the firing pattern of interneurons (Bloomfield and Sherman, 1989). Conversely, distal dendritic compartments may function as independent local processors in dendro-dendritic communication, as a simulated depolarization of the distal dendrites may be not detected in soma (Bloomfield and Sherman, 1989). Moreover, (±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid (ACPD), an agonist of metabotropic glutamate receptors, increased the release of GABA at the dendro-dendritic synapses, while ACPD did not alter the membrane potential recorded at the soma (Pape and McCormick, 1995; Cox et al., 1998), supporting the conclusion of the cell modelling study.

b. Inhibition of thalamocortical neurons by local interneurons

A possible role of interneurons in thalamic function was provided early by *in vivo* stimulation of prethalamic afferents. In cat, activation of either optic nerve or the mammillothalamic tract elicited an EPSP followed by a Cl^- mediated-IPSP with a latency short enough to propose a disynaptic feedF inhibition (Lindstrom, 1982; Paré et al., 1991). Similarly, in the rat deprived of nRt, activation of interneurons by retinal tract stimulations first evoked a fast EPSP followed by a disynaptic IPSP consisting of a $GABA_A$ and a $GABA_B$ component (Crunelli et al., 1988). In anterior thalamic nucleus, it was even possible to distinguish a triphasic IPSP with a first, fast $GABA_A$ component mediated via the dendro-dendritic synapses of the glomerulus, and the second and the third components is produced by both $GABA_A$ and $GABA_B$ receptors via the axo-dendritic synapses (Paré et al., 1991).

A second putative mechanism of inhibition by the interneurons or by the nRt is the calcium-dependent presynaptic inhibition of the retinogeniculate synapses by activation of $GABA_B$ receptor, presumably by spillover of GABA from the interneurons-TC synapses or reticulothalamic synapses (Chen and Regehr, 2003).

PAPER 1: Pacemaker channels in mouse thalamocortical neurons are regulated by distinct pathways of cAMP synthesis

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I Introduction to the paper 1

Cyclic AMP (cAMP) is a prototypical second messenger that mediates the action of extracellular signals, such as hormones and neurotransmitters, to intracellular physiological responses, such as electrical activity and gene expression (Zagotta and Siegelbaum, 1996; Antoni, 2000; Hanoune and Defer, 2001; Kaupp and Seifert, 2002). In the central nervous system, cAMP is involved in cellular and synaptic processes related to essential neural functions, such as sleeping, waking, learning and memory (Silva et al., 1998; Mayford and Kandel, 1999; Hanoune and Defer, 2001).

The regulation of cyclic nucleotides plays a particularly important role in thalamic functions. The thalamocortical (TC) neurons possess a potent subcellular machinery controlling intracellular cAMP levels ($[cAMP]_i$) that contribute to induce the transitions between the burst and the tonic firing mode (McCormick, 1992; McCormick and Bal, 1997). Thus, these cells express numerous neurotransmitter receptors that are activated at the transition from the sleeping to the waking state (Pape and McCormick, 1989; McCormick and Pape, 1990b; McCormick and Williamson, 1991; Lee and McCormick, 1996). Activated receptors are positively coupled to an increase in $[cAMP]_i$, probably via G_s-protein-mediated activation of adenylyl cyclases (ACs) (McCormick and Pape, 1990b; McCormick and Williamson, 1991; Lee and McCormick, 1996). Conversely, receptors coupled negatively to cAMP synthesis via G_i-protein-mediated inhibition of ACs may promote the burst discharge mode of thalamic cells, thereby contributing to the generation of sleep-related oscillations (Pape, 1992).

During non-rapid-eye-movement sleep, but also during some types of epilepsy, the TC system generates a number of slow, synchronized electrical oscillations, which are responsible for the electroencephalographic (EEG) patterns used to characterize sleep and epilepsy in clinical applications. The TC neurons are part of the pacemaker circuit that underlies the generation of some of these oscillations, including sleep 'spindle waves'. These are epochs of 1-3 sec periods of 7-14 Hz network activity that appear at regular intervals of 5-30 sec (McCormick and Bal, 1997). A major factor for the slow periodicity of spindle waves may be an activity-dependent increase in $[cAMP]_i$ in the TC neurons. Increased $[cAMP]_i$ modulates the intrinsic electrical properties of these cells in such a manner that their ability to participate in the

oscillations is decreased for a period that is equivalent to the silent phase between the spindle waves (Bal and McCormick, 1996; Lüthi and McCormick, 1999). The spindles oscillations waxe and wane. *In vitro*, The waxing of the oscillations is due to an progressive increase in the recruitment of neurons that participate to the oscillation. The waning is underlaid by depolarization of the thalamic neurons that promotes the inactivation of T-current. The depolarization responsible for the waning of the oscillations could be triggered by the upregulation of the h-current (Bal and McCormick, 1996) via an increase of intracellular calcium level during the burst I_h (Lüthi and McCormick, 1998a). During spindle oscillations, rhythmic influx of calcium may regulate a Ca^{2+}-sensitive adenylyl cyclase and the resulting enhancement of cAMP levels is responsible for the persistent activation of I_h (Lüthi and McCormick, 1999). The persistence of I_h activation is also determined by the slow rate of cAMP dissociation from the CNBD at the cytoplasmic tail of the channel (Wang et al., 2002). Therefore, subcellular oscillations in intracellular messenger levels appear to contribute to the timing of rhythmic behaviors in sleep, and, perhaps, also during epilepsy.

In spite of this important role of cAMP signaling in thalamus, little is known about how thalamic $GABA_B$ receptors control cAMP turnover. Given that $GABA_B$ receptors could be involved in thalamic oscillations (Jacobsen et al., 2001), it is crucial to understand how synaptically activated receptors control cAMP synthesis, and therefore, perhaps, the timing of large-scale synchronized oscillations. In this study, we therefore aimed to characterize $GABA_B$ receptor-mediated control of cAMP synthesis, taking advantage of the fact that these cells strongly express an ionic current, the pacemaker current I_h, that can be recorded with electrophysiological techniques (McCormick and Pape, 1990a). The ionic channels underlying this current are gated directly by $[cAMP]_i$, therefore, cAMP acts as a 'first messenger' for these ionic channels (DiFrancesco and Tortora, 1991; Wainger et al., 2001). The molecular subunits constituting the pacemaker channels in thalamus show the highest sensitivity to $[cAMP]_i$ amongst the four subunits identified in the brain so far (Kaupp and Seifert, 2001; Robinson and Siegelbaum, 2003). On the other hand, I_h appears to exhibit little susceptibility to direct regulation by other intracellular factors, such as Ca^{2+}, kinases or phosphatases (Pedarzani and Storm, 1995; Accili et al., 1997; Budde et al., 1997; Larkman and Kelly, 1997). Therefore, pacemaker channels are built-in, selective reporters of submembraneous $[cAMP]_i$ produced by the membrane-bound ACs. Using

I_h recordings, we were able to continuously read-out of $[cAMP]_i$ in these cells and to quantify AC activity under physiological conditions.

1. $GABA_B$ receptor signaling in the thalamus

In situ hybridization (Liang et al., 2000) and immunochemistry studies have shown that $GABA_B$ receptors were highly expressed in the thalamus (Fritschy et al., 1999; Margeta-Mitrovic et al., 1999; Princivalle et al., 2000; Princivalle et al., 2001; Kulik et al., 2002). The expression of $GABA_B$ receptors in TC neurons was already maximal at the age of two weeks in the rat (Princivalle et al., 2000). In the ventrobasal thalamus (VB), $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits expression overlapped in neurons (Princivalle et al., 2001; Kulik et al., 2002). In the dendrites, the $GABA_{B(1a/b)}$ and $GABA_{B(2)}$ subunits were found extrasynaptically both around the GABAergic synapses and around non-GABAergic synapses (presumably corticothalamic (CT) synapses) (Kulik et al., 2002). One study suggested that $GABA_{B(1a)}$ subunits might be present at postsynaptic sites on cell bodies (Princivalle et al., 2001).

a. The effects mediated by $GABA_B$ receptors in the thalamus

Activation of $GABA_B$ receptors by the reticular neurons

The activation of $GABA_B$ receptors of TC neurons required long burst discharges in the connected nRt neurons (Kim and McCormick, 1998), which were observed when disinhibition between nRt neurons was induced pharmacologically (Sanchez-Vives et al., 1997) or genetically (Huntsman et al., 1999).

In the VB nucleus of the thalamus, blocking $GABA_B$ receptors with saclofen led to a 2.3-fold increase in the average receptive field size of VB neurons (Lee et al., 1994). As the nRt neurons are the only source of GABA in VB nucleus (Arcelli et al., 1997), this result suggests that $GABA_B$ receptors at the reticulothalamic synapses are able to control the responsiveness of TC neurons to sensory stimulus and therefore are able to control the transmission of sensory signals to the cortex.

Additional information about the role of $GABA_B$ receptors in the reticulothalamic connections was detailed in the general introduction (see chapter II, 3d).

Activation of $GABA_B$ receptors by the local interneurons

$GABA_B$ receptors activated synaptically at the GABAergic synapses formed between interneurons and TC neurons induced a hyperpolarization of the cell. In the lateral geniculate nucleus, activation of the optic tract induced a tri-phasic response (Paré et al., 1991). The first was excitatory, the second was a fast hyperpolarization and the last was a slow hyperpolarization. The potassium conductance underlying the last response was similar to the hyperpolarization induced by baclofen (Bac). Moreover, the slow hyperpolarization was insensitive to bicuculline but blocked by phaclofen, suggesting a $GABA_B$ receptor-dependent action (Hirsch and Burnod, 1987; Crunelli et al., 1988; Soltesz et al., 1988). This response was due to the excitation of the interneurons by the optic tract stimulation (Crunelli et al., 1988).

b. Activation of $GABA_B$ receptors during thalamocortical synchronization

Activation of $GABA_B$ receptors during spindle oscillations

Both $GABA_A$ and $GABA_B$ receptors contribute to the spontaneous generation of sleep-related oscillations, but their relative contributions vary dependent upon the species investigated. In ferrets, application of $GABA_B$ receptor antagonists *in vitro* only weakly modulated the frequency and amplitude of spindle oscillations (von Krosigk et al., 1993; Blumenfeld and McCormick, 2000). However, in rat slices, spindle-like oscillations that were produced in a pro-oscillatory milieu were sensitive to blockade of $GABA_B$ receptors (Jacobsen et al., 2001). The $GABA_B$ receptor-mediated hyperpolarization display slow kinetics and prolonged duration (150-300 ms) and thus may increase the time to complete a loop of activity between thalamic reticular cells and thalamocortical neurons from ~70-150 ms for 7-14 Hz sleep-associated spindle oscillations to ~300-400 ms for 3-5 Hz absence seizure-related oscillations (McCormick and Contreras, 2001).

Activation of $GABA_B$ receptors during absence seizures oscillations

Definition and characterization: Epilepsy syndromes fall into two broad categories: generalized and partial (or localization-related) syndromes. In generalized epilepsies, the seizures seem to begin simultaneously in both cerebral hemispheres and generally lead to a loss of consciousness. In partial epilepsies, by contrast, seizures originate in one or more localized foci, although they can spread to involve the entire brain.

Childhood absence epilepsy or petit mal epilepsy is a generalized epilepsy syndrome that begins between the ages of four and eight years with absence seizures.

During absence seizures, patients stare and cease normal activity for a few seconds with no convulsion, then return immediately to normal and have no memory of the event. These seizures can occur tens or hundreds of times a day. There is a classic EEG pattern of three-per-second, generalized spike-and-wave discharges (SWDs) in childhood absence epilepsy. The SWDs are composed of strong burst discharges during spikes combined with inhibition-induced silencing during waves. Absences usually appear at times of transition to or from sleep, in quiet wakefulness and in light non-REM sleep (Futatsugi and Riviello, 1998; McCormick and Contreras, 2001), suggesting that they represent a paroxysmal development of naturally occurring sleep-related oscillations occurring during these states. These 3 Hz frequency waves are recorded bilaterally in TC system of epileptic children during absence seizures (Williams, 1953). The anatomical origin of SWDs is a subject of continuous debate.

Anatomical origin of the SWDs associated to absence epilepsy: In Denis Williams's study (1953), the 3 Hz SWDs were found to start first in the thalamus and Williams concluded that "the clinical state of petit mal epilepsy is due to a disturbance in the thalamus which causes a rhythmic discharge throughout the cortex" (Williams, 1953). In addition, electrical stimulation of the thalamus in cats produced bilaterally synchronous EEG discharges that resembled the classic absence pattern (Jasper and Droogleever-Fortuyn, 1947). Thus, the thalamus was considered as the centre for the generation of absence seizures.

In contrast, penicillin, a weak $GABA_A$ antagonist, was able to induce a transformation of EEG spindles to SWDs when applied within the cat cortex (Kostopoulos et al., 1981). Penicillin did not have such effect when it was applied in the thalamus (Futatsugi and Riviello, 1998). The penicillin-induced SWD in cat was considered as a good model of absence seizures as the SWD EEG pattern was accompanied with some behavioural and pharmacological features typical for absence seizures. This model is named the Feline Generalized Penicillin-induced Epilepsy or FGPE (Kostopoulos, 2000). The development of generalized SWDs requires the presence of both a functional thalamus and a functional cortex, as evident from studies in the cat with an anatomical or functional decortication or thalamectomy, leading to no SWD generation in response to penicillin injection (Kostopoulos, 2000). Thus, the mechanism that generates absence seizures is now believed to involve the circuitry between the thalamus and the cerebral cortex. Today, it seems probable that

the increase of discharge synchrony, underlying absence seizures, starts within the cortex that secondarily recruits the thalamus.

In vivo, during spontaneous SWDs arising in the unanaesthetised WAG/Rij rats, a model of absence epilepsy (Coenen et al., 1992), multi-recordings in the cortex and the thalamus showed that the SWDs started in the somatosensory cortex and then spread into other areas of the cortex and then in the thalamus. During the first 500 ms of the SWDs, the cortex led and recruited the thalamus. After this period, the cortex and the thalamus influenced each other to increase the synchronization process (Meeren et al., 2002). In the genetic absence epilepsy rats of Strasbourg (GAERS), an other model of absence epilepsy (Marescaux et al., 1992), SWD-related discharges of TC neurons and nRt neurons occurred almost synchronously, but, again, they began ~7-8 ms after the CT SWD-related discharges (Pinault, 2003). Similarly, in anaesthetised cats, multisite recordings in the cortex and the thalamus during 3 Hz SWD-like oscillations showed that paroxysmal events were first observed in the cortex and then in the dorsal thalamus (Steriade and Contreras, 1995). The SWDs were associated with an enhanced duration of the bursts of the nRt neurons that exerted strong inhibitory drive in TC neurons. Thus, 60% of the TC neurons were hyperpolarized and silent during SWDs and that may explain the lost of consciousness associated with absence seizures (Steriade and Contreras, 1995). However, this was not confirmed in GAERS, where TC neurons kept on discharging in bursts after a phasic inhibition at each cycle of the SWDs (Pinault, 2003).

Two i*n vitro* studies in ferret showed that the frequency of oscillations generated in the thalamic network was dependent upon the strength of the CT feedback inputs (Bal et al., 2000; Blumenfeld and McCormick, 2000) in such a manner that high cortical discharge preferentially induced slow 3 Hz thalamic oscillations.

Therefore, both *in vivo* and *in vitro* evidence points towards an essential role of the cortex in the initiation of SWDs, whereas the maintenance and large-scale synchronization is also dependent upon thalamus. Similarly to spindle waves, the synchronization of TC neuron discharges within the thalamus is thought to depend on nRt neurons (Steriade and Contreras, 1995; McCormick and Contreras, 2001) and the activation of TC $GABA_B$ receptors in response to nRt neuron activity may play a role in this process.

In vivo studies of absence seizures and the involvement of $GABA_B$ receptors: In previous studies, the role of the $GABA_B$ receptors has been suggested by *in vivo* experiments. In GAERS and in the lethargic mice, a mouse model of absence seizures (Hosford et al., 1992; Hosford et al., 1995), microinjection in the thalamus or systemic injection of $GABA_B$ receptor agonists exacerbated absence seizures (Hosford et al., 1992; Liu et al., 1992; Hosford et al., 1995). In contrast, injection of $GABA_B$ receptor antagonists prevented them (Hosford et al., 1992; Liu et al., 1992; Vergnes et al., 1997). The alteration of SWDs by $GABA_B$ receptor modulators was a sign of the implication of $GABA_B$ receptors in the generation of SWDs. Nevertheless, the hypothesis of $GABA_B$ receptor implication had been refuted by other *in vivo* studies.

In the $GABA_{B(1)}$ knock-out mice, despite the lack of functional $GABA_B$ receptors, long (10 s) 3-5 Hz oscillations were observed in few mice nevertheless the oscillations were rare (less than once per day) and they were considered as atypical compare to GAERS absence-seizures oscillations (Schuler et al., 2001). Stronger evidence came from *in vivo* intracellular recordings of GAERS TC neurons during spontaneous SWDs, they did not show the presence of significant inhibitory potentials mediated by $GABA_B$ receptor but uniquely by $GABA_A$ receptor (Pinault et al., 1998). Moreover, in the WAG/Rij model, the application of CGP 55845, an antagonist of $GABA_B$ receptors, failed to change the discharges pattern of the TC neurons during 5-9 Hz SWDs (Staak and Pape, 2001). Nevertheless, CGP 55845 antagonized the effect of bicuculline application, which transformed the 5-9 Hz SWDs into 3 Hz SWDs (Staak and Pape, 2001). Therefore, the $GABA_B$ receptors seem not to be activated during spontaneous SWDs occurring in two well-accepted rat models of absence seizure but $GABA_B$ receptor-dependent inhibition is present during pharmacologically induced SWDs.

The generation of 3 Hz oscillations in the TC network has been developed in *in vitro* preparations. The *in vitro* model outcomes were in favor of a $GABA_B$ receptor role in the generation of bicuculline-induced 3 Hz oscillations.

Involvement of $GABA_B$ receptors derived from *in vitro* studies: In an *in vitro* model of ferret slices, the application of bicuculline in the nRt induced thalamic 3 Hz oscillations. These oscillations strongly resembled *in vivo* recordings of SWDs occurring during absence seizures. Importantly, application of $GABA_B$ receptor

antagonists abolished this seizure-like activity whereas block of these receptors had no effect on spindle activity (von Krosigk et al., 1993). When rendering the nRt network hyperexcitable by locally blocking $GABA_A$ receptors with bicuculline, $GABA_B$ receptor-mediated potentials in TC neurons could be evoked (Bal et al., 1995b; Kim et al., 1997; Sanchez-Vives et al., 1997). It was therefore proposed that the lack of intra-reticular inhibition allowed a cooperative activation of $GABA_B$ receptors in TC neurons (Sohal and Huguenard, 2003), contributing to hypersynchronous oscillations. Following the near complete block of $GABA_A$ receptors, the time to complete a loop of activity between thalamic nRt cells and TC neurons lengthened to ~300-400 ms, and therefore the network generated a rhythmic oscillation at ~2-3 Hz (von Krosigk et al., 1993). The role of $GABA_B$ receptor-mediated responses in the generation of absence epilepsy has also been suggested by showing that two anti-absence drugs affected $GABA_B$ receptor-mediated responses. Indeed, BZs and ethosuximide reduced the probability of burst discharges in nRt neurons, thereby indirectly provoking a reduction of $GABA_B$ receptor-mediated responses in rat TC neurons (Huguenard and Prince, 1994a, b).

The increase in the CT feedback strengths has been proposed to be the factor that increases nRt neuron activity and leads to activation of $GABA_B$ receptors in TC neurons (Destexhe, 1998; Bal et al., 2000; Blumenfeld and McCormick, 2000).

2. Regulation of cAMP synthesis by $GABA_B$ receptor signaling

The inhibition of cAMP synthesis by $GABA_B$ receptor activation is well known (Wojcik and Neff, 1984; Xu and Wojcik, 1986; Gerber and Gähwiler, 1994), nevertheless, $GABA_B$ receptor can have, surprisingly, a stimulatory action on cAMP production. Indeed, it has been shown that $GABA_B$ receptor stimulation enhances the production of cAMP induced by the activation of the G_s-coupled receptors. The negative and the positive effects of $GABA_B$ receptors on $[cAMP]_i$ were described the same year in 1984 by two different groups (Karbon et al., 1984; Wojcik and Neff, 1984). The inhibition of cAMP synthesis will be shortly presented while the unusual promoting action of $GABA_B$ receptors will be explained in greater detail.

a. Negative effect of $GABA_B$ receptors on cAMP levels

Biochemical evidences of the inhibitory effect of $GABA_B$ receptors on cAMP levels

In primary cultures of cerebellar granule cells, GABA and Bac inhibited the activity of ACs (Wojcik and Neff, 1984). A similar effect was found in rat striatum (Hashimoto and Kuriyama, 1997). In rat spinal cord slices, GABA and Bac also inhibited cAMP formation induced by forskolin, a direct activator of all the AC isoforms (Malcangio and Bowery, 1993). The effect has also been reconstituted by co-expression of purified G_i-proteins, receptors and ACs in artificial membranes (Nishikawa et al., 1997), suggesting that these three components might be necessary and sufficient for the effect observed in native membranes.

Mechanisms

The inhibition of cAMP production appears to require only the presence of $GABA_{B(2)}$ receptor subunits. Indeed, the activation of recombinant $GABA_{B(2)}$ subunit-composed receptors, in HEK293 or COS cells respectively, was sufficient to inhibit the forskolin-induced increase of $[cAMP]_i$ (Kuner et al., 1999; Martin et al., 1999). Moreover, in HEK293 cells, activation of the recombinant heterodimeric receptors did not change this effect. In contrast, the opening of potassium channels required the co-expression of the two subunits in the cells (Kuner et al., 1999). Further experiments will be required to explain these observations.

The inhibitory effect of $GABA_B$ receptor activation is sensitive to pertussis toxin showing the mediation via $G_{i/o}$ proteins (Xu and Wojcik, 1986). The inhibition of ACs by G_i-protein is due to the release of the α_i subunit. α_i subunit inhibits at least AC I, III, V and VI isoforms. In contrast, the subtypes II, VII, VIII are insensitive to the α_i subunit (Tang and Gilman, 1992; Anholt, 1994; Smit and Iyengar, 1998; Hanoune and Defer, 2001).

b. Positive effect of $GABA_B$ receptors on cAMP levels

Biochemical evidences of the positive effect of $GABA_B$ receptors on cAMP levels

A first study in 1984 showed that 100 µM Bac potentiated by 50-fold the increase of $[cAMP]_i$ induced by exposing cerebral cortical slices to a saturating concentration of noradrenaline (Karbon et al., 1984). This was confirmed by two studies where the authors also investigated the negative action of $GABA_B$ receptor

activation on $[cAMP]_i$. Thus, Bac had no or only a slight effect *per se* on $[cAMP]_i$, but it inhibited the increase of $[cAMP]_i$ induced by the application of forskolin (Hill, 1985; Knight and Bowery, 1996). Therefore, depending on the condition of activation of ACs, directly by forskolin or indirectly via G_s-coupled receptors, $GABA_B$ receptor activation mediates two opposite effects.

The synergistic action of $GABA_B$ receptors was not specific to β-adrenergic receptors but also strengthened the $[cAMP]_i$ increase provoked by the activation of vasoactive intestinal peptide, histamine, corticotrophin-releasing hormone (CRH) in cortex (Karbon and Enna, 1985; Watling and Bristow, 1986; Onali and Olianas, 2001). Similar observations were obtained with neuronal membranes of the olfactory bulb, hippocampus and hypothalamus (Watling and Bristow, 1986; Olianas and Onali, 1999). Phaclofen (a phosphonic derivate of Bac) is the first antagonist shown to antagonize the opening of potassium conductances by Bac (Dutar and Nicoll, 1988). Phaclofen also reduced the $GABA_B$ receptor-induced facilitation of the increase of $[cAMP]_i$ subsequent to β-adrenergic receptor activation, showing that Bac effect was mediated by $GABA_B$ receptors (Robinson et al., 1989). A second antagonist, CGP35348, had the same antagonistic property and blocked the potentiating effect of Bac on noradrenaline-induced stimulation of ACs in rat cortical slices (Olpe et al., 1990).

Mechanisms

Six different blockers were found to show similar potencies in antagonizing the two opposite actions of Bac, suggesting that they were mediated by pharmacologically indistinguishable receptors (Knight and Bowery, 1996).

The potentiating action of $GABA_B$ receptors is mediated by $G_{i/o}$ proteins as pertussis toxin blocks the action of Bac (Olianas and Onali, 1999; Onali and Olianas, 2001). Moreover, in cortex exposed to pertussis toxin injected intracerebroventricularly, the potentiating activity of $GABA_B$ receptors on the actions of isoproterenol (Iso), an agonist of the β-adrenergic receptor, was reduced. Therefore, this effect, as the other $GABA_B$ receptor actions, is likely mediated by G_i-proteins (Wojcik et al., 1989).

Downstream of the activation of G_i-proteins, several hypotheses have been proposed to explain the increase of cAMP production subsequent to the interaction between G_i-coupled receptors and G_s-coupled receptors.

-Increase of the coupling between the G_s-coupled receptor and the G_s-proteins: A study with cortical membranes preparation suggested that the promoting influence of $GABA_B$ receptors was mediated by an increase of the affinity between the β-adrenergic receptor and the G_s-protein (Scherer et al., 1989).

-Involvement of arachidonic acid synthesis: The potentiating effect of Bac was reduced by exposing cerebral cortical slices to EGTA, a chelator of divalent cations, or quinacrine, a nonselective inhibitor of phospholipase A_2 suggesting an involvement of calcium and phospholipase A_2 (Duman et al., 1986). Moreover, the potentiation by Bac was partially inhibited by blocking the 5-lipoxygenase, which metabolizes arachidonic acid, with the inhibitor nafazatrom (Schaad et al., 1989).

-Involvement of adenylyl cyclases: The first hypothesis that the ACs were the intermediate where cross-talked the $GABA_B$ receptors and the β-adrenergic receptors was put forward in 1985. In this study, it was shown that Bac increased the amplitude of Iso-induced $[cAMP]_i$ enhancements but it did not modify the time course of the $[cAMP]_i$ increase. This suggested that Bac did not interfere with the affinity between the β-adrenergic receptor and its agonist. Moreover, the $GABA_B$ receptor-induced potentiating effect was independent of the phosphodiesterase activity. Thus, the authors proposed that the 'cyclic-nucleotide generating system' could be the point where $GABA_B$ receptor and β-adrenergic receptor cascades interacted (Karbon and Enna, 1985). The description of the different subtypes of ACs and their specific profile of modulation supported this idea.

Nowadays, a profile of modulation that could explain most directly the synergistic action of $G_{i/o}$-coupled receptors upstream of cAMP synthesis is presented by two types of AC isoforms, type II and IV (and perhaps AC VII isoforms). The activity of these enzymes is dramatically enhanced upon binding of βγ subunits from G_i-proteins in the presence of α_s subunits released from G_s-complexes. Several nM of βγ subunits activate AC II, IV isoforms but only in the presence of α_s subunit (Gao

and Gilman, 1991; Tang and Gilman, 1991; Federman et al., 1992). Activation of G_s-proteins alone may not be sufficient to activate ACs by this process because of a relatively low abundance of G_s-proteins (Milligan et al., 1998). In contrary, $G_{i/o}$ proteins are around 10-fold more abundant in the cells, permitting the release of sufficient $\beta\gamma$ subunits to potentiate the action of α_s subunits (Tang and Gilman, 1991, 1992; Anholt, 1994; Smit and Iyengar, 1998; Hanoune and Defer, 2001). In *Xenopus* oocytes, in which G_i- and G_s -coupled receptors were co-expressed, the activation of G_i–coupled receptors enhanced the cAMP production elicited by the G_s-coupled receptor stimulation (Uezono et al., 1997; Ulens and Tytgat, 2001b). In the first study, it was observed that the positive action of Bac was dependent on the AC II isoform but not on AC III isoform (Uezono et al., 1997). The interaction between G_i-and G_s-proteins was also described in ventricular myocytes (Belevych et al., 2001), in neurons of olfactory bulb (Olianas and Onali, 1999) and cortex (Onali and Olianas, 2001). Indeed, in the three studies, blocking the interaction between $\beta\gamma$ subunits and α_s subunits impaired the promotion of cAMP synthesis by G_i-coupled receptors. The $\beta\gamma$ subunits were successfully inhibited with the GDP-bound form of the α subunit of transducin, a scavenger of G-protein $\beta\gamma$ subunits (Federman et al., 1992), or with a peptide (QEHA) that binds free G-protein $\beta\gamma$ subunits (Chen et al., 1995).

c. Effects of $GABA_B$ receptors on cAMP levels in native neurons

The inhibitory and the synergistic effects of $GABA_B$ receptors on $[cAMP]_i$ were also recognized in 'living' neurons where $GABA_B$ receptor actions target ionic channels or gene expression.

The calcium-dependent potassium current (I_{AHP}) mediates the afterhyperpolarization of hippocampal PCs where it entails spike frequency adaptation. This current is reduced by $[cAMP]_i$ (Pedarzani and Storm, 1993). In CA1 PC, Bac potentiated the ability of Iso, an agonist of the β-adrenergic receptor, to decrease I_{AHP} and thus to increase $[cAMP]_i$ (Andrade, 1993). Similarly to $GABA_B$ receptors, the enhancement of $[cAMP]_i$ by β-adrenergic receptors was also promoted by activation of α-adrenergic receptors (Pedarzani and Storm, 1996). In contrast, in CA3 PCc, Bac reduced $[cAMP]_i$ and led to an increase of the I_{AHP} but it inhibited the reduction of I_{AHP} mediated by Iso (Gerber and Gähwiler, 1994), indicating a

differential coupling of $GABA_B$ receptors in CA1 versus CA3 neurons. Thus, in CA1 neurons, G_i-coupled receptors interact positively with β-adrenergic receptors to promote the synthesis of cAMP but not in CA3 neurons.

In rat midbrain synaptosomes, Bac inhibited the cAMP/protein kinase A (PKA) pathway, which, in turn, enhanced the calcium current passing through ionotropic P2X receptor opened by ATP (Gomez-Villafuertes et al., 2003). The decrease in cAMP synthesis by Bac also prevented the inhibition of $GABA_A$ receptors by forskolin in rat cerebellar granule (Barila et al., 1999).

$GABA_B$ receptor-mediated control of cAMP turnover also affects the regulation of gene expression. In primary cultures of cerebellar granule neurons, forskolin increased the expression of the chloramphenicol acetyl transferase reporter (CAT), the transcription of which is under the control of the cAMP pathway through a sequence CRE (cAMP responsive element). Bac reduced the enhanced expression of CAT by forskolin, but not the increase of CAT expression induced by overexpression of the PKA showing that the effect of $GABA_B$ receptor activation is downstream to PKA (Barthel et al., 1996). This effect could also be explained by a direct interaction between $GABA_B$ receptors and the activating transcription factor type 4 or ATF4, a transcription factor that binds to the CRE sequence (Nehring et al., 2000).

II PAPER 1

Pacemaker channels in mouse thalamocortical neurones are regulated by distinct pathways of cAMP synthesis

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Key words: I_h, cAMP, β-adrenergic receptors, $GABA_B$ receptors, supralinear, thalamocortical

Summary

A crucial aspect of pacemaker current (I_h) function is the regulation by cyclic nucleotides. To assess the endogenous mechanisms controlling cAMP levels in the vicinity of pacemaker channels, I_h regulation by G-protein-coupled neurotransmitter receptors was studied in mouse thalamocortical neurons. Activation of β-adrenergic receptors with (-)-isoproterenol (Iso) led to a small steady enhancement of I_h amplitude, whereas activation of $GABA_B$ receptors with (±)-Baclofen (Bac) reduced I_h, consistent with an up- and downregulation of basal cAMP levels, respectively. In contrast, a transient (τ_{decay}~200 s), supralinear upregulation of I_h was observed upon co-application of Iso and Bac that was larger than that observed with Iso alone. This upregulation appeared to involve a cAMP synthesis pathway distinct from that recruited by Iso, as it was associated with a reversible acceleration in I_h activation kinetics and an occlusion of modulation by photolytically released cAMP, yet showed an 11 mV as opposed to a 6 mV positive shift in the activation curve and an at least seven-fold increase in duration. GABA, in the presence of the $GABA_A$ antagonist picrotoxin, mimicked, whereas N-ethylmaleimide, an inhibitor of G_i-proteins, blocked the upregulation, supporting a requirement for $GABA_B$ receptor activation in the potentiation. Activation of synaptic $GABA_B$ responses via stimulation of inhibitory afferents from the nucleus reticularis potentiated Iso-induced increments in I_h, suggesting that synaptically located receptors couple positively to cAMP synthesis induced by β-adrenergic receptors. These findings indicate that distinct pathways of cAMP synthesis target the pacemaker current and the recruitment of these may be controlled by GABAergic activity within thalamic networks.

Introduction

The autonomous beating of the heart and a considerable number of rhythmic activities in the brain are controlled by the hyperpolarization-activated cation currents I_h, also termed pacemaker currents (for review, see Pape, 1996; Lüthi & McCormick, 1998; Santoro & Tibbs, 1999; Robinson & Siegelbaum, 2003). Pacemaker channels are gated upon hyperpolarization and generate a depolarizing

drive back towards threshold, thereby facilitating the next rhythmic firing episode. Pacemaker currents are enhanced when intracellular concentrations of cAMP are increased (for review, see Santoro & Tibbs, 1999; Kaupp & Seifert, 2001; Robinson & Siegelbaum, 2003). The cAMP-mediated augmentation of cardiac I_h is crucial for the accelerating effects of sympathetic activity on the heartbeat (Brown *et al*., 1979). Moreover, cAMP-mediated enhancement of I_h in the brain contributes to control the slow periodicities in neuronal network activities related to sleep and epilepsy (Bal & McCormick, 1996; Lüthi & McCormick, 1999).

Pacemaker channels are composed of subunits from the family of the hyperpolarization-activated cation non-selective (HCN) channels. In addition to a voltage sensor contained within the six transmembrane segments, HCN channels possess a C-terminal cyclic nucleotide-binding domain (for review, see Santoro & Tibbs, 1999; Kaupp & Seifert, 2001; Wainger *et al*., 2001), which has a high selectivity for cAMP (Kaupp & Seifert, 2001). This modular structure provides the molecular basis for the dual gating of HCN channels by both voltage and cyclic nucleotides. Direct cAMP-dependent modulation constitutes a major regulatory pathway of native pacemaker channels as well, as evident from the similarity in the concentration-dependence and kinetics of cAMP-modulation to cloned channels (DiFrancesco & Tortora, 1991; Ludwig *et al*., 1998; Lüthi & McCormick, 1999; Seifert *et al*., 1999).

In spite of this important role of cAMP in the direct regulation of pacemaker channel function, little is known about the strength and the type of cAMP signals generated in the vicinity of pacemaker channels in intact cells. Here, we have addressed the diversity of cAMP signaling by studying the regulation of I_h in mouse thalamocortical (TC) cells, which shows a high sensitivity to cAMP (Lüthi & McCormick, 1999; Seifert *et al*., 1999). In TC neurons, multiple neurotransmitter receptor systems coupled both positively and negatively to cAMP synthesis (via G_s- and G_i-proteins, respectively) control the electrophysiological activities related to sleep and arousal (for review, see McCormick & Bal, 1997). We find that I_h is steadily up- and downregulated by neurotransmitter receptors coupled positively or negatively to cAMP synthesis, as described previously (for review, see Pape, 1996). However, the most vigorous modulation of I_h is observed upon co-activation of G_s- and $G_{i/o}$-coupled receptors, which produces a supralinear

cAMP signal. These data demonstrate that, in mouse TC neurons, I_h is modulated by several cAMP signals differing both in strength and time course. This suggests that the pacemaker channels may be surrounded by distinct cAMP synthesis pathways, perhaps incorporating distinct adenylyl cyclases (ACs), that are recruited according to the type and timing of neurotransmitter stimuli.

Methods

Slice preparation

Mice of either sex between 16-21 days were anesthetized by intraperitoneal injection of 90 mg/kg ketamine and 21 mg/kg xylazine and decapitated according to the Guidelines of the Veterinary Institute of the Canton Basel-Stadt. Coronal slices containing the dorsal lateral geniculate nucleus and the ventrobasal nucleus were prepared on a vibratome (VT1000S, Leica, Germany) in an ice-cold oxygenated solution containing (in mM): 63 NaCl, 107 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 18 dextrose. The slices were allowed to recover for 5 min in a home-made interface-type chamber at 35.0°C in the cutting solution, before being transferred to a sucrose-free solution containing 126 mM NaCl instead. After an additional 30 min, slices were incubated at room temperature for 1-2 h before recordings commenced.

Electrophysiological recordings

Whole-cell voltage-clamp recordings were obtained from visually identified TC neurons in the dorsal lateral geniculate and the ventrobasal nucleus of the thalamus (BX51WI microscope, Olympus, Germany) at 33.5-35.0°C. No difference in cAMP-dependent modulation of I_h was found for neurons in these two nuclei and the data were pooled. The bath solution contained (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.5 $CaCl_2$, 2 $MgCl_2$, 1.5 $BaCl_2$, 18 dextrose, 1.7 L(+)ascorbic acid. Unless stated otherwise, 1.5 mM Ba^{2+} ions were present to prevent activation of K^+ currents by (±)-Baclofen (Bac). In some experiments, tetrodotoxin (0.5 µM) was included to reduce spontaneous synaptic activity. Patch pipettes (2.5-3.5 MΩ, WPI, Sarasota, FL) were filled with (in mM): 130 KGluconate, 10 HEPES, 10 KCl, 2 K_2-ATP, 0.2 Na-GTP, 10 Phosphocreatine, 2 $MgCl_2$, pH 7.25, 290 mOsm. GTP was

freshly added daily from 100-fold concentrated stock solutions. This solution yielded a liquid junction potential of 12 mV that was taken into account for all voltages. Recordings yielded series resistances between 5-15 MΩ that were electronically compensated by 40-70% and the capacitive transient monitored in parallel with the I_h responses. When the series resistance was < 9 MΩ, no compensation was applied, but the stability of the capacitive transient checked before each voltage step. Data were discarded if the capacitive transient changed by >20% of the original amplitude.

The h-current was activated in whole-cell voltage-clamp mode by applying 5 s hyperpolarizing voltage commands from a holding potential of –62 mV to a test potential of –92 mV, the half-maximal activation voltage, at interstimulus intervals of 12 s. Recordings were selected when current amplitudes reached levels of at least -100 pA. This voltage protocol ensured that, on the one hand, >85% of the steady-state current amplitude at this potential could be activated and the current deactivated completely upon return to -62 mV. On the other hand, current amplitudes could be measured frequently enough to monitor the temporal development of current modulation. To follow the time course of transient upregulation of I_h induced by focal application of neurotransmitter agonists or by flash photolysis of caged cAMP, I_h activation was limited to 2.5 s.

Bath application of neurotransmitter receptor agonists was limited to one per experiment due to the incomplete wash-out of the effects on I_h. (-)-Isoproterenol (Iso) and 8-Bromo-cAMP (8Br-cAMP) solutions were prepared fresh daily from frozen stocks. Drugs were applied in the bath (4 ml/min) or via pressure application through a patch pipette placed in the vicinity of the cell recorded from. As the efficacy of the large number of $GABA_B$ antagonists on Bac-induced cAMP formation is characterized incompletely (Cunningham & Enna, 1996; Knight & Bowery, 1996), the action of antagonists on Bac effects or on those induced by synaptically activated $GABA_B$ receptors on the Iso-induced potentiation of I_h was not systematically evaluated. Data were collected through an Axopatch200B amplifier (Axon Instruments, Foster City, CA), digitized at 1 kHz, and analyzed off-line using PClamp8.0 software. Monoexponential time constants were analyzed by fitting the first 1.5 s of the I_h transient elicited at –92 mV ($V_{1/2}$) using the Chebychev fitting routine, while leaving away the initial lag in the onset of activation (~120 ms). Activation curves (Fig. 1, Fig. 3) were fit to the Boltzmann function, with $I/I_{max}= (1 +$

exp $[(V-V_{1/2})/s])^{-1}$, with $V_{1/2}$ the voltage for half-maximal activation, and s the slope factor. Normalization of tail current amplitudes was always done with respect to the maximal tail current observed under control conditions. Origin software (Version 4.1) was used for the fits to the data presented in Fig. 5. Data are presented as mean±S.E.M. Paired or unpaired t-tests as appropriate were used for statistical analysis and a value of $P<0.05$ was considered statistically significant.

Electrical stimulation of nucleus reticularis (nRt) afferents

Electrical stimulation of afferents from the nRt was achieved via bipolar tungsten electrodes (115 µm spacing, Frederick Haer & Co., Bowdoinham, ME) positioned within the nRt cell layer and exposed to constant current pulses (300-700 µA, 100 µs). To isolate $GABA_B$ receptor-mediated responses, the bathing solution contained DL-2-Amino-5-phosphonopentanoic acid (APV, 100 µM), 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX, 10 µM) to block glutamatergic receptors and picrotoxin (100 µM) to block $GABA_A$ receptors. In these experiments, Ba^{2+} was omitted from the extracellular solution until a synaptic $GABA_B$ response was identified.

Flash photolysis of caged cAMP

For flash photolysis, caged cAMP ((P^1-(2-Nitrophenyl)ethyl ester, 100 µM) was added to the patch solution from a 100-fold concentrated stock solution in dimethylsulfoxide. Flashes were applied via a UV-lamp attached to the epifluorescence pathway of the microscope and discharged via the capacitive discharges of the FlashMic (Rapp Optoelectronics, Germany), set at 4 V. Trains of 10 flashes at 10 Hz produced maximal responses and were used for the occlusion experiments (Fig. 4). UV-light applied to cells free of caged cAMP induced no change in I_h (n=2). The magnitude of the response to photolyzed cAMP was not significantly different for at least three flashes delivered at 1-2 min intervals (n=3, $P>0.05$), indicating that similar amounts of cAMP could be released at least three times within one cell.

Pharmacological agents used in this study were purchased from SIGMA (St. Louis, MO), except for caged cAMP (Calbiochem, Germany), tetrodotoxin (Latoxan, France), CGP54626 and NBQX (Tocris, UK).

Results

Regulation of I_h by G_s- and $G_{i/o}$-coupled neurotransmitter receptors in mouse TC cells

We initially confirmed the regulation of I_h by cAMP following activation of GPCRs the coupling of which to cAMP synthesis is well documented. We used the selective agonist Iso to activate β-adrenergic receptors, known to lead to stimulation of cAMP synthesis (Bloom *et al*., 1975; Madison & Nicoll, 1986; McCormick & Pape, 1990) and Bac to stimulate $GABA_B$ receptors, which inhibit cAMP production (Wojcik & Neff, 1984; Knight & Bowery, 1996). Both β-adrenergic and $GABA_B$ receptors are expressed in TC neurons (Rainbow *et al*., 1984; Princivalle *et al*., 2001; Kulik *et al*., 2002) and contribute to the control of intrinsic and synaptic processes related to thalamic function during sleep and arousal (for review, see McCormick & Bal, 1997; Huguenard, 1998).

In agreement with previous reports, bath application of Iso (0.5 μM), a selective β-adrenergic agonist, induced a gradual enhancement of the amplitude of I_h to 128.0±4.3% of control (n=6, P<0.001; Fig. 1*A*). This enhancement showed a shallow dose dependence with a maximal value reached at 135.8±5.7% of control amplitude for 5 μM Iso, and a half-maximal effect around 1 nM (116.2±5.3% of control). In the continuous presence of Iso, the enhancement of I_h was maintained (less than 10% decay) for at least 5 min following start of the bath application. The Iso-induced increase in I_h amplitude corresponded to a positive shift in the activation curve of I_h from -94.4±0.6 mV to -87.6±1.0 mV with no change in maximal conductance (n=4, P<0.05; Fig. 1*B*; see Methods), similar to values reported previously (McCormick & Pape, 1990). Monoexponential fitting of current traces at –92 mV revealed time constants of 543±97 ms in Iso compared to 726±120 ms in control (n=6, P<0.02). The Iso-induced enhancement of current amplitude, the shift in the activation curve and the acceleration of the activation time course typically reflect increased binding of h-channels to cAMP (for review, see Pape, 1996; Wainger *et al*., 2001).

Conversely, alterations in the amplitude of cAMP-sensitive currents following activation of $G_{i/o}$-coupled neurotransmitter receptors have been associated with an inhibition of either basal or forskolin-stimulated AC activity (Pape, 1992; Ingram & Williams, 1994; Gerber & Gähwiler, 1994; Svoboda & Lupica, 1998). However, a

decrease in I_h has also been attributed to a shunting action of K^+ currents activated by $G_{i/o}$-coupled $GABA_B$ receptors (Watts *et al.*, 1996). In our experiments, the continuous presence of 1.5 mM Ba^{2+} blocked outward currents activated by Bac (172±14 pA in the absence, n=4; -6±7 pA in the presence of Ba^{2+}; n=18, P<0.001; Pape, 1992; Sodickson & Bean, 1996), while I_h amplitude remained unchanged (<10% decrease, n=11). The subsequent application of Bac at a concentration leading to maximal activation of K^+ currents (80 μM; Sodickson & Bean, 1996) produced a persistent decrease in the amplitude of I_h to 74.6±3.3% of the original value (n=15, P<0.02), without a decrease in input resistance (Fig. 1*C*). In contrast, no significant decrease in I_h was found when Bac was applied at 0.8 μM, close to the threshold for activation of K^+ currents (111.5±5.9% of ctrl, n=7, P>0.05; Sodickson & Bean, 1996). The Bac-induced decrease was associated with a negative shift in the activation curve of I_h from –92.0±0.3 mV to –97.0±0.4 mV (n=6, P<0.01) with no change in the maximal conductance of the current (Fig. 1*D*). Moreover, the monoexponential time constants of activation of I_h were increased from 669±31 ms to 797±35 ms (n=8, P<0.01; see Methods), consistent with a reduction of basal cAMP levels surrounding h-channels. In support of this possibility, inclusion of a saturating concentration of a non-hydrolyzable analog of cAMP in the patch pipette, 8Br-cAMP (5-10 μM), prevented the Bac-induced reduction of I_h amplitude (93.8±3.1% of control amplitude, n=6, P>0.05; Fig. 1*E*), indicating an occlusion of Bac-mediated inhibitory effects by cAMP previously bound to h-channels. Furthermore, we studied the effects of 3-isobutyl-1-methyl-xanthine (IBMX, 100 μM), a general phosphodiesterase (PDE) inhibitor, to prevent hydrolysis of cAMP molecules. Bath application of IBMX for 4-6 min induced a steady increase in the amplitude of I_h to 117.8±8.8% (n=10, P<0.001; Fig. 1*F*), revealing a basal production of cAMP that is normally

counteracted by the hydrolytic activity of PDEs. In 9 cells exposed to Bac, this IBMX-induced enhancement was limited to, on average, 105.1±5.3% of control amplitude (P<0.05 compared to effects obtained with IBMX alone; Fig. 1*F*), suggesting that basal cAMP synthesis in the vicinity of h-channels was reduced. To exclude Bac-induced decreases in channel sensitivity to cAMP, we studied the effects

of photolytic release of caged cAMP on I_h before and after application of Bac. Maximally three flashes were applied per experiment (see Methods). When cAMP was applied photolytically, it induced an increase of I_h amplitude to 131.1±5.8% of control (n=4, P<0.05; Fig. 1*F*). Following application of Bac (80 µM), the increase amounted to 133.7±9.1% (n=4, P>0.05; Fig. 1*F*), indicating that the sensitivity of I_h to exogenously applied cAMP was not affected by Bac. Taken together, our data strongly suggest that Bac leads to an inhibition of on-going AC activity in a concentration range covering that of $GABA_B$-mediated activation of K^+ currents (Sodickson & Bean, 1996). Activated $GABA_B$ receptors thus reduce cAMP levels and provoke an unbinding of cAMP tonically bound to h-channels.

In summary, pharmacological activation of G_s- and $G_{i/o}$-coupled GPCRs on TC neurons led to time-invariant changes in I_h that are consistent with an accumulation and a decrease of available cAMP in the vicinity of h-channels, respectively.

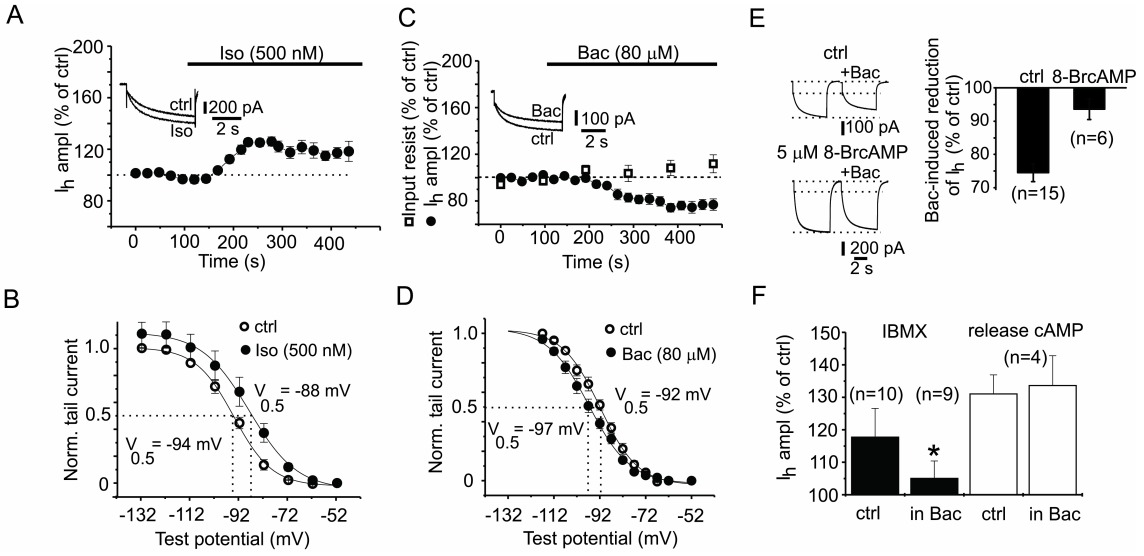


Figure 1. Iso and Bac modulate I_h in a manner consistent with the coupling of β-adrenergic and $GABA_B$ receptors to adenylyl cyclase.

A, Bath application of Iso (500 nM) induced a small steady enhancement of I_h amplitude to 128.0±4.3% of control (n=6, P<0.001). *Inset* shows an overlay of the I_h current activated during a voltage step from –62 to –92 mV in ctrl and during Iso application at steady-state. *B*, Activation curve of I_h in the absence (○) and in the presence (●) of Iso. Activation curves were constructed from tail current analysis and normalized to the maximal current under control conditions. This yielded $V_{0.5}$ = –94.4±0.6 mV in control and $V_{0.5}$ = -87.6±1.0 mV in Iso, respectively (n=4, P<0.05). *C*, Bath application of a saturating concentration of Bac induced a steady reduction in I_h amplitude (●) to 74.6±3.3% (n=15,

P<0.02). This effect was not associated with a decrease in the input resistance of the neuron (□), but rather a small increase to 111.6±9.0% of control (n=7, P>0.05), likely due to decreased I_h amplitude. *Inset* shows an overlay of the I_h current activated during a voltage step from –62 to –92 mV in ctrl and during Bac application in steady-state. *D*, Evaluation of the relative tail current amplitudes of I_h shows that Bac induced a leftward shift in $V_{0.5}$ of the activation curve from –92.0±0.3 mV to –97.0±0.4 mV (n=6, P<0.01) with no change in the maximal conductance. *E*, *Left*, Bac effects on I_h were occluded when 5-10 μM 8Br-cAMP were present in the pipette solution *Right*, Pooled data illustrating the Bac-induced decrease in I_h in control (74.6±3.3% of control amplitude, n=15, P<0.02) and with 8Br-cAMP present in the pipette (93.8±3.1% of control, n=6, P>0.05). *F*, Pooled data illustrating the effects of bath application of IBMX (*filled columns*) on the amplitude of I_h in control (117.8±8.8%, n=10, P<0.001) and during preceding exposure to Bac (105.1±5.3% of control, n=9, *P<0.05 compared to values in control) and of uncaging cAMP (*open columns*) on the amplitude of I_h in control (131.1±5.8% of control, n=4, p<0.05) and during preceding exposure to Bac (133.7±9.1% of control, n=4, P>0.05 compared to values in control). In *E* and *F*, step voltages from –62 to –92 mV were used to evoke I_h.

Co-activation of G_s- and $G_{i/o}$-coupled neurotransmitter receptors strongly upregulates I_h

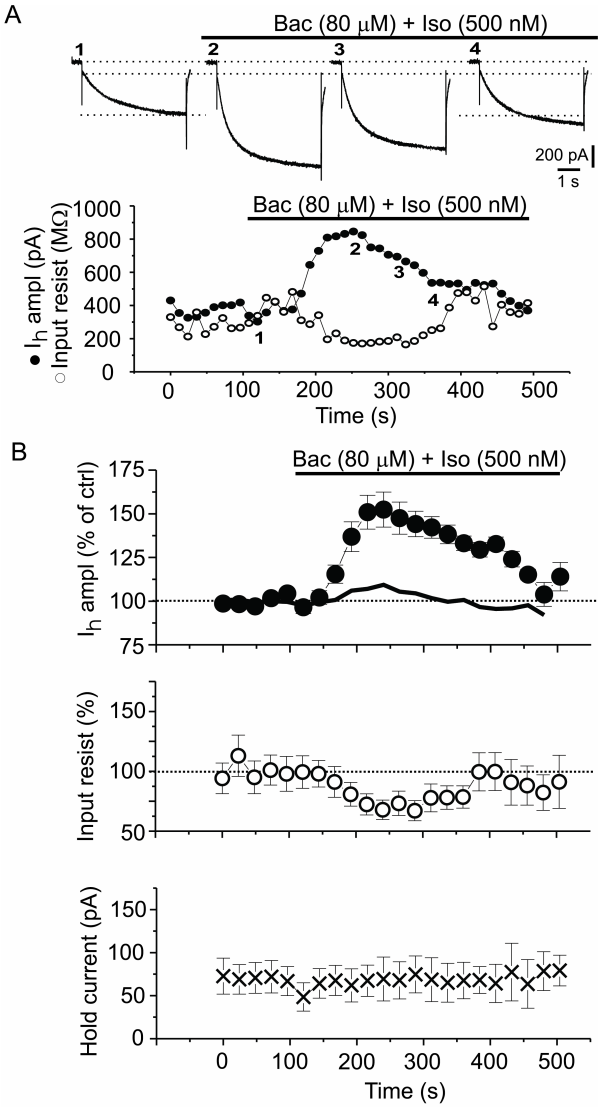
There is evidence that $G_{i/o}$-coupled receptors can strongly modulate cAMP-dependent regulation of ionic currents via G_s-coupled receptors in intact cells, either in an antagonistic (Hartzell, 1988; Pape, 1992; Gerber & Gähwiler, 1994) or in a potentiating fashion (Andrade, 1993; Pedarzani & Storm, 1996). However, the direction and physiological role of cross-talk between these two types of GPCRs has never been examined in the TC system, in which β-adrenergically mediated cAMP turnover is intimately involved in cellular activities related to states of arousal (Cirelli *et al.*, 1996; McCormick & Bal, 1997). The expected time course of change in I_h amplitude, assuming an antagonistic effect of Bac on the stimulatory action of Iso, is demonstrated in Fig. 2*B* (*thick line*). In this case, I_h amplitude should deviate by <10% from control at all time points. Superimposed are the experimentally measured values of I_h amplitude. In sharp contrast to the expected time course, the amplitude of I_h was strongly enhanced during co-application of Iso and Bac. As illustrated in the example in Fig. 2*A*, I_h amplitude approximately doubled (from –400 to –800 pA) upon co-application of Iso and Bac, but then gradually decayed back to control with a monoexponential time constant of 164 s. In 6 cells, co-application of Iso and Bac yielded a 152.6±9.6% enhancement of I_h at the peak (range 132-222%) which decayed with τ=281±80 s (measured in n=5 of 6 cells). During the co-application,

holding current levels showed no significant change (73±21 pA before vs. 63±24 pA, n=6, P>0.05; Fig. 2*B*; *lower panel*). The input resistance, measured from the instantaneous current response during the hyperpolarizing step, decreased by ~30% (from 302±56 MΩ to 212±38 MΩ, n=6, P<0.005; Fig. 2*B*, *middle panel*) at the peak of I_h enhancement, and was restored to control before I_h enhancement had fully decayed (272 s after the start of agonist exposure, I_h amplitude at this point: 129.4±4.2%, n=6, P<0.01). Therefore, the increase in I_h could not be explained based on alterations in passive cellular properties, and the decrease in input resistance most likely resulted from a contribution of strongly enhanced I_h to instantaneous current amplitudes. The results from the co-application suggested that Iso and Bac effects on cAMP synthesis displayed a marked divergence from linear summation during an initial period, whereas they canceled each other during a delayed steady-state phase.

The effect of Iso and Bac on I_h showed properties distinct from that induced by Iso alone. Thus, the maximal enhancement of I_h obtained in the presence of Iso and Bac was markedly larger than that produced by Iso alone (n=11 for Iso; n=6 for Iso and Bac, P<0.05). Furthermore, similar strong enhancements of I_h were found when 500 nM Iso were co-applied with low concentrations of Bac (0.8 μM; 182.3±23.9%, n=5, P<0.05; cf. Fig. 5), which, when applied alone, did not reduce I_h (see above). The co-application of Bac with Iso therefore induced a potentiating effect on I_h that differed with respect to strength and concentration dependence from that of Iso alone. This indicates the presence of a distinct regulatory pathway of I_h modulation induced by a synergistic action of Bac and Iso.

Figure 2. Co-application of Iso and Bac induces a marked potentiation of I_h.

A, Raw data showing the transient, strong increase in I_h upon bath application of Iso together with Bac at a concentration (80 μM) that reduced I_h when applied alone. *Dotted lines* are presented to facilitate comparison of instantaneous and I_h current amplitudes between the four traces. The time course of the potentiation is illustrated at the bottom (●), together with the input resistance of the neuron (○). The data points deduced from the traces presented at the top (1-4) are indicated in the plot. *B*, Pooled data from 6 cells illustrating the time course of the potentiation (*top panel*), of the input resistance (*middle panel*) and the holding current (*lower panel*). The *thick line* in the top panel depicts the linear sum of the effects of Iso and Bac illustrated in Fig. 1.



The synergistic effect is mediated by cAMP

The enhancement of I_h in the presence of Iso and Bac could be explained either by 1) a cAMP-dependent upregulation of I_h, for example via an increased synthesis of cAMP triggered by the co-application or 2) alternative modulatory pathways targetting I_h that do not involve cAMP (see e.g. Accili *et al*., 1997; Pan, 2003). We first determined the activation curve of I_h during the peak of the potentiation (Fig. 3*A*, *B*). The activation curve was shifted by 11.1±1.7 mV (from –95.7±0.7 mV to –84.6±0.9 mV, n=6, P<0.05) towards more positive values (Fig. 3*B*), whereas the amplitude of the maximal currents elicited by voltage steps to –132 mV remained unchanged (-1701±181 pA in ctrl vs. -1786±207 pA in Iso+Bac, n=6, P>0.05; Fig.

3*A*). Thus, the upregulation could be described by a simple positive shift in the voltage dependence of I_h that was larger than that produced by Iso alone ($P<0.05$) and consistent with a modulation of I_h by cyclic nucleotides.

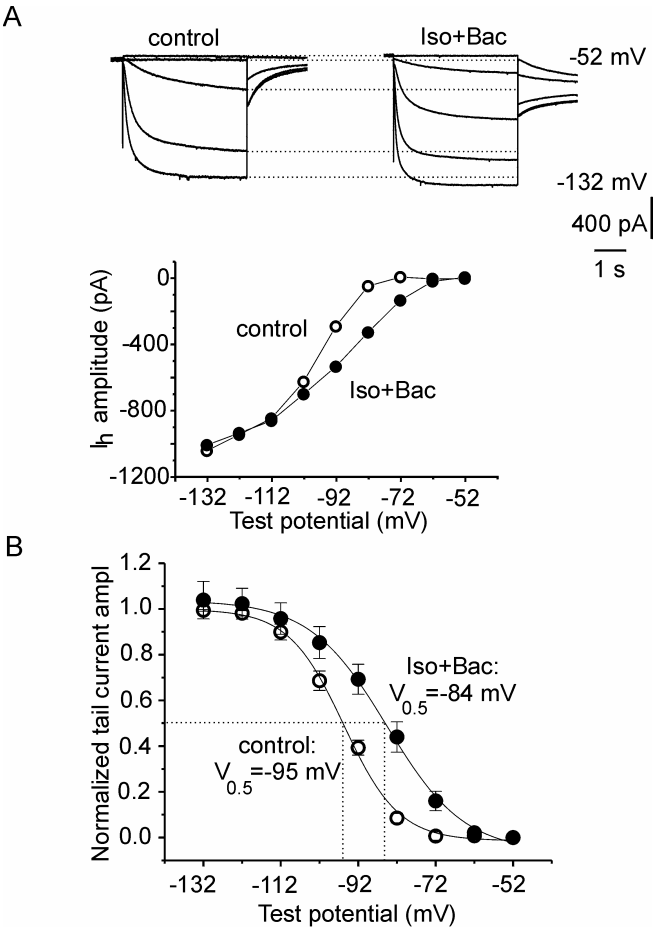


Figure 3. The potentiation of I_h by co-application of Iso and Bac is associated with a strong positive shift in the activation curve with no change in maximal conductance.

A, *Top*, Family of I_h currents during control (*left*) and in the presence of Iso and Bac (*right*). Note the pronounced increase in current amplitude at intermediately hyperpolarized potentials. *Bottom*, Graph of I_h current amplitudes as a function of test potential in control (○) and in the presence of Iso and Bac (●). Maximal current amplitudes are not changed by Iso and Bac (-1701±181 pA in ctrl vs. -1786±207 pA in Iso+Bac, n=6, P>0.05). *B*, Activation curves in control (○) and in the presence of Iso and Bac (●). All tail currents were normalized with respect to the maximal tail current under control conditions. Co-applied Iso and Bac produced an 11 mV positive shift in the activation curve (from –95.7±0.7 mV to –84.6±0.9 mV, n=6, P<0.05) with no change in the maximal activation of the current.

To further substantiate an involvement of cAMP, we used flash photolysis of caged cAMP to address the sensitivity of I_h to cyclic nucleotides at different time

points during the modulation. When cAMP was photolytically released under control conditions, it produced an increase in amplitude to 128.9±6.3% of control (n=8, P<0.005; Fig. 4*A* and *B*). In contrast, when caged cAMP was photolyzed during the peak of the enhancement produced by Iso and Bac, the effect was reduced to 106.6±2.5% of current amplitude preceding the flash (n=5, P>0.05; Fig. 4*A* and *B*), indicating that the response to cAMP was fully occluded when the action of Iso and Bac was maximal. In the continuous presence of Iso and Bac, the sensitivity to cAMP was restored upon complete decay of the enhancement, such that photolytically released cAMP increased I_h amplitude to 123.6±4.1% of control (n=11, P>0.05 vs. flash-induced I_h increase before application of Iso and Bac; Fig. 4*B*). Thus, enhancement of I_h by co-application of Iso and Bac occluded the response to cAMP, whereas its decay restored responsiveness. We also compared the properties of I_h at the peak of the enhancement with those induced following exposure to Forskolin (Forsk), a general AC activator. When bath-applied at a saturating concentration of 10 μM, Forsk produced a steady increase in I_h amplitude equaling 148.8±5.3% of control (n=4, P<0.01), close to the enhancement produced by the co-application of Iso and Bac (152.6±9.6%, n=6, P>0.05). Monoexponential fitting of the time course of activation of I_h in the presence of Forsk yielded an acceleration of the time constant from 825±114 ms to 561±86 ms (n=4, P<0.05; Fig. 4*C*), reflecting increased cAMP binding to the channels. Similarly, the co-application of Iso and Bac accelerated the time constant of I_h from 780±57 ms to 533±23 ms (n=6, P<0.01; Fig. 4*C*). Following the decay of the enhancement, however, the time constant recovered to 723±82 ms (P>0.05 compared to control). Using lower concentrations of Forsk (1 μM), we tested whether Bac could alter the sensitivity of interaction of h-channels with cAMP. Forsk alone enhanced I_h to 129.0±2.8% of control (n=4, P<0.05), while the combination of Forsk and Bac yielded an enhancement of 114.0±1.2% (n=4, P<0.05), slightly, but not significantly smaller than that observed with Forsk alone (P>0.05). Taken together, the alterations in the activation properties of I_h, the full occlusion of cAMP effects at the peak of the potentation, and the lack of a potentiating effect of Bac on the actions of Forsk are indicative of a mechanism dominated by cAMP that mediated the potentiation of I_h, most likely via a stimulation of cAMP synthesis (see Discussion).

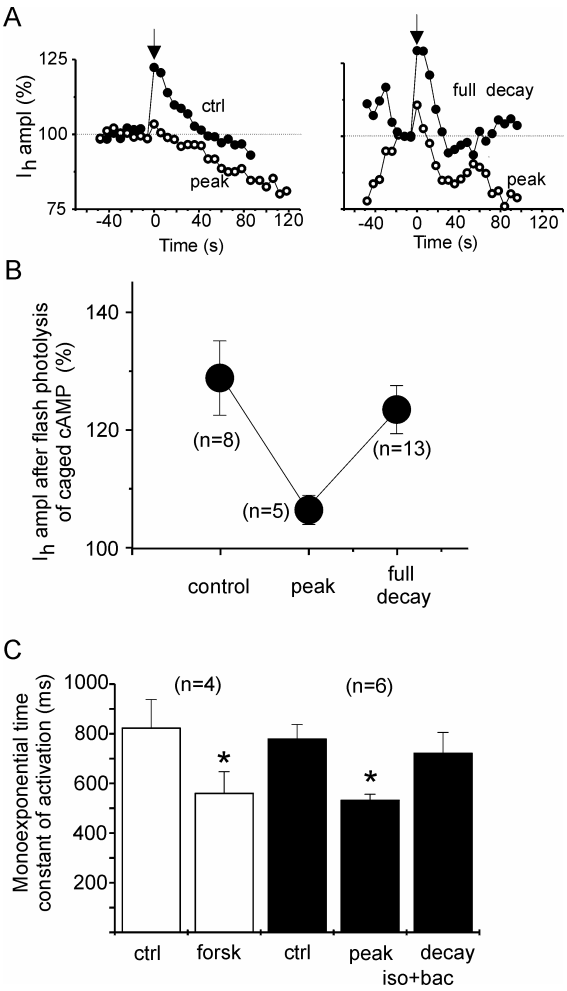


Figure 4. The potentiation of I_h by co-application of Iso and Bac leads to a reversible occlusion of I_h modulation via photolytically released cAMP.

A, Data from two separate experiments showing the response to uncaged cAMP (photolysis was initiated at the time depicted by the *arrow*). In the experiment presented on the *left*, cAMP was photolytically released during control (●) and during the peak of the potentiation induced by Iso and Bac (○). In the experiment presented on the *right*, cAMP was photolytically released at the peak of the potentiation (○) and after its full decay (●). To facilitate comparison of the extent of the potentiation during the different periods, data were normalized to the average of the three data points preceding flash application. *B*, Pooled data illustrating the increase in I_h amplitude following flash photolysis of caged cAMP during control (128.9±6.3% of basal I_h amplitude, n=8, P<0.005), at the peak of the potentiation (106.6±2.5% of I_h amplitude preceding the flash, n=5, P>0.05), and after its full decay (123.6±4.1% of basal I_h amplitude, n=11, P>0.05 vs. increase before application of Iso and Bac). *C*, Histogram of the time constants of activation of I_h during exposure to Forsk (825±114 ms vs. 561±86 ms, n=4, *P<0.05) and during the response to co-application of Iso and Bac (780±57 ms in control; 533±23 ms at the peak, n=6, *P<0.01; 723±82 ms during recovery, P>0.05 compared to control).

The presence of Bac transforms the time course of the cAMP signal induced by Iso

The potentiation induced by the co-application of Iso and Bac suggests that a coincidental activation of β-adrenergic and $GABA_B$ receptors recruited a powerful pathway of cAMP synthesis distinct from that targeted by the individual GPCRs. To further characterize this pathway, we investigated how the presence of Bac affected the time course of the response induced by a brief application of Iso. For this purpose, we combined focal application of Iso with bath application of Bac. When Iso (500 nM in a pressure ejection pipette) was applied during baseline recording, it produced a 132.5±4.1% enhancement of I_h amplitude (n=13, P<0.001) that changed by <5% during subsequent applications in control (n=3) and decayed with a time course of 32±6 s. When Bac was applied at a saturating concentration of 80 μM, focal application of Iso produced a potentiation of 175.8±10.0% of control amplitude (n=5, P<0.02; Fig. 5*A*). The potentiated response decayed with a time constant of 241±30 s (measured in n=4 cells; Fig. 5*B*), which was markedly slower than the control response (P<0.005). When Bac was applied at 0.8 μM, focal application of Iso produced an enhancement equaling 190.6±29.2% (n=7, P<0.02 compared to control responses which yielded 141.9±11.0% increase of I_h; Fig. 5*C*). The decay of these responses was even further decelerated, with a remaining 152.7±19.5% increase of I_h at 4 min after the application of Iso (Fig. 5*D*). Thus, the cAMP synthesis pathway requiring both Iso and Bac showed a distinct temporal profile of cAMP synthesis that depended on the strength of activation of Bac receptors. Interestingly, the time course of the potentiated response greatly outlasted the duration of the stimulus mediated by Iso alone. Thus, the presence of Bac allows the transformation of a transient positive input for cAMP synthesis into a more persistent cAMP signal.

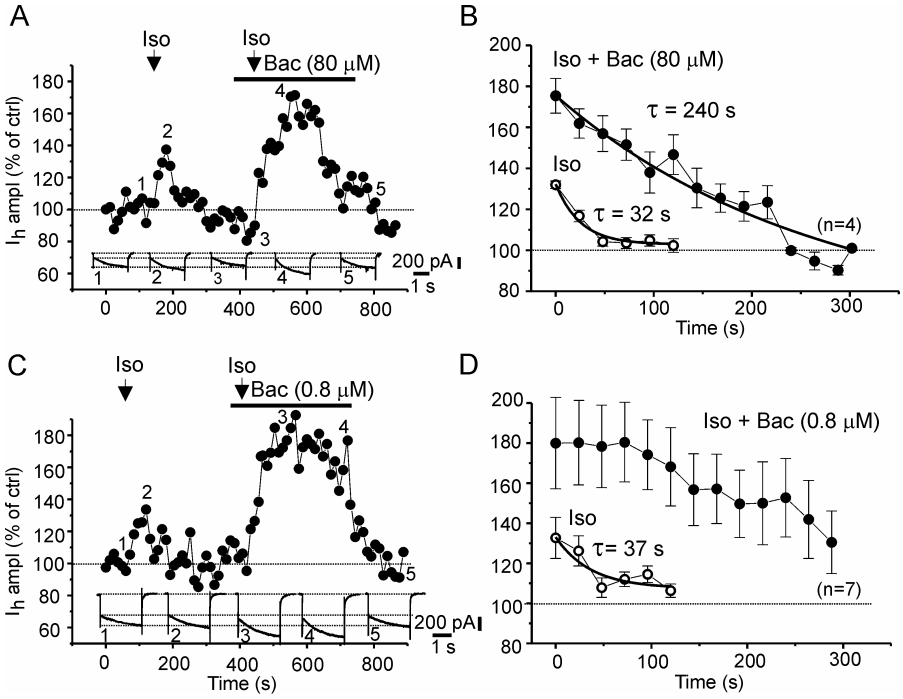


Figure 5. The presence of Bac changes the time course of the cAMP transient induced by Iso.

A, Data from a single experiment, illustrating the time course of I_h amplitudes following focal exposure to Iso before and after bath application of Bac. Bac alone reduced I_h in this cell by 15%. Selected I_h recordings are presented in the *inset* (1-5). *B*, Averaged decay time course of the potentiation of I_h by local application of Iso alone (○) and in the presence of Bac (●). Lines show the non-linear least square fit of a monoexponential curve to the data, yielding a time constant τ = 241±30 s for Iso+Bac vs. τ = 32±6 sec for Iso (n=4, P<0.005). *C, D*. Same experiment as in *A, B* but with Bac applied at 0.8 μM. Note that the decay time course was further decelerated by low concentrations of Bac, such that monoexponential fitting was not possible.

Pharmacological properties of the receptors involved in the potentiation

We next verified whether γ-aminobutyric acid (GABA), the natural ligand for $GABA_B$ receptors, could induce the potentiation of Iso effects by Bac. The potentiation was mimicked when Bac was replaced by GABA (1 mM) in the presence of the $GABA_A$ receptor antagonist picrotoxin (100 μM) in 5 of 7 cells tested (145.8±9.4% in ctrl vs. 183.6±11.4% in GABA+Iso, P<0.05; Fig. 6*A* and *D*), indicating that the endogenous agonist for $GABA_B$ receptors could induce a potentiation of β-adrenergic responses. To further address the involvement of G_i-proteins in the potentiation of I_h, we used N-ethylmaleimide (NEM), a membrane-permeable inhibitor of pertussis-toxin sensitive G-proteins (Winslow *et al.*, 1987;

Shapiro *et al*., 1994; Hirono *et al*., 2001), to selectively interfere with G_i- but not G_s-proteins. Bath application of NEM for 2 min fully antagonized Ba^{2+}-sensitive outward currents induced by Bac at –50 mV (data not shown), while it did not interfere with Iso-induced enhancements of I_h (92.2±0.9% of responses without NEM, n=4, P>0.05), consistent with a selective inhibition of G_i-proteins. When Iso was locally applied in the presence of Bac (0.8 μM) and NEM, the potentiation was fully abolished (151.1±4.0% in Iso vs. 150.3±6.8% enhancement in Iso and Bac, n=4, P>0.05; Fig. 6*B*). Moreover, the time constants of the decay of the cAMP transient in NEM were rapid, with a monoexponential decay of 90±43 s (compared to 47±16 s in control, P>0.05; Fig. 6*C*). These data point to a requirement of G_i-proteins activated by $GABA_B$ receptors in the potentiation and prolongation of the cAMP stimulation mediated by Iso.

We then investigated whether activation of GPCRs other than baclofen-sensitive receptors also enhanced Iso responses. The A1 agonist N^6-cyclopentyladenosine (CPA, 50 μM) was tested because adenosine receptors are functionally expressed in TC neurons and share common mechanisms of action with $GABA_B$ receptors in these cells (Pape, 1992). When applied in the bath, CPA did not affect the amplitude of control responses induced by puff application of Iso (146.4±16.4% during control vs. 143.0±8.3% during CPA, n=3, P>0.05; Fig. 6*D*), suggesting that A1 receptors did not undergo synergistic interactions with β-adrenergic receptors. We then used [D-Ala-2, NMe-Phe-4, Gly-5-ol]-enkephalin (DAMGO, 1 μM) to activate $G_{i/o}$-coupled μ-opioid receptors that are widely expressed in TC neurons (Brunton & Charpak, 1998). Interestingly, application of DAMGO alone caused a rapid, transient increase in the amplitude of I_h to 148.0±16.1% of control amplitude (n=4, P<0.02; Fig. 6*D*), which was associated with an acceleration in the monoexponential time constant from 803±92 ms to 680±99 ms (n=4, P<0.01), suggesting that activation of μ-opioid receptors alone coupled positively to cAMP production detected by I_h (see Discussion). Thus, distinct $G_{i/o}$-coupled neurotransmitters appear to couple differently to cAMP synthesis pathways in the vicinity of I_h, both when activated alone or in conjunction with β-adrenergic receptors.

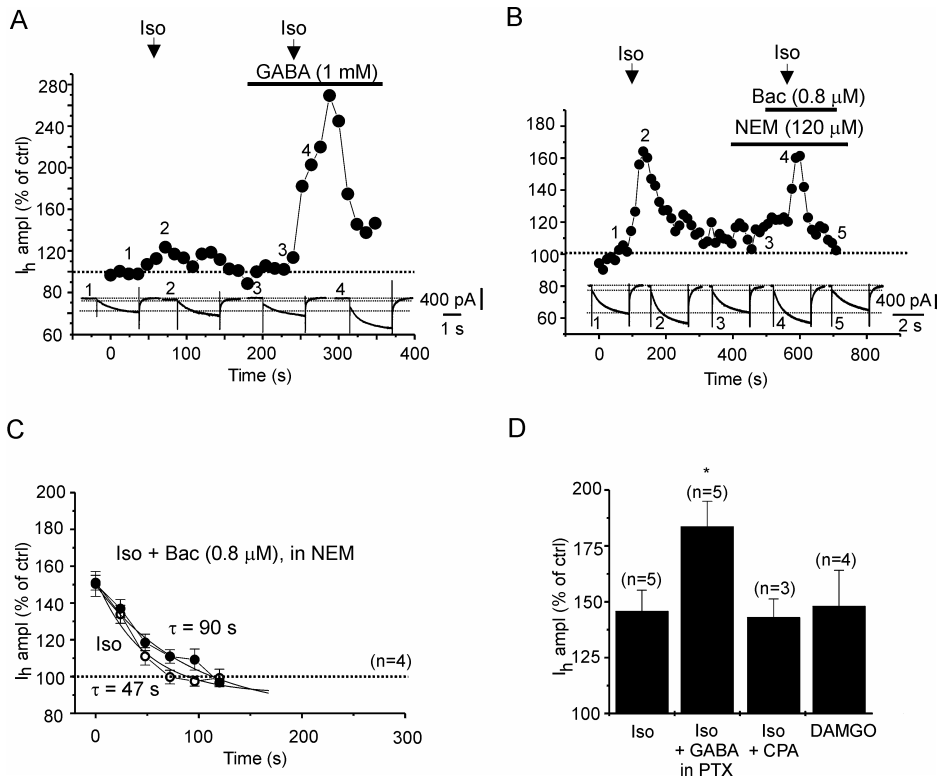


Figure 6. Pharmacological characterization of the upregulation.

A, The natural transmitter for $GABA_B$ receptors, GABA, induced a potentiation of Iso responses in the presence of picrotoxin (100 μM). The graph illustrated the time course of I_h amplitudes in a single experiment. Selected I_h recordings are presented in the *inset*. *B*, The modulation of I_h by Iso remained unaltered in the presence of Bac (0.8 μM), when NEM (120 μM) was pre-applied for 2 min. Time course of I_h amplitudes in a representative experiment. Selected I_h recordings are presented in the *inset* (1-5). *C*, Average time course of decay of Iso-induced modulation of I_h before (○, τ = 47± 16 s, n=4) and after (●, τ = 90 ± 43 s, n=4, P>0.05) application of Bac in the presence of NEM. *D*, Histogram summarizing the effects of different combinations of agonists for GPCRs. The responses to Iso were potentiated in the presence of GABA (1 mM) and picrotoxin (PTX, 100 μM) (183.6±11.4% vs. 145.8±9.4% of control in 5 of 7 cells tested, *P<0.05), but not in the presence of CPA (50 μM) (146.4±16.4% vs. 143.0±8.3% of control, n=3, P>0.05), an A1 agonist. DAMGO (1 μM), a μ-opioid receptor agonist, increased I_h in the absence of Iso to 148.0±16.1% of control (n=4, P<0.02).

Actions of synaptically activated $GABA_B$ receptors on I_h modulation

In thalamic networks, synaptic activation of $GABA_B$ receptors on TC neurons occurs during both sleep-related and during pathological hypersynchronous activity *in vitro* resembling generalized epilepsies (Blumenfeld & McCormick, 2000; Bal *et al.*,

2000), and can result from a hyperexcitation of GABAergic afferents arising in the nRt. The effect of postsynaptically activated $GABA_B$ receptors on cAMP metabolism in TC neurons is, however, unknown. To address the coupling of synaptically activated $GABA_B$ receptors to cAMP-mediated modulation of I_h, we studied the effects of $GABA_B$ receptor-mediated synaptic currents evoked via electrical stimulation in the nRt (see Methods). In the presence of glutamatergic and $GABA_A$ergic receptor antagonists (APV, 100 μM, NBQX, 10 μM, Picrotoxin, 100 μM), electrical stimulation evoked slow outward current responses that peaked at a delay of 80±4 ms (range 59-100 ms) and reached amplitudes of 12±3 pA (range 2-50 pA, n=18; Fig. 7*A*), similar to responses described previously in rat (Ulrich & Huguenard, 1996). The highly selective $GABA_B$ receptor antagonist CGP54626 (500 nM) was tested in 4 cells and completely blocked these outward currents, indicating that they were mediated by $GABA_B$ receptors (Fig. 7*A*). Iso was then applied locally while concomitantly eliciting $GABA_B$ responses (10 stimulations, 5 Hz in the presence of 1.5 mM Ba^{2+}) and the modulation of I_h amplitude was monitored. Synaptic activation of $GABA_B$ receptors during simultaneous local application of Iso induced a significant potentiation of I_h amplitude (I_h amplitude in Iso: 118.5±4.3% of control; I_h amplitude in Iso, with $GABA_B$ receptors activated synaptically: 140.2±12.0% of control, n=7, $P<0.05$; Fig. 7*B* and *C*). In contrast, synaptic stimulation alone induced a minor enhancement of I_h (101.7±2.4% of control amplitude at 24 sec after application of the stimulation; n=4, $P>0.05$; Fig. 7*B*) and these stimulation-dependent effects were subtracted from the responses obtained during concomitant stimulation and Iso application. Our results thus suggest that synaptically activated $GABA_B$ receptors can contribute to the control of cAMP turnover in TC neurons, while a modulation of I_h via released compounds other than GABA appears to play a minor role.

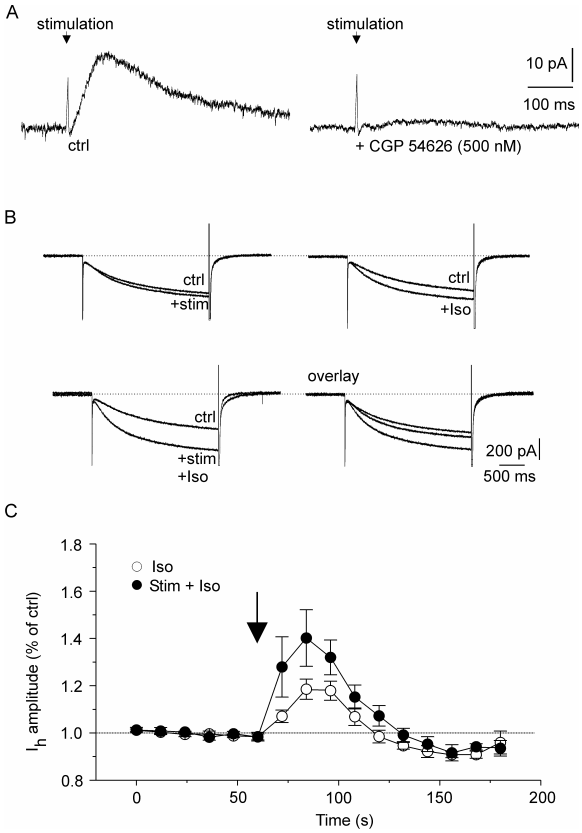


Figure 7. Examination of the role of synaptic $GABA_B$ receptors in the potentiation of I_h responses by Iso.

A, CGP 54626-sensitive outward currents elicited by stimulation of afferent nRt fibers via bipolar tungsten electrodes (300-700 μA, 100 μsec). *B*, In a different cell, representative I_h responses were monitored under control conditions (*ctrl*) and after Iso was applied with (+ *stim*, + *Iso*) and without (+ *Iso*) concomitant activation of $GABA_B$ receptors (10 stimuli at 5 Hz). Overlay shows control I_h, the current response to application of Iso alone, and the response following conjoint Iso application and electrical stimulation. During this experiment, Ba^{2+} ions (1.5 mM) were present to prevent activation of outward K^+ currents. *C*, Averaged data for seven experiments, indicating a significant increase in the Iso sensitivity of I_h amplitude after electrical stimulation (118.5±4.3% of control I_h amplitude during Iso application; 140.2±12.0% of I_h amplitude during Iso application and co-activation of $GABA_B$ receptors, n=7, $P<0.05$). (○): Control responses; (●): Responses with $GABA_B$ receptor activation.

Discussion

Here we describe distinct types of GPCR-induced cAMP signals that modulate the pacemaker current of TC neurons. The strength and time course of modulation of I_h varied considerably depending on the pattern of activation of GPCRs. Steady increases or decreases in I_h amplitude occurred upon activation of single receptor types coupled positively or negatively to cAMP production. Thus, a basal cAMP turnover, which is pronounced in thalamus compared to other regions of the brain (Matsuoka *et al*., 1997; Ihnatovych *et al*., 2002), can be up- and downregulated steadily under the tonic influence of neurotransmitter receptors. In contrast, co-exposure to two agonists was integrated in a supralinear manner to produce a strong, transient increase in I_h, likely mediated by a pharmacologically and kinetically distinct cAMP synthesis pathway (see below). Thus, the pacemaker current can be exposed to cAMP signals originating from diverse sources, suggesting that pacemaker channels are surrounded by cAMP synthesis pathways with distinct molecular properties.

A crucial point in our study was to demonstrate that the dynamics of the modulation of I_h by agonists for GPCRs primarily reflected the time course of intracellular cAMP concentrations. While increases in I_h via Iso have been attributed to synthesis of cAMP in TC neurons (McCormick & Pape, 1990), Bac-mediated decreases of I_h were proposed to occur through a pathway independent of cAMP in several neuronal cell types (Jiang *et al*., 1993; Watts *et al*., 1996; Pape, 1996). In TC neurons, a highly cAMP-sensitive isoform of I_h is expressed (Seifert *et al*., 1999) and basal cAMP synthesis rate in TC neurons is comparatively pronounced (Matsuoka *et al*., 1997; Ihnatovych *et al*., 2002), two factors advantageous for detecting decreases in cAMP via I_h. Indeed, the inhibition of I_h identified here showed characteristics typical for cAMP-dependent actions on h-channels, including a negative shift in the activation curve and decelerated activation kinetics (for review, see Pape, 1996; Wainger *et al*., 2001). In addition, a saturating cAMP concentration largely occluded Bac effects, the activity of ACs appeared reduced in the presence of Bac, and there was no measurable change in the sensitivity for cAMP in the presence of Bac. These findings therefore indicate that $G_{i/o}$-coupled $GABA_B$ receptors, in addition to adenosine A1 receptors (Pape, 1992), primarily reduce I_h via inhibiting basal cAMP synthesis in TC neurons. However, a minor Bac-induced contribution to cAMP-

independent modulation of I_h, for example via decreases in the concentration of cGMP (Fedele *et al*., 1997; Pape & Mager, 1992), can not be excluded.

A vigorous synthesis of cyclic AMP likely mediated the transient potentiation of I_h by Iso and Bac. First, the modulation of I_h was associated with a maximal shift in the activation curve of I_h, similar to that observed with exogenous addition of high cAMP concentrations intracellularly (McCormick & Pape, 1990; Lüthi & McCormick, 1999). Second, modulation of I_h, induced by photolysis of caged cAMP, was occluded during the peak of the potentiation induced by Iso and Bac, but fully reinstated following the decay of the potentiation. Third, reversible accelerations in the time course of activation of I_h, which are widely used hallmarks of cAMP-dependent actions on h-channels, paralleled the enhancement. Fourth, the characteristics of the potentiation could be mimicked by stimulation of endogenous ACs with Forsk. Fifth, Bac did not appear to alter the sensitivity of h-channels for cAMP generated in the presence of non-saturating concentrations of Forsk. Taken together, the enhancement of I_h by co-application of Iso and Bac shows characteristics that are consistent with an exposure of h-channels to a powerful elevation of cAMP, which represents the most widely described pathway of pacemaker current modulation (for review, see Pape, 1996; Santoro & Tibbs, 1999; Kaupp & Seifert, 2001; Robinson & Siegelbaum, 2003). Alternate, more complex modulations of pacemaker channel function, such as decreases in the concentrations of cAMP required for channel gating, can, however, not be excluded at this point and would require experiments under cell-free conditions.

A cross-talk between Bac and Iso receptors in cAMP signaling could be induced at the level of the receptors, the G-proteins, the ACs and the PDEs. As application of Bac alone inhibited rather than enhanced I_h, an involvement of alternate $GABA_B$-receptor induced modulatory pathways, such as Ca^{2+} release (Hirono *et al*., 2001) and an associated cAMP synthesis (Lüthi & McCormick, 1999) are unlikely to be involved in the synergism. Moreover, Bac failed to potentiate the action of low concentrations of Forsk, indicating that AC activity was not stimulated directly by Bac. Furthermore, inhibition of PDEs and decreasing cAMP degradation led to a weak augmentation of I_h that could not account for the strength of the potentiation. Therefore, the synergism appears to arise at a point upstream of cAMP synthesis. Accordingly, we were able to interfer with the potentiation by using NEM, an inhibitor of G_i-proteins that interferes with multiple $GABA_B$ receptor-mediated effects

on ionic currents (Sodickson & Bean, 1996; Hirono *et al*., 2001). Activation of G_i-proteins, likely induced by ligand-bound $GABA_B$ receptors showing a high apparent affinity for Bac, appears thus to be a primary requirement for inducing a potentiation of cAMP synthesis, induced in conjunction with stimulation of G_s-proteins.

In biochemical assays of cAMP levels in neural tissue, activation of $G_{i/o}$-coupled neurotransmitter receptors, including $GABA_B$, α-adrenergic and μ-opioid receptors has been reported to potentiate cAMP accumulation induced by G_s-coupled neurotransmitter receptors by severalfold (Perkins & Moore, 1973; Sattin *et al*., 1975; Karbon & Enna, 1985; Makman *et al*., 1988). This paradoxical action of Bac is sensitive to pertussis toxin (Wojcik *et al*., 1989), and was proposed to include a Bac-induced strengthening of receptor coupling to AC (Scherer *et al*., 1989) and arachidonic acid metabolism (Duman *et al*., 1986; Schaad *et al*., 1989). However, a profile of modulation that could explain most directly the synergistic action of $G_{i/o}$-coupled receptors upstream of cAMP synthesis is presented by two types of AC isoforms, type II and IV. The activity of these enzymes is dramatically enhanced upon binding of βγ-subunits from G_i-proteins in the presence of α subunits from G_s-proteins (Tang & Gilman, 1991; for review, see Tang & Gilman, 1992; Anholt, 1994; Smit & Iyengar, 1998; Hanoune & Defer, 2001). These ACs may be involved in $G_{i/o}$-stimulated cAMP production in Xenopus oocytes (Uezono *et al*., 1997; Ulens & Tytgat, 2001), in ventricular myocytes (Belevych *et al*., 2001) in olfactory bulb (Olianas & Onali, 1999), and in cortex (Onali & Olianas, 2001), but their activity in intact neurons has not been addressed. In TC neurons, both type II and type IV AC are expressed, and type IV activity is up to 10-fold more pronounced than that of other ACs (Ihnatovych *et al*., 2002). This suggests that the marked cAMP production and the dependence on synergistic activation of both G_s- and G_i-proteins described here may be, at least in part, explained by the activation of this molecularly distinct AC in TC cells.

The activation of different types of $G_{i/o}$-coupled receptors showed variable capability to potentiate the action of Iso on I_h. Whereas A1 agonists failed to induce a potentiation, μ-opioid receptor activation induced a potentiation of I_h in the absence of β-adrenergic receptor activation. Thus, in mouse TC neurons, the coupling of $G_{i/o}$-coupled receptors to cAMP turnover detected by I_h, whether positive or negative, depends on the receptor type. Interestingly, in *Xenopus* oocytes, it was observed that

μ-opioid receptors unexpectedly potentiate HCN2-mediated currents in ~10% of the oocytes in a manner that is sensitive to AC blockers and limited by the availability of free α_s-subunits (Ulens & Tytgat, 2001). Thus, these GPCRs appear to preferentially couple to synthesis of cAMP that is detected by isoforms of HCN channels expressed in thalamus (Moosmang *et al*., 1999; Monteggia *et al*., 2000; Santoro *et al*., 2000).

Electrical stimulation in the nRt cell layer, which comprise the principal inhibitory afferents into the dorsal thalamus (for review, see Guillery *et al*., 1998; Crabtree, 1999), produced a $GABA_B$ receptor-mediated postsynaptic response on TC neurons, with properties similar to those found in rat (Ulrich & Huguenard, 1996). To study the effect of synaptically activated $GABA_B$ receptors on cAMP formation, the activation of K^+ currents was prevented and stimulation applied repetitively within the frequency range of thalamic oscillations (McCormick & Bal, 1997). This protocol induced minor changes in I_h, indicating that $GABA_B$ receptor activation was not strong enough to inhibit cAMP synthesis. Moreover, stimulation per se appeared not to induce release of other neurotransmitters that led to strong modulation of I_h. However, when stimulation occurred concomitantly with activation of β-adrenergic receptors, a marked potentiation of I_h amplitude was observed. Thus, the interaction of β-adrenergic receptors with $GABA_B$ receptors to control cAMP synthesis extends to the synaptic level, although additional factors released upon stimulation that may interact synergistically with β-adrenergic receptors can not be excluded (see e.g. Pedarzani & Storm, 1996). The present data therefore suggest that the GABAergic tone exerted by nRt cells may control the strength of cAMP synthesis induced by afferent neuromodulatory pathways. Interestingly, locus coeruleus neurons discharge synchronously with sleep-related EEG rhythms in the TC system, while they fire in isolation during states of waking (Aston-Jones & Bloom, 1981). Thus, activation of β-adrenergic receptors could take place over an increased level of activated $GABA_B$ receptors during states of sleep and during the transition between sleeping and waking, perhaps associating these phases with intracellular cAMP signals distinct from those during waking. Norepinephrine plays an important role in the control of cAMP-dependent gene expression during states of arousal in the TC system (Cirelli *et al*., 1996; Cirelli & Tononi, 2000) and discrete temporal profiles of cAMP transients contribute to determine the patterns of gene expression (Bacskai *et al*., 1993; Kaang *et al*., 1993). The physiological role of various types of cAMP signals induced by

GPCRs and via synergistic interactions between these could therefore extend into the determination of state-dependent patterns of gene expression.

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PAPER 2: Selective GABAergic control of higher-order thalamic relays

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I Introduction to the paper 2

The thalamus is composed of a dual system composed of first-order nuclei and the higher-order nuclei (HOn). Besides the defining difference in the origin of the excitatory inputs that drive the firing properties of the TC neurons (see chapter, II, 1b), there is emerging evidence for an increased diversity of GABAergic signaling, both in terms of neuronal subtypes in nRt (Pinault, 2004) and in terms of extrareticular inputs into HOn. For example, an extrareticular inhibitory input from the zona incerta innervates specifically HOn (Barthó et al., 2002).

As mentioned in the introduction (see chapter, II, 1a), the driving functions accorded to the cortical layer V are derived from morphological and physiological studies. The layer V corticothalamic terminals originate from thick axons and large terminals that contain round and large vesicles. The terminals innervate the proximal dendrites of TC cells in latero-posterior nucleus (LP), a visual HOn. Moreover, the synapses are encapsulated in glomeruli limited by glial cells (Bourassa and Deschênes, 1995; Feig and Harting, 1998; Van Horn and Sherman, 2004). Thus, these synaptic formations remind the arrangements made by the retinal terminals in the dLGN (see chapter II, 1b). They are different from the modulatory inputs arising from the neurons of cortical layer VI that innervate the distal dendrites of TC neurons of the HOn and relay nuclei. The synapses have small and round vesicles (Vidnyanszky and Hamori, 1994; Van Horn and Sherman, 2004). The role of cortex in the receptive fied of the pulvinar neurons was shown by making lesions in primate visual cortex. The lesions eliminated visual response in the neurons of the pulvinar (Bender, 1983). Therefore, the synapses formed by the CT axons arising from the cortical layer V have the arrangement of driving synapses and the responses of LP neurons to sensory stimulus are dependent on the cortex. The role of the pulvinar and its classification as higher-order nuclei has been reviewed in several publications (Casanova et al., 2001; Sherman and Guillery, 2002).

1. The Zona Incerta: a novel inhibitory afferent

As the nRt, the zona incerta (ZI) is an embryological derivate of the ventral thalamus. It forms a distinct nucleus at the base of the dorsal thalamus. The ZI has been subdivided into the rostral, dorsal, ventral and caudal groups (Nicolelis et al.,

1992), each having a largely different cytoarchitecture and neurochemical characteristics.

The ZI receives topographically organized cortical projections (Shaw and Mitrofanis, 2002) from the layer V exclusively, via collaterals passing to the dorsal thalamus (Levesque et al., 1996) or from axons passing to the brainstem or spinal cord (Mitrofanis and Mikuletic, 1999). They receive ascending inputs from collaterals of sensory pathways as the trigeminothalamic axons that arise from the whiskers and that terminate in the thalamic posterior nucleus (Po) (Veinante and Deschenes, 1999). ZI is also engaged in reciprocal connections with the pretectum and projects to the dorsal thalamus (Roger and Cadusseau, 1985; Shammah-Lagnado et al., 1985; Barthó et al., 2002). Moreover, the anterior pretectal nucleus provides a dense projection to the ventral part of the ZI. The ventral and dorsal ZI (ZIv) neurons project to the dorsal thalamus as shown by microelectrophoretic injections of tritiated proline and leucine (Ricardo, 1981). The result has been confirmed later with different techniques (Watanabe and Kawana, 1982; Power et al., 1999; Barthó et al., 2002). Interestingly, the preferential ZIv innervations of the dorsal thalamus are restricted to the HOn that are also specifically targeted by cortical layer V (Power et al., 1999; Barthó et al., 2002). The symmetrical synapses made by the ZIv innervations described above are primarily GABAergic, contact the proximal dendrites of the HOn neurons and vary considerably in size and shape (Barthó et al., 2002). The GABAergic nature of the terminals arising from the ZIv is also supported by a study showing that GABA and GAD immunoreactive cells are mostly found within the ventral sector of the ZI (Kolmac and Mitrofanis, 1998).

The characteristics and the functional role of ZI - HOn synapses remain to be investigated.

2. The anterior pretectum group: a novel afferent projecting to the higher-order nuclei

Besides the ZI, the anterior pretectum (APT) was found to project GABAergic afferents into the thalamus (Bokor et al., 2004). The anterograde fluorescent tracer microruby (a fluorescent dye, the tetramethylrhodamine, coupled to biotinylated dextran) was injected into APT and permitted to visualize the axonal projections of

APT cells. These were found to terminate exclusively in HOn in a topographic manner. Postembedding immunogold staining of GABA revealed the co-localization of GABA with the dye, demonstrating the GABAergic nature of the synapses made between the APT and the TC neurons. GABAergic terminals in the Po formed symmetrical synapses with multiple release sites on the proximal dendrites of TC neurons. An anterograde tracer was injected in nRt and APT. Thus, the terminals formed in thalamic Po by the two GABAergic nuclei could be compared. The terminals from APT were larger (surface area 1.2-7.2 μm^2) than the nRt terminals (0.6-1.2 μm^2). In addition, the APT terminals established up to 10 release sites preferentially on single proximal TC cells dendrites while the nRt terminals established mostly a single release site on distal TC cells dendrites. The properties of the APT terminals showing a higher number of release sites and a location on more proximal dendrites suggests a powerful control of relay cell firing by extrareticular GABAergic afferents (Bodor et al., 2004). To my knowledge, in HOn such as Po or LP, investigations of the inhibitory responses evoked by the direct stimulation of nRt have not been reported.

To obtain a functional correlate for the morphological evidence of a GABAergic projection from APT to HOn, we developed an *in vitro* preparation that preserved the connections between the APT and the thalamus. The development of this preparation was facilitated because the anterograde staining had revealed a strict horizontal path of projection between APT and HOn. Moreover, APT afferents were fluorescently stained *in vivo* prior to slice preparation. Thus, the positioning of stimulation and recording electrodes in the slice was guided by the signals in fluorescent microscopy. The stimulation electrode was placed close to the site of the injection and the recording trials were performed on cells co-localized with the labeled terminals.

II Paper 2

Selective GABAergic Control of Higher-Order Thalamic Relays

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Running title: Novel GABAergic afferent system in the thalamus

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Summary

GABAergic signaling is central to the function of the thalamus and has been traditionally attributed primarily to the nucleus reticularis thalami (nRT). Here we present a previously undisclosed GABAergic pathway, distinct from the nRT that exerts a powerful inhibitory effect selectively in higher order thalamic relays of the rat. Axons originating in the anterior pretectal nucleus (APT) innervated the proximal dendrites of relay cells via large GABAergic terminals with multiple release sites. Stimulation of the APT in an in vitro slice preparation revealed a $GABA_A$-receptor-mediated, monosynaptic IPSC in relay cells. Activation of presumed single APT fibers induced rebound burst firing in relay cells. Different APT neurons recorded in vivo displayed fast bursting, tonic or rhythmic firing. Our data suggest that selective extrareticular GABAergic control of relay cell activity will result in effective, state dependent gating of thalamocortical information transfer in higher order but not in first order relays.

Introduction

The inhibitory control of thalamocortical neurons has so far been attributed primarily to two neuronal subtypes; neurons of the nucleus reticularis thalami (nRT) and local interneurons. Reticular neurons innervate all dorsal thalamic nuclei and act as a global pacemaking structure in the generation of sleep-related thalamocortical oscillations (Pinault, 2004; Steriade et al., 1993) and as modifiers of the efficacy of thalamocortical transmission (Guillery et al., 1998). Interneurons are integrated in local circuits and involved in stimulus specific feedforward inhibitory actions (Sherman, 2004; Steriade, 2004).

Several thalamic nuclei receive giant, cortical excitatory afferents arising from pyramidal neurons located in layer V. These inputs are functionally and morphologically surprisingly similar to the peripheral inputs (Hoogland et al., 1991; Reichova and Sherman, 2004; Vidnyanszky et al., 1996). Based on these findings, nuclei receiving layer V afferents were distinguished as higher order thalamic relays as opposed to first order thalamic relays which receive driving input solely from peripheral sources (Guillery and Sherman, 2002; Sherman and Guillery, 2001). In

higher order thalamic relays cortical inactivation renders relay cells unresponsive to peripheral input, suggesting strong cortical drive in these nuclei, whereas response properties in first order relays are hardly affected (Bender, 1983; Diamond et al., 1992). Furthermore, higher order relays apparently do not participate in simple peripheral information transfer but in more complex functions (reward dependent firing, binding, attention, complex sensory coding) that necessitate cortical involvement (Ahissar and Arieli, 2001; Kinomura et al., 1996; Komura et al., 2001; Ward et al., 2002). Selective damage to higher order thalamic nuclei (also known as non-specific nuclei) in humans results in sensory neglect, or in more severe cases a persistent vegetative state, even when first order nuclei and the neocortex are relatively intact (Kinney et al., 1994; Llinás and Paré, 1997; Schiff et al., 2002). This strongly suggests that cortical integration of first order and higher order thalamic input is critical for cognitive functions.

Whether the characteristic layer V excitatory input in higher order nuclei is paralleled by a distinct inhibitory control is presently not known. Indeed, recent studies indicated that inhibitory inputs to higher order nuclei may have multiple origins (Bartho et al., 2002; Trageser and Keller, 2004). In the present study, we identified a previously unknown inhibitory pathway to the thalamus that originates from the anterior pretectal nucleus (APT) and effectively controls relay cell activity selectively in thalamic regions that are considered as higher order thalamic relays.

Results

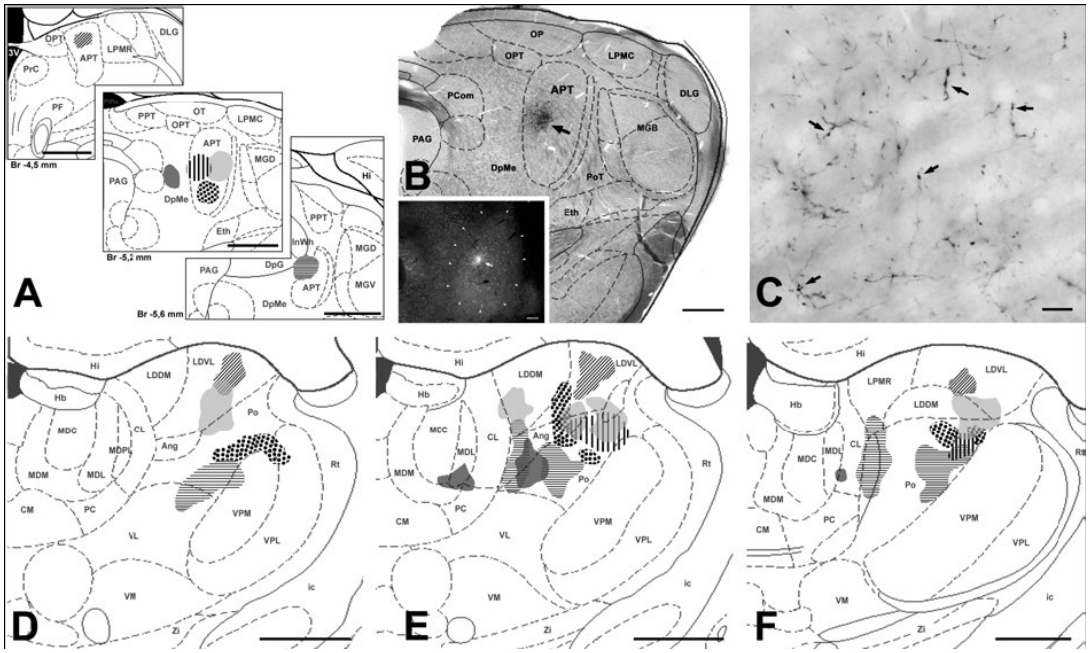
The APT-Thalamic Pathway

Small injections of biotinylated dextran amine (BDA) or Phaseolus vulgaris leucoagglutinin (PHAL) into various parts of the APT (Fig. 1A,B) resulted in dense patches of varicose fiber labeling in the ipsilateral thalamus (Fig 1C). Contralateral projections were negligible. The patches were surrounded by a more loosely organized network of terminals. The position of all injection sites relative to the APT was determined using double fluorescent labeling for the tracers and for parvalbumin, a neurochemical marker for the APT (Celio, 1990). The anterogradely labeled APT axons were confined to those thalamic nuclei that are considered as higher order relays (Sherman and Guillery, 2001). These included the posterior thalamic (Po),

ethmoid and posterior triangular nuclei (somatosensory), the laterodorsal (LD) and lateral posterior nuclei (visual), ventromedial nucleus (motor), the mediodorsal nucleus (associative), the centrolateral, paracentral and parafascicular nuclei (intralaminar) and the suprageniculate-limitans complex. First order relays including ventral posterolateral, ventral posteromedial, dorsal lateral geniculate, ventral medial geniculate nuclei and the anterior nuclear group were devoid of APT fibers. No APT fibers were found in the nRT. The APT-thalamic pathway was organized in a focal manner, suggesting a point-to-point rather than a diffuse information transfer. Dorsal injections resulted in dense clusters of terminals in laterodorsal and lateral posterior nucleus, whereas more ventral injections labeled patches of terminals in the more ventral nucleus posterior (Fig. 1D-F). APT injections always labeled a dense intranuclear recurrent collateral system within the APT. Sectioning of the brain in the horizontal plane following BDA injection into the APT revealed that many fibers from the APT to the thalamus ran mainly horizontally, facilitating the preparation of a pretecto-thalamic slice in which intact connections between the two structures could be studied in vitro (see below). Control injections caudally into the superior colliculus (n=5) resulted in a different innervation pattern that was focused on intralaminar nuclei. Rostral injections that were confined to thalamus (n=2) resulted in no axonal labeling in other thalamic nuclei.

Figure 1. Projection pattern from the anterior pretectal nucleus (APT) to higher order thalamic relays.

A) Six small injection sites (gray patterned patches) are shown on coronal maps (modified from Paxinos and Watson, 1998) at three rostrocaudal levels. Four of the six injection sites are located within the APT. One is situated near the medial border of the APT, another lies at the border of the APT and the adjacent deep mesencephalic region. (B) The light microscopic image of the injection site labeled by vertical stripes in A. In the inset the same injection site is depicted in a composite fluorescent image for BDA (indicated by a white arrow) and parvalbumin (fluorescently labeled cells within the area bordered by white arrowheads), a neurochemical marker for the APT. (C) High-power light photomicrograph demonstrating a patch of anterograde terminal labeling in the posterior thalamic nucleus after BDA injection into the APT. Arrows depict large axon terminals. In D-F the afferent fiber labeling is shown in coronal thalamic maps at three rostrocaudal levels following BDA injections at the locations presented in A with the same grayscale patterned coding. Note the focal terminal labeling in all types of higher order thalamic relays including visual (LPMR, LDDM, LDVL), somatosensory (Po, Ang) intralaminar (CL) and mediodorsal (MD) nuclei. Scales: A, D, E, F: 1 mm; B: 500 μm; inset in B: 200 μm; C: 20 μm. Abbreviations according to Paxinos and Watson (1998).



The ultrastructure of APT thalamic terminals was examined in five animals (3 BDA, 2 PHAL), in three of which quantitative analysis of GABA content was performed (2 BDA, 1 PHAL, n=91). The APT-thalamic terminals were large and elongated (long axis up to 5 μm, short axis 1μm), and they established multiple release sites (up to 10, examined in serial sections) on the proximal dendrites of relay cells (Fig. 2A,B). Almost all boutons possessed multiple rows of puncta adhaerentia-like specializations and were ensheathed by glial processes. Several GABAergic APT terminals were frequently clustered along a single dendrite, each establishing multiple contacts, suggesting a powerful control of relay cell activity (Fig. 2C,D). Postembedding GABA reaction revealed that the majority (82%; n=91) of the labeled terminals were GABAergic (for details see: Supplementary Table 1). In summary, our tract tracing experiments revealed a direct, focal, GABAergic APT-thalamic pathway that is strategically positioned to exert a strong inhibitory action selectively on thalamic neurons of higher order relays.

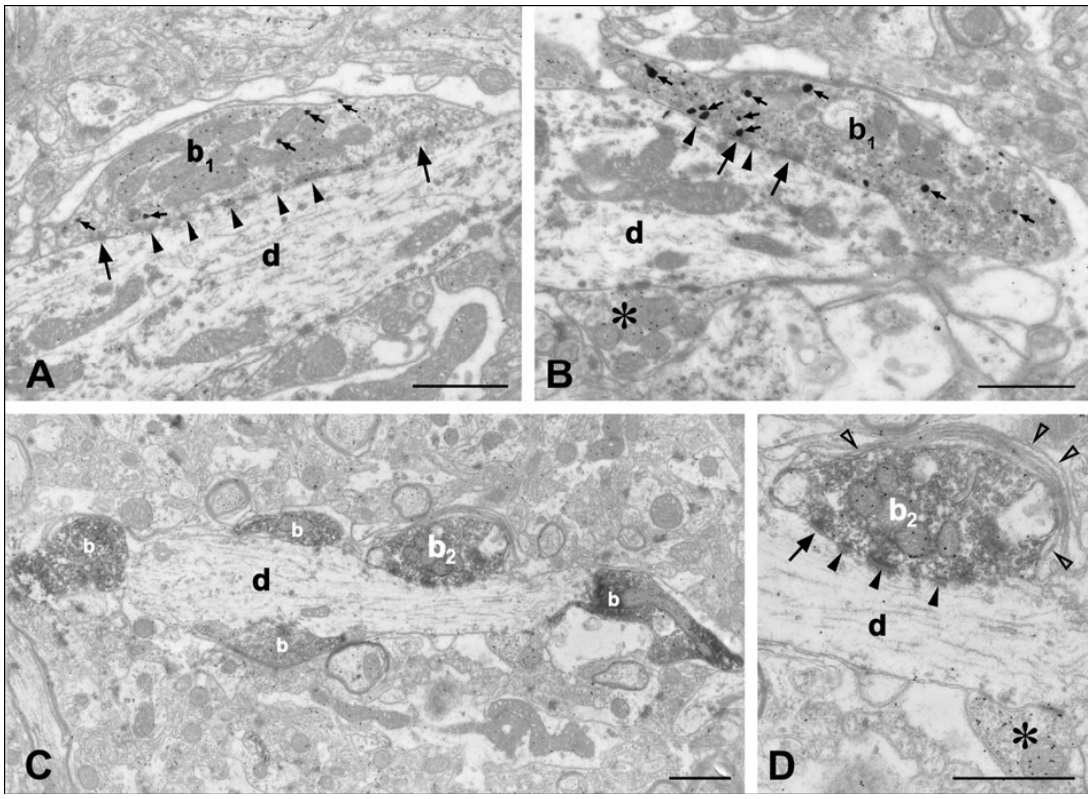


Figure 2. Anterior pretectal (APT) terminals are GABAergic in the thalamus.

(A, B) Electron micrographs of BDA labeled APT terminals in the nucleus posterior. Silver intensified preembedding gold staining (larger silver deposits, small arrows) was used to visualize the anterograde tracer, BDA (biotin dextran amine), whereas postembedding immunogold staining (small gold particles, as black dots) indicates GABA-immunoreactivity. A giant GABAergic terminal (b_1) is shown on two different electron microscopic sections (approximately 600 nm apart). The bouton establishes symmetrical synapses with multiple release sites (arrows) on the proximal dendrite (d) of a relay cell. Arrowheads show a row of puncta adhaerentia. Note also the glial coverage on the whole non-synaptic surface of the bouton.

(C) Low power electron micrograph depicts Phaseolus vulgaris leuco-agglutinin (PHAL) labeled GABAergic (small gold particles, black dots) boutons terminating on a thick proximal dendrite (d) in the nucleus posterior following PHAL injection into the APT. In this case PHAL was revealed using DAB as a precipitate (dark deposit within the labeled boutons: b, b_2). Note that almost all GABAergic terminals contacting the dendrite are labeled by the tracer. The bouton b_2 is shown at higher magnification in D. Arrow points to a symmetrical synaptic contact in D, arrowheads show puncta adhaerentia. Open arrowheads label the glial coverage of the non-synaptic surface of the bouton. The asterisks in B and D label GABAergic tracer-negative boutons terminating on the same dendritic profiles. Scales, A-D: 1 μm.

APT Control of Relay Cell Activity In Vitro

To physiologically characterize the synaptic contacts from APT to higher order nuclei, a horizontal in vitro slice preparation was developed that maintained the connections between the two areas. Slices were prepared from young rats (≤ 1 month) injected in vivo with the anterograde tracer micro-ruby into the APT (see Methods). The injection resulted in a patch of anterogradely labeled terminals in higher order nuclei similar to the adult animals (Fig. $3A_1$). Whole-cell patch-clamp recordings were obtained from cells located among the anterogradely labeled fibers (Fig. $3A_2$). Thalamocortical cells had a resting membrane potential of -63.8±1.7 mV (n=13) and showed rebound burst discharges upon transient negative current injections.

Electrical stimulation (0.2 Hz, 300-900 μA, 100 μs) of the injection site located in the APT in slices bathed in glutamate receptor antagonists (see Methods) evoked an outward current with an average amplitude of 66±11 pA (n=26, range 9-178 pA) (Fig. $3A_3$). This outward current was blocked completely and reversibly by the $GABA_A$ receptor antagonist bicuculline (25 μM), (Fig. $3B_1$, B_2, 1.2±1.0% of control amplitude, n=7, $p<0.001$), indicating that it was an IPSC mediated by activation of $GABA_A$ receptors. The average response latency, measured from the peak of the stimulus artefact to the onset of the response (Fig. 3C, inset) was 2.9±0.3 ms (n=26, range 1.0-6.6 ms). The time for the response to rise from 10% to 90% was 1.07±0.11 ms (n=14).

To determine whether APT neurons formed monosynaptic connections with neurons of higher order nuclei, the variation in response latency and the dependence of response amplitude on stimulation intensity was determined. Response latencies showed a narrow unimodal distribution with a standard deviation <0.4 ms (Fig. 3C, analyzed in 39-272 successive sweeps from n=6 cells). IPSC amplitude showed an all-or-none behavior as a function of stimulation intensity, with an abrupt appearance of the maximal response amplitude following increments in stimulation currents of 10-20 μA (Fig. 3D). This step-like intensity-response curve was found in 3 out of 4 connections tested, indicating that, in these cases, single APT-thalamic fibers were causing the observed functional effects. Taken together, both the stability in latency and the all-or-none characteristics of the response size are indicative of a monosynaptic projection between APT and higher order nuclei neurons.

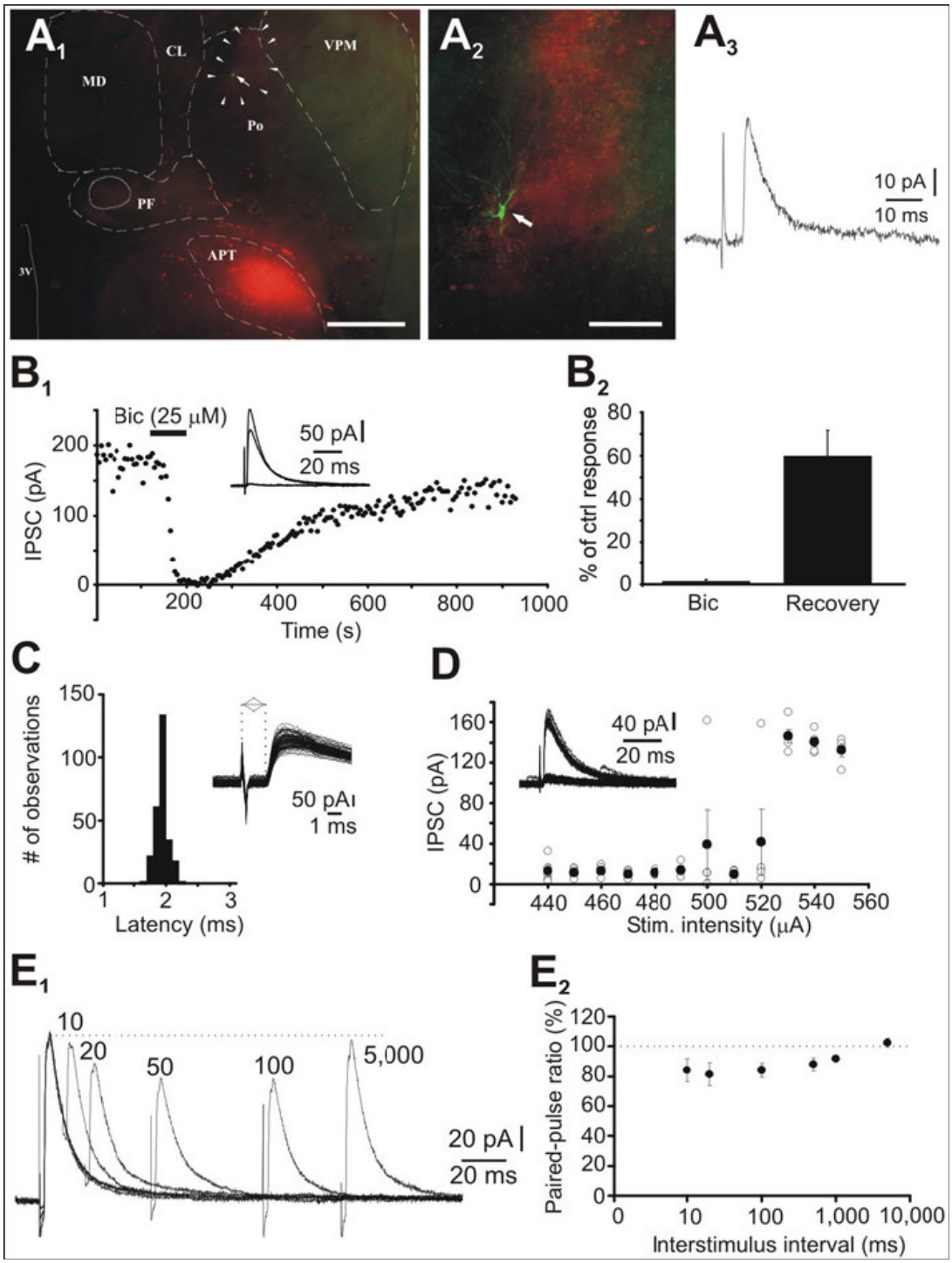
Given that APT neurons show a diverse pattern of ongoing spontaneous action potential discharge (see below), it was important to determine how the strength of inhibition exerted on thalamocortical neurons depended on prior activity. Here, the short-term plasticity of APT synapses was examined using dual extracellular stimulation within the APT at interstimulus intervals between 10 and 5000 ms (Fig. $3E_1$,E_2). Stimulation intensities were chosen such that the first pulse yielded a maximal synaptic response and that no failures were observed with the second pulse, such that alterations in the paired-pulse ratio could be attributed predominantly to modifications in presynaptic release processes. Under these conditions, each stimulus evoked a measurable IPSC and the APT-thalamic pathway showed a weak paired-pulse depression of <20% at intervals between 10 ms and 1s, but not after 5 s. Thus, short-term plasticity of the APT-thalamic synapse is characterized by a weak depression over a large range of time intervals and helps to sustain high-frequency inhibition of thalamic relay cells.

Figure 3. Projections from the anterior pretectum (APT) to higher order nuclei are GABAergic, monosynaptic and exhibit weak paired-pulse depression.

(A_1) Low power composite fluorescent micrograph demonstrating the injection site in the APT (red signal) and the micro-ruby labeled axon terminals in the thalamic nucleus posterior (Po) (arrowheads) in a horizontal slice. An arrow points to the recorded and filled thalamocortical cell labeled with a green fluorescent marker for biocytin. The same cell is shown in A_2 at higher magnification (green signal). Note that the cell is located within the micro-ruby-labeled axons (red signal). (A_3) Synaptic response evoked in the cell presented in A_1 upon extracellular stimulation in the APT. The holding potential was -50 mV for this and all subsequent recordings presented in this figure.

(B_1) Representative data from a single cell showing the time course of blockade of the inhibitory postsynaptic current (IPSC) by bicuculline (Bic, 25 μM). The inset shows 3 superimposed traces, each averaged from 10 consecutive sweeps during control, in the presence of bicuculline, and during recovery. (B_2) Histogram showing the average block of the synaptic response and the partial reappearance of the IPSC upon washout (n=7, $p<0.001$ for Bic vs. control).

(C) Latency histogram of responses from a representative cell, determined from the peak of the stimulation artefact to the beginning of the upward deflection of the IPSC. Note the narrow distribution of latencies of totally 272 synaptic responses within a time window <1 ms. Inset shows the superposition of 79 sweeps, with the dotted lines and the double-headed arrow indicating the latency.



(D) Plot of the amplitude of individual responses (open circles) and mean amplitudes (closed circles) versus stimulation intensity. The synaptic response shows a step-like dose-response curve, with a sharp threshold for stimulations intensities between 500 and 520 μA. There is no further increase with increasing stimulation intensity. Inset shows 50 consecutive sweeps obtained at stimulation intensities from 450 to 540 μA (10 μA steps, 5 responses per intensity). (E_1) Paired-pulse characteristics of IPSCs elicited by stimulation in the APT. Representative traces from a single cell are shown for 5 different

interstimulus intervals, indicated at the top of the traces. For compactness of the figure, the response obtained after the 5.000 ms interval is appositioned closely to the response after 100 ms. Dotted horizontal line indicates the peak response to the first of the two stimuli. Small variations in the amplitude of the first response (<10%) were compensated for by scaling the traces. (E_2) Time course of recovery from paired-pulse depression, plotted as the paired-pulse ratio (ratio of second vs. first IPSC in %) against interstimulus interval. Each data points represents the average of values obtained from 5-9 cells, with at least 5 interstimulus intervals tested per cell ($p<0.05$ for all values except 10ms).

Scales: A_1: 1mm, A_2: 200 µm, Abbreviations according to Paxinos and Watson (1998).

To assess the functional impact of APT activity on the discharge properties of neurons in higher order nuclei, APT afferents were stimulated while thalamic neurons were held in the current-clamp configuration. For IPSCs with amplitudes >50 pA at –50 mV, APT activity modulated firing characteristics of thalamic cells at both resting and depolarized membrane potentials (Fig. 4). When 1-10 IPSPs were evoked at 10 Hz, a frequency typical for IPSP barrages occurring during natural inhibitory input from the nRT (Bal et al., 1995; Steriade et al., 1985), a rebound depolarization (n=3) occasionally associated with a burst of action potentials (Fig. 4A) was produced. Similar to nRT-dependent rebound burst discharges in vitro (Bal et al., 1995), the probability of rebound burst discharge increased with the number of stimuli applied (Fig. 4A,B), strongly suggesting that the rebound response was due to the recruitment of a low-threshold calcium spike. However, in contrast to nRT, such stimulations never led to secondary, rhythmic bursts of IPSPs typical for intrathalamic oscillations in somatosensory thalamus (Huguenard and Prince, 1994), consistent with a lack of reciprocal excitatory-inhibitory loops between the APT and higher order nuclei. When thalamic cells were induced to discharge action potentials by injection of a suprathreshold current, APT activity completely prevented or attenuated on-going action potential discharge (n=3, Fig. 4C,D). Notably, in two cases, this effective control of neuronal discharge was also observed in presumed single-fiber connections. Thus, APT afferents, even single axons, can control both burst and tonic modes of action potential discharge of thalamic relay cells.

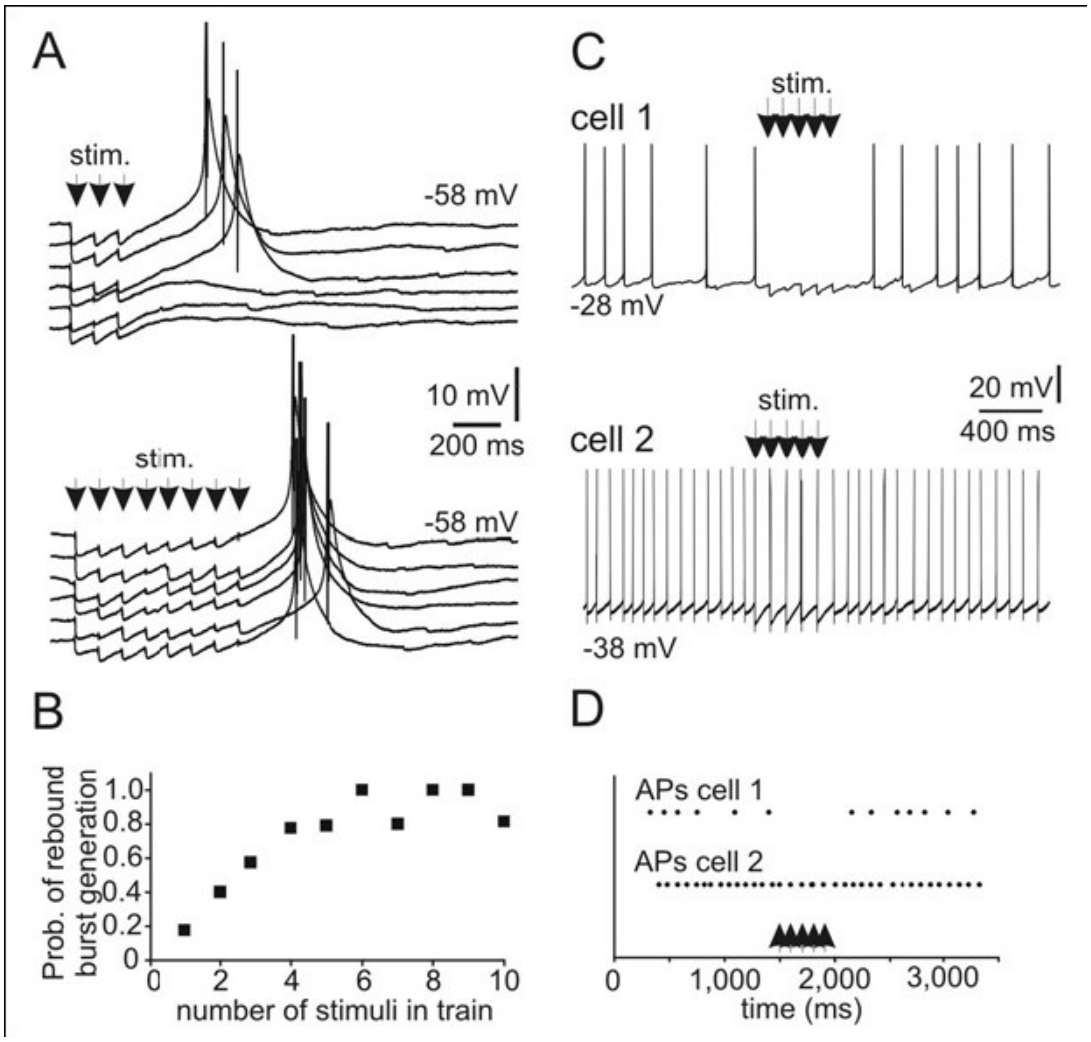


Figure 4. Control of thalamic cell firing by synaptic inputs from the anterior pretectum (APT). (A) Rebound burst responses of a representative cell upon application of 3 (top six traces) or 8 repetitive stimuli (bottom six traces) at a frequency of 10 Hz. Note the failure of rebound burst generation in 50% of the sweeps when only 3 stimuli were applied. Top and bottom group of traces are displaced vertically for clarity. Resting membrane potential is indicated to the right of the top trace of each group (-58 mV), but accounts for all traces. (B) Graph illustrating the probability (Prob.) of rebound burst generation as a function of the number of stimuli for the cell presented in A. (C) Inhibition of tonic action potential generation in response to repetitive APT stimulation. In cell 1 (top), discharge was fully prevented, whereas in cell 2 (bottom), the discharge rate was decreased. The cells were depolarized to potentials indicated upon d.c. injection of 360 pA (cell 1) and 530 pA (cell 2) to induce tonic firing. (D) Graphic illustration of the action potential discharge patterns of the two cells presented in C. Each action potential is symbolized as a point. Arrows indicate the time points at which stimulation was applied to the APT. Action potential amplitudes were truncated for clarity (see Methods).

Single Cell Activity in the APT In Vivo

To identify the natural pattern of inhibitory input a relay cell may receive in vivo, juxtacellular recording and labeling was performed in the APT with simultaneous EEG recording in the S1 cortex of urethane-anaesthetized rats. During recordings (5-15 minutes per neuron) the cortical EEG activity was dominated by a slow, low frequency (1-3 Hz), large amplitude oscillation, indicating deep anesthesia (Figure 5, Supplementary Fig 1-3). In order to examine the firing patterns of the APT neurons during desynchronized cortical activity, the slow oscillation was disrupted by applying tail-pinch for 15-60 seconds. This resulted in an immediate change in the power spectrum of the cortical EEG, the power of the 1-3 Hz component decreased by at least 10-fold and a faster 20-60 Hz band gained place in the spectrum. After termination of the tail pinch, the slow oscillation fully recovered. The rhythmicity of the cortical slow oscillation was also demonstrated by the multiunit activity of cortical neurons recorded by the EEG electrodes. During the slow oscillation, cortical units were active only on the upstates of the EEG, while in desynchronized states the cortical multiunit activity lost its rhythm.

APT neurons were found to display a surprisingly heterogeneous firing pattern. Based on neuronal firing patterns, 23 out of 27 recorded neurons in the APT could be classified into three distinct populations. Major differences were observed among these categories in baseline activity, coherence with EEG and in the response to tail pinch-induced cortical activation (Fig. 5, see also Supplementary Fig. 1-3 and Supplementary Table 2 online).

Fast bursting neurons (n=6) were characterized by high-frequency discharges of 4-16 action potentials during the slow oscillation, mixed with irregular single spikes (baseline firing: 9-26 Hz, for details see: Supplementary Table 2) (Fig. 5A). All neurons reached intraburst frequencies higher than 350 Hz (up to 600 Hz). The activity of most of these neurons (5 out of 6) displayed a moderate correlation with the cortical EEG, as demonstrated by the spike-triggered averages (Fig 5A, Supplementary Fig 1). Spontaneous or tail-pinch-induced EEG desynchronization decreased bursting activity, and the correlation with cortical activity was also abolished (Fig. $5A_1$).

Tonic cells (n=8) displayed monotonous or irregular single spike activity during the slow oscillation (baseline firing 7-21 Hz, for details see: Supplementary

Table 2). Very rarely, tonic cells fired spike doublets but they never displayed high frequency bursts, which characterizes fast bursting cells. The activity of tonic cells had no noticeable correlation with the simultaneously recorded slow cortical oscillation, as shown by their flat spike triggered EEG averages (Fig. 5B, Supplementary Fig 2). Cortical activation induced a slight (about 10%) increase of the firing frequency without changing the firing mode (Fig. $5B_1$).

Slow rhythmic cells (n=9) were characterized by a prominent slow (0.7-8 Hz, for details see: Supplementary Table 2) rhythmic activity, consisting of single spikes, doublets or bursts. Firing of all slow rhythmic cells was intimately related to the ongoing cortical slow oscillation and all action potentials were locked to the up-states of the EEG (Fig. 5C, Supplementary Fig 3). Slow rhythmic cells rarely fired during cortical down states unlike fast bursting or tonic cells. The intraburst frequency of slow rhythmic cells rarely exceeded 150 Hz (max 290 Hz), which is much slower than the bursts of fast bursting cells. EEG desynchronization changed the rhythmic activity into irregular single spiking (Fig. $5C_1$). In six of these neurons the firing ceased for some seconds following tail pinch or decreased to 0.1-3 Hz. Three slow rhythmic neurons responded to the cortical activation with elevated, 8-16 Hz tonic firing. All neurons regained their slow rhythmic firing pattern when the cortical slow oscillation reappeared.

Four of the 27 recorded neurons located in the APT could not fit into in either of the above mentions groups. Two of these displayed a highly irregular firing pattern, completely different from other observed activities in the APT. Both of them were located in the dorsalmost part of the APT, and thus might be part of a so-far-unidentified subpopulation of APT cells. The other two neurons showed mainly the characteristics of the tonic type, but occasional ~100-200 Hz doublets or short bursts were observed.

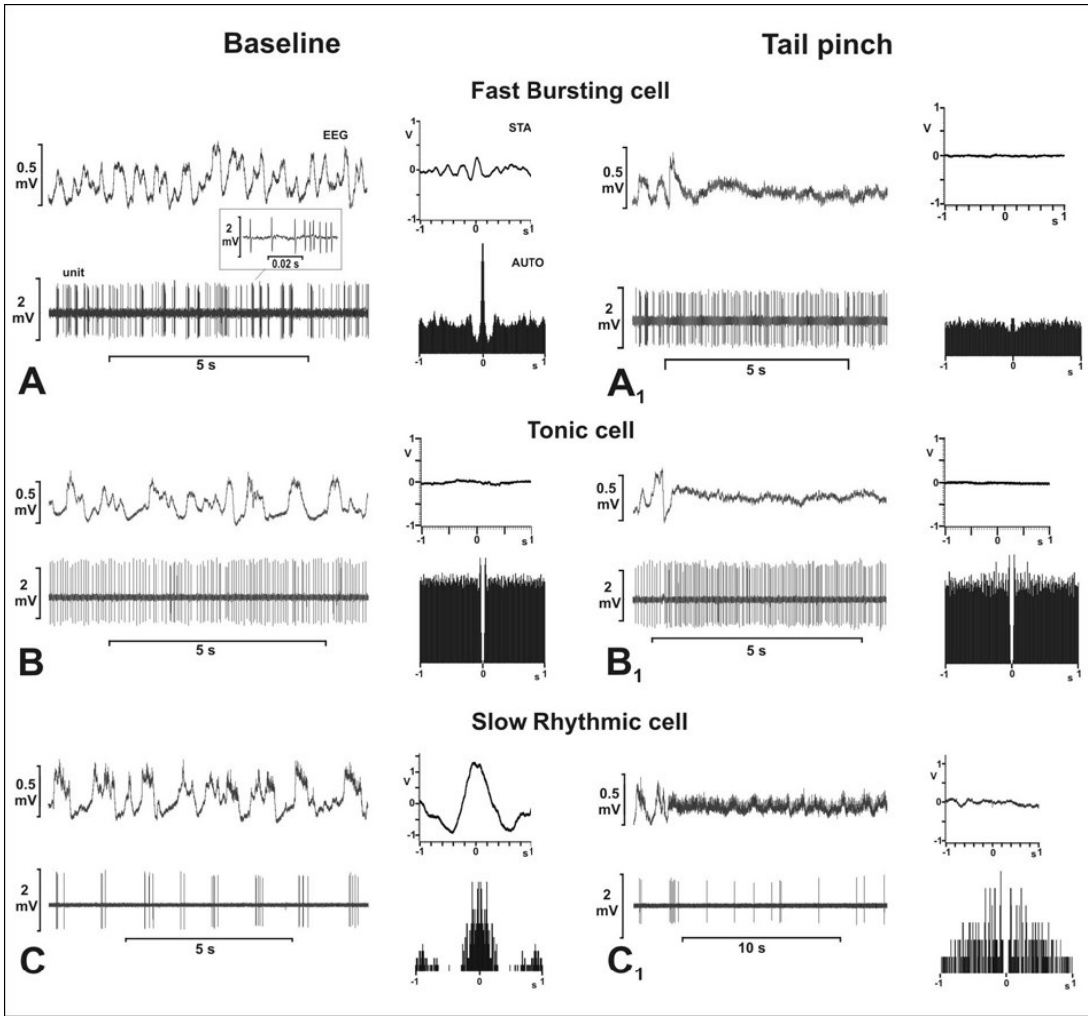


Figure 5. Three distinct types of neuronal firing patterns in the APT under urethane anesthesia. Fast bursting firing pattern during urethane slow oscillation (A) and tail pinch induced cortical activation (A_1). Autocorrelograms (AUTO) and spike triggered averages (STA) are shown. Note the prominent peak in the autocorrelogram indicating rhythmic bursting activity. In the inset in A typical burst pattern is shown. Intraburst frequency of the neuron can exceed 500 Hz. Note also that there is a moderate correlation of the EEG and the unit activity as shown by the STA, which was absent during tail pinch induced EEG desynchronization. Cortical activation was also accompanied by a great reduction in bursting activity, as shown by a drop in the central peak of the autocorrelogram.

(B) Tonic APT unit activity during urethane slow oscillation (B) and tail pinch induced cortical activation (B_1). The flat STA indicates a lack of correlation between the unit and the EEG. The unit displayed no apparent burst activity or rhythmicity, which is also demonstrated by the flat autocorrelogram without a central peak. Firing rate slightly increases during EEG desynchronization.

(C) Firing pattern of a slow rhythmic neuron in APT during urethane slow oscillation and tail pinch induced cortical activation (C_1). The autocorrelogram indicates the rhythmic spike clusters of this

cell type. The firing pattern of slow rhythmic cells was highly coherent with the EEG oscillation as shown by the high amplitude STA. During cortical activation (C_1) the rhythmic firing changed to a very slow irregular activity, or ceased for seconds. Note the changes in the autocorrelogram, indicating irregular activity, and the lack of correlation with the EEG as shown by the flat STA.

In a subset of the recorded APT neurons (n=14) light and electron microscopic examination were performed to identify the neurochemical and morphological properties of the cells, and to establish the type of synapses they form on their postsynaptic targets. The location of these cells within the APT and their reconstructed dendritic tree and local axonal arbor are shown in Supplementary Fig 4 online. All three neuron types had fusiform or irregularly shaped soma with 4-5 primary dendrites and a dense local axon arbor network (Fig. $6A_1$,B_1,$C_{1,2}$). The dendrites of fast bursting neurons had nearly twice as many branchpoints (average: 21; range: 19-24) compared to slow rhythmic (average: 12; range 6-15) or tonic cells (average: 6.5; range: 2-11). In addition, the distal dendrites of fast bursting cells were covered by peculiar, filopodial, spine-like structures (Fig. $6A_1$), which were found to be contacted by vesicle-filled terminals at the electron microscopic level (n=4; data not shown). The dendrites of tonic and slow rhythmic cells were only sparsely spiny. As the APT is rich in parvalbumin-immunoreactive neurons, we tested whether any difference in the parvalbumin content of the physiologically different cell populations could be observed. Parvalbumin-immunoreactivity was successfully performed in a subset of unequivocally identified cells. All fast bursting cells (n=3) were strongly parvalbumin-positive (Fig. 6A), whereas slow rhythmic cells (n=4) were parvalbumin-negative (Fig. 6C). Tonic cells (n=5) consistently displayed weak immunoreactivity for parvalbumin (Fig. 6B). These data demonstrate that neuronal cell classes established by physiological criteria correlate with distinct morphological characteristics, substantiating the relevance of the classification.

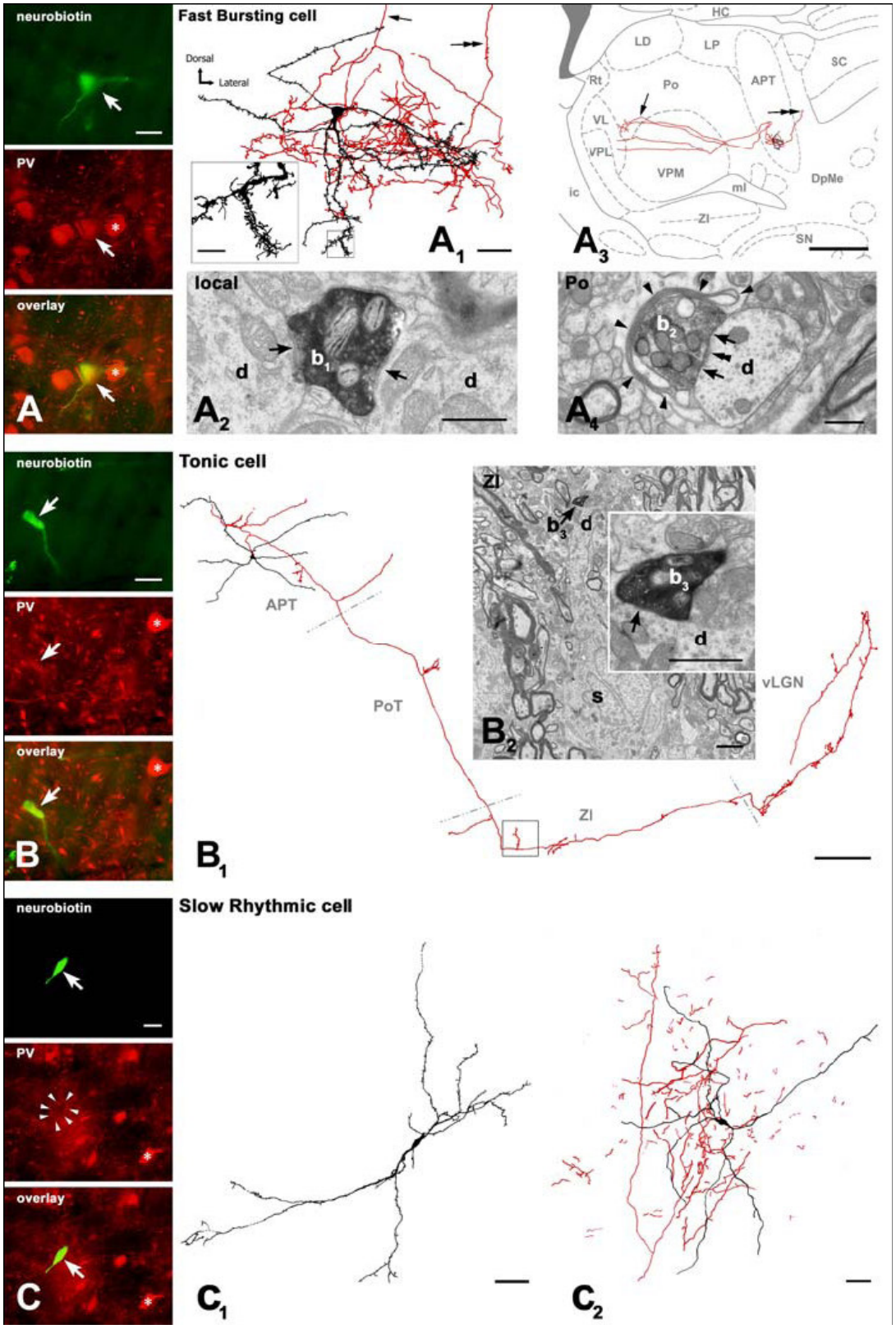
The ascending axon collaterals of two fast bursting cells could be followed to the thalamus (Fig. $6A_3$). In one of them, a cluster of (70-80) labeled terminals was recovered in the posterior thalamic nucleus. Electron microscopic analysis of these boutons demonstrated that the examined terminals established multiple symmetrical synapses on the thick, proximal dendrites of relay cells (n=8). Seven of these terminals also showed the ultrastructural characteristics established by the tracing

(large size, multiple puncta adhaerentia, glial ensheathment) (Fig. $6A_4$). Local axon terminals of the same fast bursting cell also established symmetrical synapses on somata or proximal dendrites of APT cells (n=12), but these terminals were smaller and had no puncta adhaerentia (Fig. $6A_2$). The terminals of a tonic cell were analyzed at the electron microscopic level in the case of a neuron that had collaterals in the posterior triangular thalamic nucleus (n=9 terminal), the zona incerta (n=3), the ventral lateral geniculate nucleus (n=5) as well as locally (n=3). All 20 terminals established single symmetrical synapses (Fig. $6B_2$). No puncta adhaerentia were observed. The identification of these cells provides the morphological basis for the monosynaptic inhibitory connection between APT and thalamus.

Figure 6. Morphological characteristics of the three physiologically identified cell types. APT-thalamic projection at the single cell level.

(A) A fast bursting cell is visualized with a green fluorescent marker for neurobiotin. Parvalbumin immunostaining of the same section (PV, in red) demonstrates that the neurobiotin filled cell (arrow) is strongly PV-positive. (A_1) Cell body, dendrites (in black) and local axonal arbor (in red) of the same cell are shown. Note complex filopodial spine like structures accumulating in distal dendritic regions (see inset). (A_2) A local axon terminal of the labeled cell establishes two symmetrical synaptic contacts (arrows) with non-labeled dendrites (d) in the APT. (A_3) Projecting axons of the reconstructed cell are manually mapped to parasaggital plane using Paxinos and Watson (1998). Four ascending main axons reach the thalamus. Terminals were recovered in the nucleus posterior. A descending axon collateral projects towards the superior colliculus (double arrow). (A_4) At the electron microscopic level the labeled terminals of the bursting cell establishes symmetrical synapses (arrows) onto thick, proximal dendrites in the thalamus. Arrowheads label glial coverage, double arrowhead points to a punctum adherens. Note that this thalamic terminal had similar characteristics to those visualized by anterograde tracing.

(B) A neurobiotin filled tonic cell (green) is weakly parvalbumin positive (red). Asterisk labels a neuron strongly positive for parvalbumin. (B_1) Soma and dendrites are shown in black, projecting axon is in red in a coronal plane. The neuron is incompletely filled, but a major ascending axon was recovered, which reaches the posterior triangular nucleus of the thalamus (PoT), the dorsal part of the zona incerta (ZI) and ventral lateral geniculate nucleus (vLGN). At the electron microscopic level the axons of tonic cell established symmetrical synapse onto thick, proximal dendrites in all the three target areas. (B_2) A neurobiotin filled bouton (b_3) is demonstrated here forming symmetrical synaptic contact (arrow) with a proximal dendrite (d) in the zona incerta in a low power electron micrograph. The same bouton is visible at higher magnification in the inset.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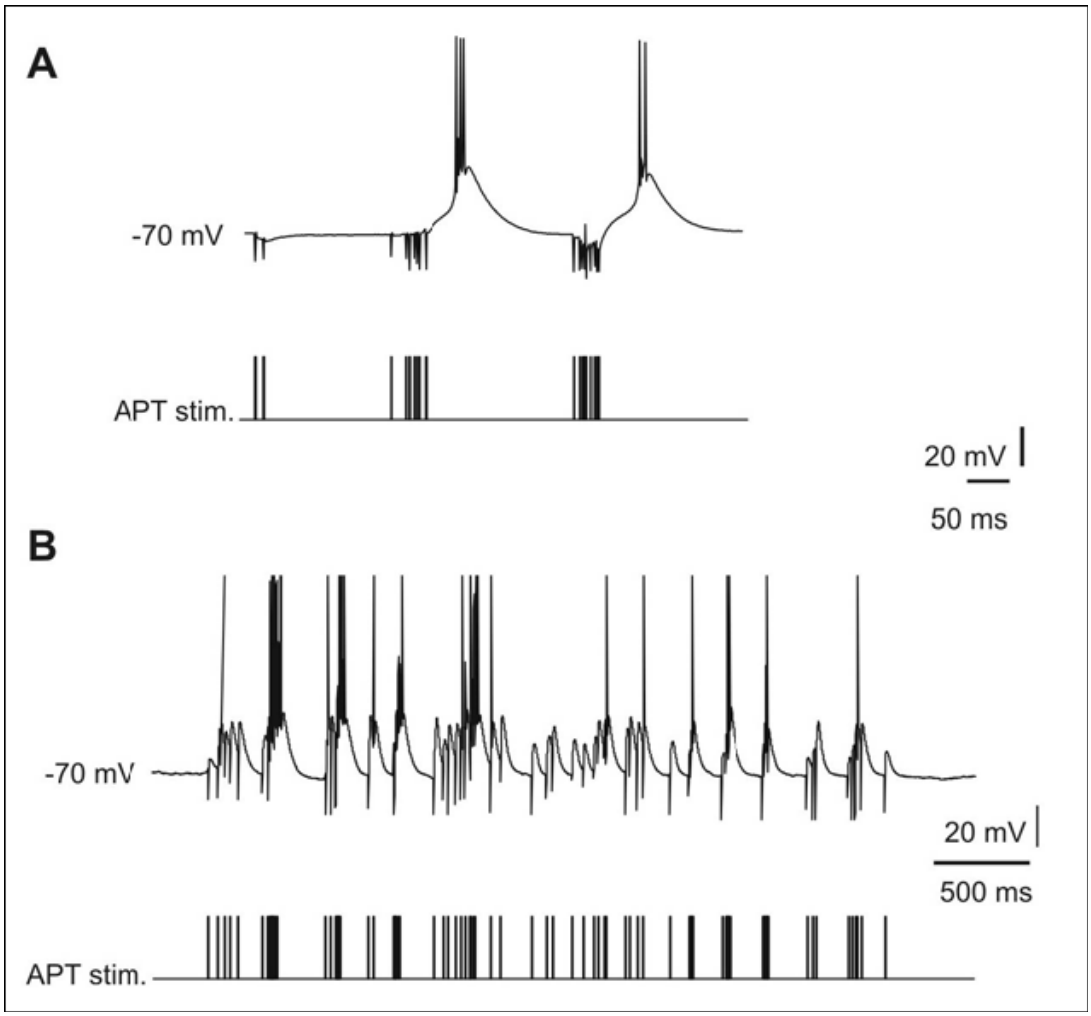
(C) Slow rhythmic cells were parvalbumin negative. The neurobiotin labeled, parvalbumin–negative cell body of the cell C_1 is shown. C_1 and C_2 demonstrate two reconstructed slow rhythmic cells, cell bodies and dendrites are in black, axons are shown in red. Dense local axon arbor extended with similar dimensions to the dendrites. White arrows in A, B, C point to neurobiotin filled cells visualized by a green fluorescent marker. Asterisks labels PV-positive unfilled neurons. Scales: A-C: 20 µm; A_1, $C_{1,2}$: 50 µm; $A_{2,4}$: 500 nm; A_3: 1 mm; B_1: 200 µm; B_2: 2 µm, inset 1 µm. Abbreviations according to Paxinos and Watson (1998).

In Vitro Stimulation With In Vivo Firing Pattern

Finally, the impact of natural APT discharges on the behavior of thalamic cells was addressed. APT discharge patterns monitored in vivo were therefore used as stimulation protocols in vitro (see Methods). The neuronal activity of a fast bursting cell (see Fig. 5A) was applied to the APT while recording from relay cells held in current-clamp around resting membrane potential. Using a short stimulus train consisting of a doublet and two burst discharges clearly indicated that rebound burst firing of relay cells could be evoked following application of burst-like stimuli (Fig. 7A). In a stimulus train mimicking the natural firing of a fast bursting cell (Fig. 7B), the probability of rebound burst firing was determined for three periods of 3-32 Hz (5-14 stimuli) and for 6 periods of 240-300 Hz (5-10 stimuli). When evaluating traces from 3 cells, the average probability of rebound burst discharge was 67.2±5.6% for high-frequency stimuli, whereas it was limited to 20.2±11.0% after a tonic period ($p<0.002$). This indicates that natural burst firing of APT cells may be a particularly effective means of promoting rebound activity in higher order nuclei.

Figure 7. Action potential discharge patterns elicited in thalamic cells of higher order nuclei upon stimulation of the anterior pretectum (APT) according to single-unit recordings in vivo. (A) Burst discharges in the APT elicit rebound burst discharges in a thalamic cell. (B) APT stimulation patterned by the in vivo activity of a fast bursting cell elicit rebound bursts in thalamic cells, preferentially following bursting in the APT. Action potential amplitudes were truncated for clarity (see Methods).



Discussion

In the present study, we demonstrate that higher order thalamic nuclei are under the control of a powerful GABAergic afferent pathway originating in APT. This inhibitory system is morphologically and functionally distinct from the GABAergic innervation arising from the nRT. Therefore our data suggest that the inhibitory control of relay cell activity is qualitatively different in first order and higher order nuclei.

Identification of a GABAergic APT-thalamic projection is consistent with previous tracing studies (Cadusseau and Roger, 1991; Terenzi et al., 1995) and the abundant glutamic acid decarboxilase mRNA signal in the APT (Benson et al., 1992; Esclapez et al., 1994). Our tract tracing data were confirmed at the single cell level

and by physiological data, excluding major confounding effects by spurious tracer labeling. The APT-thalamic pathway was found to be highly focal, unlike many other ascending inputs to the thalamus from subcortical centers (Hallanger et al., 1987; Jourdain et al., 1989) This implicates a localized and specific inhibitory action rather than a diffuse modulatory system. Interestingly, a GABAergic pretecto-thalamic pathway from the non-retinorecipient pretectum has also been described in reptiles (Kenigfest et al., 2000), suggesting the evolutionarily conservative nature of this pathway. An APT-higher order projection has already been revealed in the cat as well (Berman, 1977; Graham and Berman, 1981; Robertson et al., 1983), although its GABAergic nature remains to be verified.

It has to be stressed that the pathway described here is distinct from that arising mainly from another pretectal nucleus in carnivores, the nucleus of the optic tract (Cucchiaro et al., 1993). These latter fibers innervate the first order dorsal lateral geniculate nucleus, which is always avoided by APT fibers. Afferents from the nucleus of the optic tract contact local interneurons with terminals resembling nRT boutons (Cucchiaro et al., 1993; Wang et al., 2002), unlike APT-thalamic fibers forming terminals with multiple release sites on the proximal dendrites of relay cells.

Comparison of Reticular and Extrareticular Inhibition

The present data and previous studies indicate that reticular and extrareticular (i.e. originating outside nRT) GABAergic inputs in the thalamus are organized according to different morphological and functional principles. Small sized nRT boutons form single inhibitory synaptic contacts via single active zones mainly with the distal dendritic regions of relay cells in all thalamic nuclei (Cucchiaro et al., 1991; Liu et al., 1995; Montero and Scott, 1981). In contrast, APT terminals innervate exclusively higher order relays, and selectively target the proximal dendritic region of relay cells via multiple release sites (Bodor, Bokor, Acsády, unpublished data). Reticular input evokes a slow $GABA_B$-mediated inhibition besides fast $GABA_A$ IPSCs, which play a crucial role in determining the strength and latency of rebound burst responses in thalamocortical oscillations (Huguenard, 1998). In contrast, in our hands, extrareticular input showed only $GABA_A$ responses, even after repetitive stimulation, which suggests that it exerts fast, phasic control of relay cell activity.

Under urethane anesthesia, nRT cells are characterized by a tonic or burst firing pattern, and they can shift between modes (Pinault, 2004). Morphologically, nRT neurons are quite homogeneous and display only subtle differences (Lubke, 1993). In contrast, in vivo juxtacellular recording under urethane anesthesia in this study disclosed three types of neurons in the APT with distinct state dependent firing patterns and morphology.

The afferent-efferent connectivity pattern of nRT and APT is significantly different. The former have few intranuclear collaterals (Pinault and Deschenes, 1998) whereas all three types of APT neurons in this study had a profuse local axon network. In addition, the nRT is reciprocally connected to the thalamus, whereas APT does not receive direct thalamic feedback. The sole output of the nRT is the thalamus, whereas APT has also been shown to innervate brainstem motor centers (Terenzi et al., 1995). nRT receives collaterals from cortical layer VI (Steriade et al., 1997), whereas APT is contacted by cortical layer V (Cadusseau and Roger, 1991; Foster et al., 1989); thus the layer V-APT-thalamus circle represents a separate cortico-thalamic pathway, parallel to the layer VI-nRT-thalamus loop. Finally, APT but not nRT receives peripheral inputs (Veinante and Deschenes, 1999), which enables the APT to gate ascending sensory information in a feedforward inhibitory manner.

The above analysis strongly suggests that the principles of operation are different when comparing reticular vs. extrareticular thalamic inhibition, and that the extrareticular system represents a structurally and functionally novel component in thalamocortical systems. The extensive recurrent collateral system, the ascending and the descending projections of the APT indicate complex information processing and integrative control of functionally related thalamic and brainstem regions.

Effective Control of Relay Cell Activity by Extrareticular Inhibition

In vitro experiments in the slice preparation containing interconnected APT and higher order nuclei clearly revealed the presence of monosynaptic GABAergic IPSCs that showed unitary characteristics in the majority of cases. The large size of APT terminals with multiple release sites and their proximal dendritic location are morphological indicators of a synapse capable of maintaining transmission at high presynaptic firing rates, and thus can play a crucial role in the control of neuronal activity (Xu-Friedman and Regehr, 2004). Indeed, activation of a single APT fiber

was able to induce rebound burst firing in relay cells. Interestingly, in the cerebellum corticonuclear synapses show similar ultrastructural features, including multiple adjacent presynaptic release sites, and they exert a powerful inhibition of nuclear cells (Telgkamp et al., 2004). Due to the confluence of GABA from these multiple release sites to a shared pool of postsynaptic receptors, synaptic depression is minimal even at artificially high release probabilities. In the case of APT-thalamic synapses, the comparatively slow rise time of IPSCs, the weak paired-pulse depression at all interstimulus intervals and the lack of a temporal structure of recovery from paired-pulse depression are features consistent with other studies on GABAergic synapses with multiple release sites (Kraushaar and Jonas, 2000; Telgkamp et al., 2004).

Trains of stimuli showed that activity in the APT is able to influence thalamic information transfer in both burst and tonic firing modes. High-frequency discharges of APT cells proved particularly efficient in evoking rebound bursts in relay cells. Altogether, the APT input appears functionally as effective as the well-known inhibitory interface of thalamocortical networks, the reticular nucleus, although additional characteristics critical for nRT function, such as synaptic facilitation during burst discharges (Kim and McCormick, 1998) and the exact conditions required for the possible synaptic activation of $GABA_B$ receptors, remain to be determined.

High-frequency discharge in GABAergic inputs from the APT may help to induce coherent oscillations and/or correlated rebound responses in a group of higher order relay neurons that can synchronize simultaneously active thalamocortical neuronal ensembles. This activity is then conveyed to cortical areas and will be integrated in the cortex together with the specific information ascending through first order thalamic relays (Jones, 2001; Llinás and Paré, 1997). The relative timing of these two types of thalamocortical activity will dynamically change the way cortical networks integrate thalamic inputs from various sources.

The Extrareticular System

We previously described a GABAergic projection from the zona incerta to higher order thalamic nuclei that has similar morphologically characteristics to the APT-thalamic pathway (Bartho et al., 2002). Recent data indicate that the zona incerta effectively controls the response properties of higher order thalamic relays (Trageser and Keller, 2004). Zona incerta is reciprocally connected with the APT (May et al.,

1997; Terenzi et al., 1995), both innervate brainstem motor centers, and both receive layer V cortical input. These data indicate that rather than being localized to a single nucleus, the extrareticular GABAergic system may encompass several interconnected nuclei at the mesencephalic/diencephalic junction.

Our conclusion is that the peculiar characteristics of extrareticular GABAergic inputs in the thalamus enable this system to impose efficient state-dependent gating mechanisms on thalamocortical and corticothalamic information processing selectively in higher order thalamic nuclei.

Experimental Procedures

Male Wistar rats (≤ 1 month, old or adults (300-400g); Charles River, Hungary) were used for all experiments. All experimental procedures were performed according to the ethical guidelines of the Institute of Experimental Medicine Hungarian Academy of Sciences and approved by the Ethical Committee.

Tract Tracing Experiments

Two different anterograde tracers, biotin-dextran amine (BDA; 10000 MW, Molecular Probes, Leiden, The Netherlands, 10% in saline; n=10) and Phaseolus vulgaris leuco-agglutinin (PHAL, Vector Labs. Burlingame CA, 2,5% in 0.1 M phosphate-buffer (PB); n=5) were used to describe the APT-thalamic pathway. Since contralateral labeling was very sparse, unilateral (n=9) and bilateral (n=6) injections were also used. Animals were mounted in a stereotaxic frame and iontophoretic injections of BDA or PHAL (10 min, 0.5–4.0 μA, 2–7 s on/off duty cycle) were made via a glass capillary (tip outer diameter: 5-20 μm) at the following coordinates: 4.8-5.2 mm posterior, 1.7-2.0 mm lateral and 4.5-5.5 mm ventral to the Bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

After a survival time of 4-7 days, rats were deeply anaesthetized by Equithesin (chlornembutal, 0.3 ml/100 g), then perfused through the heart first with physiological saline (2 min), then with 100 ml fixative containing 2% paraformaldehyde (TAAB, UK) and 1% glutaraldehyde (TAAB) in acetate buffer (pH=6.0; 3 min), followed by 400 ml fixative containing 2% paraformaldehyde and 1% glutaraldehyde in borate

buffer (pH=8.5; 50 min). Coronal or horizontal sections (50-60 μm thick) were cut on a Vibratome, washed, cryoprotected in 30% sucrose in 0.1 M PB overnight, and freeze-thawed in aluminum-foil boat over liquid nitrogen. The position of all injection sites was localized using double fluorescent methods for the tracer and parvalbumin. For the mapping of the APT-thalamic pathway, only those injections were considered in which no retrograde labeling was found in other nuclei in order to minimize spurious collateral labeling (n=6). For fluorescent labeling of parvalbumin neurons rabbit anti-parvalbumin (1:200, Baimbridge and Miller, 1982) was used. The second layer was Alexa 594 conjugated goat anti-rabbit (1:200; Molecular Probes). BDA was visualized with Alexa 488 conjugated streptavidin (StA 488; 1:1000; Molecular Probes). PHAL was incubated first with goat anti-PHAL (1:10000; Vector) overnight, then with FITC-conjugated donkey anti-goat (1:100; Jackson, West Grove, PA). The sections were mounted and covered by Vectashield and evaluated by a fluorescent microscope (Zeiss Axioscope). Injection sites and labeled fibers were then visualized also with 3,3'-diaminobenzidine (DAB) or nickel intensified DAB (DABNi) reaction. In the case of BDA, sections were incubated with avidin biotinylated-horseradish peroxidase complex (ABC, Vector Laboratories, 1:300) in TBS for 2 hours, then developed with DABNi. Sections from animals injected with PHAL were first incubated with rabbit anti-PHAL (1:10000; Vector) overnight, the second layer was biotin-SP-donkey anti-rabbit F_{AB} fragment (1:300; Jackson) for 2 hours, the third layer was ABC. In our experimental conditions postembedding GABA immunogold labeling was not always reliable for quantitative purposes if DAB was used as a chromogene for the tracers. The DAB precipitate could not be etched from heavily labeled terminals, which increased the chance of identifying a terminal as falsely GABA-negative. To overcome this difficulty the tracer was developed using the preembedding gold method. For preembedding immunogold staining following ABC incubation, the ABC signal was amplified by Biotinyl tyramide reagent (1:50, 15 min.; PerkinElmer Life Sciences, Boston, USA), then sections were incubated in 1 nm gold conjugated streptavidin (1:50; Aurion, Wageningen, The Netherlands) dissolved in TBS containing 0.8% BSA and 0.1% gelatin overnight, postfixed in 2% glutaraldehyde in TBS, then silver intensified with Aurion R-Gent intensification kit. All sections were treated with OsO_4 (1% for 45 min. for DAB staining, 1% 1 min. and 0.5% for 20 min. in 4 ºC for immunogold staining in 0.1M PB), dehydrated in ethanol

and propylene oxide, and embedded in Durcupan (ACM, Fluka, Buchs Switzerland). During dehydration the sections were treated with 1% uranyl acetate in 70% ethanol for 40 min. Selected blocks containing identified thalamic nuclei were reembedded and 65 nm thick ultrathin sections were cut with an Ultramicrotome (Reichert), and alternate sections were mounted on copper or nickel grids. Postembedding GABA immunostaining was carried out on nickel grids according to the protocol of Somogyi et al. (Somogyi et al., 1985). Light microscopic images were scanned with a digital camera (Olympus, DP 70). The electron micrographs were taken on a HITACHI 7100 electron microscope, the negatives were scanned, and brightness and contrast were adjusted if necessary using Adobe Photoshop 7.0.

In Vitro Electrophysiological Experiments

Horizontal slices (400 μm thick) were prepared from rats labeled in vivo at postnatal day 16 with the anterograde fluorescent tracer micro-ruby (Molecular Probes) in the APT and rats were allowed to survive for 2-14 days before slices were prepared. Gas-anesthesia and decapitation occurred according to the guidelines of the Veterinary Institute of the Canton Basel-Stadt. Slices were prepared following standard procedures. For electrophysiological recordings, slices were constantly perfused at 2-3 ml/min with a solution containing (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 1.5, $MgCl_2$ 2, dextrose 18, L(+)-ascorbic acid 1.7, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f] quinoxaline-7-sulfonamide disodium salt (NBQX) 0.01, D,L-2-amino-5-phosphonovalerate (APV) 0.1, adjusted to pH 7.4 by constantly bubbling with 95% O_2/5% CO_2 and to an osmolarity of 310 mOsm. Whole-cell voltage-clamp recordings were obtained from neurons located in the fluorescently labeled area in higher order nuclei using patch pipettes (2.5-3.5 MΩ, WPI, Sarasota, Florida, pulled on a Narishige Puller PP-83) filled with (in mM): KGluconate 130, HEPES 10, KCl 10, K_2-ATP 2, Na-GTP 0.2, Phosphocreatine 10, $MgCl_2$ 2, adjusted to pH 7.25 and to an osmolarity of 290 mOsm. Data were acquired using an AxoPatch200B amplifier (Axon Instr., Union City, CA), filtered at 2 kHz and digitized at 10 kHz. Liquid junction potential (12 mV) was not taken into account for the recordings presented. Series resistance (8-15 MΩ) was monitored for stability throughout the experiments and data were not included in the analysis if series resistance changed by more than 20%. The positioning of a bipolar tungsten

stimulation electrode (115 μm spacing, Frederick Haer & Co., Bowdoinham, ME) was guided using the fluorescent signal in the APT and electrical shocks (100 μs each) were applied using a stimulus isolator (A360, WPI, Sarasota, FL). Stimulation waveforms derived from in vivo discharges of APT neurons were applied to the isolator via stimulus files generated in Clampex 9.2. Baseline stimulation frequency was 0.2 Hz, paired stimuli were applied at 0.1 Hz. At least 5-10 sweeps per interstimulus interval were averaged to obtain an accurate quantification of IPSC amplitudes. For all recordings presented in voltage-clamp, the holding potential was -50 mV. Note that the properties of action potentials and associated afterhyperpolarizations may be distorted due to the electronic design of the Axopatch 200B amplifier (Magistretti et al., 1996). For display purposes, action potential but not afterhyperpolarization amplitudes were truncated. Electrophysiological data were analyzed using PClamp 9.2. software. Data are presented as means±s.e.m. unless indicated otherwise. Statistical significance ($p<0.05$) was assessed using paired two-tailed t-tests.

For histological recovery of the recorded cells, 0.2% biocytin (Sigma-Aldrich, Sinsheim, Germany) was included in the recording solution and the patch pipette was carefully withdrawn at the end of the recordings. Slices were immediately transferred to a fixative solution (4% paraformaldehyde with 15% picric acid, 4 °C). Slices were fixed for 30 minutes, washed twice in phosphate buffer (0.1 M), before being transferred to a cryoprotective solution (30% sucrose in 0.1 M PB, 0.05% NaN_3) and stored at -20 °C. In vitro biocytin labeled cells were visualized by StA 488, then by ABC-DABNi reaction (for details: see above).

In Vivo Juxtacellular Recording and Labeling

Rats were implanted in the S1 cortex with a bipolar tungsten EEG electrode (surface and 1.5 mm depth, in vitro impedance: 0.8-1.2 MΩ) under urethane anesthesia (20% in saline, 0.76 ml/100g). The recorded signal was amplified, band pass filtered (0.1 Hz to 5 kHz; Supertech BioAmp, Supertech, Hungary) and digitized at 16.6 kHz (micro 1401 mkll, CED, UK). APT unit activity was recorded by glass microelectrodes (in vivo impedance of 20-40 MΩ) pulled from borosilicate glass capillaries (1.5 mm OD, 0,86 mm ID, Sutter Inc., USA) and filled with 0.5 M NaCl and 2% neurobiotin (Vector Labs, USA). Electrodes were lowered by a piezoelectric

microdrive (6000 ULN, Burleigh-EXFO, USA). Neuronal signals were amplified by a DC amplifier (Axoclamp 2B, Axon Instruments, USA), filtered between 0.1-5 kHz by a signal conditioner (Supertech) and digitized at 16.6 kHz (CED). Juxtacellular labeling of the recorded neurons was done as described by Pinault (Pinault, 1996). Injection current intensity was increased until the modulation of neuronal firing (up to 10 nA, for 2-10 min) occurred. Following labeling and a survival period (15 min to 5 hours) animals were perfused transcardially first with saline (2 min) then with 100 ml fixative containing 2% paraformaldehyde and 3.6 % acrolein (Sigma-Aldrich) and finally with 300 ml fixative containing 2% paraformaldehyde in 0.1 M PB. 50 μm coronal sections were cut on a Vibratome. Double immunofluorescence was used to visualize the neurobiotin-filled cell (by StA488) and the parvalbumin content of the cell (for details see above in Methods). The neurobiotin was then developed using DABNi as a chromogen, and the sections were osmicated, dehydrated and flat-embedded in Durcupan for light and electron microscopy (for details see above in Methods). Filled cells were reconstucted by camera lucida using a 100x oil immersion lens. For electron microscopic investigation sections were reembedded and ultrathin (60 nm) sections were then cut and examined in the electron microscope.

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Supplementary data

Supplementary Table 1

The majority of the APT–thalamic terminals are GABAergic.

	GABA positive APT terminals in the thalamus	
tracer/thalamic nu	**Po**	**MD**
BDA	81 % (n=33)	100 % (n=20)
PHAL		74 % (n=38)

Table 1 demonstrates the percentage of the GABA-positive anterogradely labeled APT terminals in different higher order thalamic nuclei following injection of BDA (2 animals) or PHAL (1 animal) into the APT.

Abbreviations: BDA, Biotin dextran amine; LD, laterodorsal thal nu; MD, mediodorsal thal nu; PHAL, Phaseolus vulgaris leucoagglutinin; Po, posterior thal nu

Supplementary Table 2

Firing rate (Hz) of the recorded APT neurons			
cell	Fast Bursting	Tonic	Slow Rhythmic
1	8.73±2.21	17.16±2.00	2.48±1.76
2	26.06±5.56	21.60±1.36	1.25±1.85
3	19.05±10.80	15.05±2.42	8.48±4.28
4	18.92±6.02	6.58±1.65	2.06±1.54
5	13.25±5.14	9.33±1.13	4.28±2.14

6	11.31±8.48	7.03±1.74	3.48±1.95
7	-	7.98±2.90	2.80±2.63
8	-	14.62±2.94	0.71±0.85
9	-	-	4.85±1.54

Firing frequencies of the recorded APT neurons during cortical slow oscillation. Mean firing rate ± SD is given.

Discharge rate of the fast bursting and tonic neurons are variable, but as shown in Supplementary Figures 1-3, their firing pattern is very consistent within a group. The larger SD values of the fast bursting neurons is due to the irregularly appearing high frequency bursts, whereas in tonic cells, which exhibit a more balanced, single spike firing pattern, the SD is much smaller. Slow rhythmic cells displayed the lowest firing rate. The rhythmic clustered spiking, characteristic of this cell type, is indicated by the large SD values relative to the mean frequencies.

Discussion

The diversity of the GABAergic signaling has a primordial role in the functional homeostasis of the brain. In cortex, the GABAergic inhibition is driven by a huge diversity of interneurons. In the thalamus, the traditional view highlighted the relative homogeneity of the sources of GABA, from the nucleus reticularis neurons or from the local interneurons.

In this thesis, I present two studies derived from *in vitro* electrophysiological experiments that shed novel light on GABAergic function in the thalamus:

1) At the level of biochemical signaling, in particular the control of cAMP turnover, suggesting that the nRt can control the regulation of thalamic pacemaker currents and of other cAMP-dependent processes related to the state of arousal (for example, the state-dependent expression of genes and the control of selective attention).

2) At the level of architectural innervation of thalamic nuclei, suggesting that extrareticular inhibitory input can control the discharge mode of TC neurons and thus the gating function of higher-order nuclei (HOn).

I First paper discussion

By recording the modulation of the biophysical properties of I_h, we could determine the dynamics of the $[cAMP]_i$ regulation in TC neurons upon the activation of G-protein-coupled receptors.

We first observed that $[cAMP]_i$ turnover, which is pronounced in thalamus compared to other regions of the brain (Matsuoka et al., 1997; Ihnatovych et al., 2002), can be up- and downregulated steadily under the tonic influence of the G_s- and G_i-coupled receptors, respectively. A steady increase of $[cAMP]_i$ occurred upon the activation of the β-adrenergic receptors by isoproterenol (Iso), as it was already shown (Pape and McCormick, 1989). Conversely, the activation of $GABA_B$ receptors with baclofen (Bac) led to a steady decrease of I_h. The hyperpolarized shift in the activation curve and the slower activation kinetics of I_h induced by Bac, the occlusion of Bac-induced inhibition of I_h by saturating concentrations of 8-Br cAMP in the pipette solution, and the decreased IBMX-mediated enhancement of I_h in presence of Bac strongly argues that I_h reduction was due to the inhibition of $[cAMP]_i$ levels by Bac.

1. The interaction between G_i- and G_s-coupled receptors is mediated by cAMP.

The co-exposure to agonists of both G_s- and G_i-coupled receptors was integrated in a supralinear manner to produce a strong, transient increase in I_h. We performed several experiments to show that the strong and transient enhancement of I_h showed the properties of a regulation by $[cAMP]_i$.

First, co-application of Iso and Bac led to modifications of the voltage- and time-dependent properties of I_h similar to alterations induced by a direct modulation of I_h by addition of cAMP analogs in the intracellular solution or by application of forskolin (McCormick and Pape, 1990b; Lüthi and McCormick, 1999). Second, the increase of $[cAMP]_i$ at the peak of the modulation appeared to reach a saturating concentration for cAMP binding properties to the HCN channels. Thus, flash photolysis of caged cAMP, had no effect on I_h during the peak of the potentiation induced by Iso and Bac, but the flash-induced increase of I_h recovered following the decay of the offset of the potentiation. Third, Bac did not appear to alter the sensitivity of h-channels for cAMP generated in the presence of non-saturating concentrations of forskolin.

Thus, concomitant activation of $GABA_B$ receptors transformed the amplitude and the temporal properties of the Iso-mediated up-regulation of $[cAMP]_i$. In native cells, evidence for such $[cAMP]_i$ modulation by the interactions between G_i- and G_s-coupled receptors has been presented in two cases. The calcium-dependent potassium currents has been shown to be up-regulated by co-activation of $GABA_B$ receptors, serotoninergic receptors or α-adrenergic receptors with β-adrenergic receptors in hippocampal CA1 pyramidal neurons (Andrade, 1993; Pedarzani and Storm, 1996). In ventricular cardiomyocytes, co-activation of M_2 muscarinic receptors with the β-adrenergic receptors promoted the increase of the cAMP-dependent Cl^- currents and the L-type calcium currents (Zakharov and Harvey, 1997; Belevych et al., 2001). Nevertheless, in cardiomyocytes, the interaction of the G_i- and G_s-coupled receptors was masked by the strong inhibitory actions of G_i- coupled receptors. The washout of the muscarinic receptor agonists or the block of the receptors by an antagonist was required to unmask the inhibition and then to observe a rebound supralinear

stimulation of the 'reporter' currents. In contrast, in our study, similarly to CA1 neurons, the positive effect of Bac masked the negative actions of Bac.

2. The up-regulation of $[cAMP]_i$ is mediated by β-adrenergic and $GABA_B$ receptors

The activation of the β-adrenergic receptors was required to observe synergistic interactions between Bac and Iso. Indeed, the blockade of β-adrenergic receptors with the antagonist propranolol (10μM) prevented the increase of I_h by local application of Iso (n=3, data not shown). The synergistic action of Bac on I_h was also not observed anymore after co-application of Iso and Bac in the presence of propranolol (n=2, data not shown).

Similarly, to show that the $GABA_B$ receptors mediated the positive effect of Bac, we tested a selective antagonist of $GABA_B$ receptors. Surprisingly, we were not able to block the potentiation action of Bac by CGP 54626, a potent antagonist of $GABA_B$ receptors (Brugger et al., 1993). In our hands, CGP 54626 did not fully prevent the hyperpolarizing G-protein inward rectifier (GIRK) currents activated by Bac (data not shown), while the antagonist blocked the $GABA_B$ receptor mediated IPSCs evoked by stimulation in the nRt. Futher investigations on the new $GABA_B$ antagonists may be required as the efficacy of the large number of $GABA_B$ antagonists on Bac-induced cAMP formation is characterized incompletely (Cunningham & Enna, 1996; Knight & Bowery, 1996)

We showed that the facilitation of cAMP synthesis provoked by Bac was mimicked, in the presence of $GABA_A$ receptor antagonists, by GABA applied in the bath or released synaptically, indicating that the natural ligand for $GABA_B$ receptors could induce a potentiation of β-adrenergic responses. Moreover, we were able to interfere with the Bac-induced GIRK current opening or with the Bac-induced potentiation by using the sulfhydryl alkylating agent N-ethylmaleimide (NEM), a blocker of the G_i-proteins (Sodickson and Bean, 1996; Hirono et al., 2001).

The experiments -1) GABA application and 2) synaptic activation of $GABA_B$ receptors potentiated β-adrenergic receptors effects and 3) NEM prevented the synergistic action of Bac on cAMP synthesis- suggest that the strong increase of

cAMP by co-application of Bac and Iso is mediated by the interaction between the $GABA_B$ receptors and the β-adrenergic receptors.

3. Mechanisms of the up-regulation of $[cAMP]_i$ by G_i-coupled receptors

As discussed in the introduction (chapter II, 2), biochemical investigations in neuronal tissue (Olianas and Onali, 1999; Onali and Olianas, 2001) showed that two AC isoforms, the AC II and the AC IV, could be stimulated by βγ subunits in condition of the presence of free α_s subunits (Gao and Gilman, 1991; Tang and Gilman, 1991; Federman et al., 1992). The AC II and IV isoforms were attractive candidates to explain the paradoxical potentiation of G_s-mediated cAMP synthesis by receptors coupled to G_i-subunits in TC neurons. However, the involvement of these enzymes in physiological processes in intact cells has so far not been investigated thoroughly.

In cardiac cells, Belevych (2001) succeeded to describe the molecular cascades for the two actions, inhibitory and excitatory, of M_2 receptors. In heart, the release of acetylcholine by the parasympathetic system generally reduces the heart rate and contractility and this action is partially mediated by an inhibition of cAMP synthesis (Harvey and Belevych, 2003). Nevertheless M_2 receptors have an additional but positive effect via βγ subunits. He showed that βγ subunits mediated the stimulation of ACs by M_2 receptors by the addition of a peptide composed of 27 amino-acids (QEHA peptide) in the recording pipette solution. This peptide trapped the βγ subunits and prevented the stimulated potency of muscarinic agonist without affecting the inhibitory potency of M_2 receptors (Belevych et al., 2001). Thus, by inducing βγ subunit-induced potentiation of cAMP synthesis, M_2 receptors may provoke post-vagal tachycardia (Belevych et al., 2001). In humans, this rebound increase in heart rate and contractility is observed following the termination of parasympathetic stimulation (Prystowsky and Zipes, 1985).

Inspired by Belevych's study, we also investigated the effects of the inclusion of QEHA peptide in the patch pipette. Unfortunately, we did not manage to get good recordings of TC neurons when the peptide was added to the pipette solution. The intracellular solutions used in the two studies were similar, ruling out insufficient solubility of the peptide in the pipette solution. One hypothesis is the discrepancy in

the pipette resistance between the two studies. Belevych reported pipette resistance of ~1.5 MΩ while our pipettes had a resistance ~3 MΩ. Higher pipette resistances provide a better diffusion between the pipette solution and the soma thus may help for the passage of a peptide of 27 amino-acids. So far, the hypothesis that $GABA_B$ receptors potentiate the action of β-adrenergic receptor via the release of βγ subunits, and consequently activation of AC II and IV isoforms, is supported by the strong expression of these two isoforms in thalamus (Matsuoka et al., 1997; Ihnatovych et al., 2002).

4. Synaptic regulation of $[cAMP]_i$ by $GABA_B$ receptors

To assess the functional significance of the $GABA_B$ receptor-mediated potentiation of cAMP synthesis, we examined whether synaptic inhibitory inputs on TC neurons could also influence cAMP. The thalamic ventrobasal nucleus (VB) is devoid of interneurons (Arcelli et al., 1997), therefore the only source of GABAergic inputs is the nRt. In a VB TC neuron, the postsynaptic activation of CGP 54626-sensitive GIRK currents was evoked by external stimulation of the nRt tracts. The activation of K^+ currents was then prevented by application of barium. We found that synaptic stimulation of $GABA_B$ receptor up-regulated significantly the cAMP enhancement induced by local application of Iso. Thus, the $GABA_B$ receptors located at the nRt-TC synapses can influence the generation of cAMP signal in the vicinity of HCN channels.

Besides the direct control of ionic conductance gating, reports on the regulation of second messenger levels by metabotropic receptors activated synaptically are rare. In the cerebellum synaptic activation of $GABA_B$ receptors has been described to modulate calcium release from the intracellular store (Hirono et al., 2001). The metabotropic glutamate receptors, synaptically activated, control biochemical signaling cascades (Heuss et al., 1999) and cAMP-dependent long-term plasticity (Tzounopoulos et al., 1998).

The synaptic activation of $GABA_B$ receptors revealed a potentiating, but not an inhibiting effect on $[cAMP]_i$ metabolism. This result suggests that a higher stimulation of $GABA_B$ receptors is required to observe the inhibition of $[cAMP]_i$ that is detected by I_h. Similarly, we found that higher concentrations of Bac (80 μM) leading to a

saturation of potassium currents were required to observe an inhibitory action on ACs, whereas low concentration (800 nM) were sufficient to observe the synergistic interactions between $GABA_B$ and β-adrenergic receptors. One possibility to explain this observation is that HCN channels are spatially co-localized with AC II/IV isoforms and not with the other AC isoforms. The co-localization of ionic channels with the cAMP metabolism machinery has already been shown in hippocampal neurons (Davare et al., 2001).

5. Functional implications of the up-regulation of cAMP by $GABA_B$ receptors

Norepinephrine plays an important role in the control of cAMP-dependent gene expression during states of arousal in the thalamocortical system (Cirelli et al., 1996; Cirelli and Tononi, 2000) and in the modulation of long-term potentiation, a cellular form of learning and memory (Wang et al., 1999; Watabe et al., 2000). Distinct temporal profiles of cAMP transients contribute to determine the patterns of gene expression (Bacskai et al., 1993; Kaang et al., 1993). Therefore, the distinction between cAMP 'spikes' induced by Iso alone and the slowly decaying plateaus produced by Iso and Bac could be involved in determining gene expression patterns. Interestingly, locus coeruleus neurons discharge synchronously with sleep-related EEG rhythms in the thalamocortical system (Aston-Jones and Bloom, 1981), to which a partial activation of $GABA_B$ receptors contributes (Huguenard, 1998; Blumenfeld and McCormick, 2000). Thus, coincidental activation of $GABA_B$ and β-adrenergic receptors, in association with a strong cAMP signal, may be typical for phases of transition between sleeping and waking. An important aspect into the role of norepinephrine-dependent cAMP synthesis in the thalamocortical system could, therefore, be found in its timing with respect to synaptically activated $GABA_B$, and perhaps other, $G_{i/o}$-coupled receptors.

6. Is I_h a good sensor for monitoring the temporal dynamics of $[cAMP]_i$?

Endogenous ion channels have been exploited previously as sensors for second messengers (Yazejian et al., 2000; Heine et al., 2002). The temporal precision of such tracking methods is limited by the kinetics of channel gating and the localization of

channels with respect to the sources of synthesis. Pacemaker channels detect rapid increases in cAMP induced by photolysis within about ten seconds (Lüthi and McCormick, 1999; Seifert et al., 1999), indicating that they are fast enough to monitor fluctuations in cAMP levels that commonly occur over hundreds of seconds (Rich et al., 2001; Gorbunova and Spitzer, 2002; Zaccolo et al., 2002). On the other hand, half-maximal modulation of I_h is achieved by cAMP levels around 0.2 µM (DiFrancesco and Tortora, 1991; Lüthi and McCormick, 1999), while intracellular cAMP levels can reach concentrations of tens of micromolar (Bacskai et al., 1993; Sudlow and Gillette, 1997; Rich et al., 2001; Heine et al., 2002). Indeed, the increases in I_h amplitude during the strong regulation by Iso and Bac correspond to those predicted from a maximal shift in the activation curve (Lüthi and McCormick, 1999), indicating that the channels may be fully bound to cAMP. Thus, h-channels are cAMP sensors with the appropriate kinetics to follow cAMP signals, but cAMP levels reached may surpass their binding capacity. Therefore, the strength of cAMP synthesis induced by co-application of Iso and Bac is underestimated by measuring I_h. The cAMP sensor I_h appears thus to be positioned intracellularly in such a manner that strong, transient cAMP signals remain distinguishable from moderate, slow effects and thereby allow to reveal distinct dynamics of cAMP signals. Indeed, the differences in kinetics, associated with the sharpened sensitivity to increases in the concentration of Iso, suggests that h-channels may be spatially closer to the signal source activated by Iso and Bac, as opposed to the slower, shallow effects of Iso alone.

II Second paper discussion

We established that besides the well-known GABAergic input arising from the reticular nucleus, HOn are under the influence of the inhibitory anterior pretectum (APT).

Morphological studies showed that the afferents from the APT are in 90% GABAergic and that they formed symmetrical synapses onto the proximal dendrites of the TC neurons of the HOn (Bokor et al., 2004). Stimulations of the APT evoked monosynaptic, bicuculline-sensitive outward currents in TC neurons of the Po and the LD. Therefore, APT-evoked IPSCs are mediated by $GABA_A$ receptors. Surprisingly,

the IPSC reversal potential observed experimentally was -82.3 ± 1.9 mV (n=15) and this value did not match the calculated reversal potential for the Cl^- (-62 mV, assuming that the carbonate conductance is considered negligible). The values of the reversal potential we observed was similar to the reversal potential (-82 mV) of the $GABA_A$-receptor-mediated currents induced by local application of GABA or by electric stimulation of the nRt and obtained using perforated patch-clamp technique, which preserves the native cytosolic milieu (Ulrich and Huguenard, 1997). Thus, in our preparation, the Cl^- gradient seemed not to be altered although we used whole-cell configuration of the patch-clamp technique. A similar negative reversal potential (-94 mV) was already observed at the synapses between nRt neurons and TC neurons in whole-cell configuration and the authors presumed that a strong extrusion of Cl^- by TC neurons could explain the negative reversal potential of the IPSCs (Huguenard and Prince, 1994a).

Due to a high driving force for Cl^- ions, a negative reversal potential of $GABA_A$-mediated response may elicit sufficient hyperpolarization to de-inactivate T-current and then the hyperpolarization induced may promote burst generation. Indeed, it was possible to induce burst of action potentials in TC neurons by evoking a single or a train IPSPs. For higher number of evoked IPSPs, the probability to observe a burst was higher. Furthermore, evoked IPSPs inhibited the production of APs in TC neurons. The IPSPs induced by APT stimulation were able to coerce the time when an AP was generated. Similarly to nRt neurons, the potency of GABAergic APT neurons to generate burst of APs or to phase-locked the TC neuron discharges indicate a powerful role of APT in the control of the firing pattern of the HOn neurons. This could be primordial in the generation of synchronized activity related to different state of arousal. As APT receive afferents from the cortical layer V (Foster et al., 1989; Cadusseau and Roger, 1991b), the cortical layer V can thus indirectly control the firing pattern of the HOn neurons via the ZI and the APT It will be important to determine the different role of these two nuclei in the relation between cortex and thalamus.

We performed paired-pulse protocols to investigate the dynamic properties of the synapses. We observed that the depression was weak and similar for frequencies between 10 and 100 Hz. The morphological studies highlighted that the APT afferents formed large size terminals with multiple release sites. This may explain the weak paired-pulse depression observed *in vitro* and the non-linearity between interstimulus

frequencies and the paired-pulse ratio. Thus, it was shown in the cerebellum at the GABAergic synapses between the Purkinje cells and the nuclear neurons that multiple release site synapses permitted 1) high response probability even for low vesicle release probability 2) to limit presynaptic depression for high-frequency stimulation. In theses studies, they realized a model of multiple release site synapses, which showed that the depression was extremely reduced (paired-pulse ratio ~1) even for high release probability (0.8) (Telgkamp and Raman, 2002).

The present experiments were done in rats nevertheless we also observed electrophysiologically the connection between APT and HOn in mice. Thus, stimulation of APT evoked $GABA_A$-receptor-mediated IPSCs in murine HOn neurons with properties similar to the ones observed in rats, such as very negative reversal potential. (-90 ± 1.7 mV, n=10).

Therefore, in rodents, the thalamic HOn are under the control of the layer VI of the cortex in two ways, excitatory via the corticothalamic tracts and inhibitory via the nRt. A similar scheme seems to be involved for the connections of the cortical layer V on the HOn. The neurons of the cortical layer V can drive the HOn neurons directly but this may be balanced by disynaptic inhibition via the ZI or the APT. Thus, the synaptic organization of the thalamus is extremely diverse and may suggest a complex role of the thalamus in brain functions besides the role of gateway between the external world and the cortex.

PAPER 3: Regulation of recombinant and native hyperpolarization-activated cation channels

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Regulation of recombinant and native hyperpolarization-activated cation channels

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1. Abstract

Ionic currents generated by hyperpolarization-activated cation channels (HCN channels) have been principally known as pacemaker currents (I_h), as they allow cardiac and neuronal cells to be rhythmically active over precise intervals in time. Nowadays, these currents are implicated in numerous additional cellular functions, including neuronal integration, synaptic transmission and sensory reception. These roles are accomplished by virtue of the regulation of I_h by both voltage and ligands. The present review summarizes recent developments on the properties and allosteric interactions of these two regulatory pathways in cloned and native channels. In addition, it discusses how the expression and properties of native channels may be controlled via regulating the transcription of the HCN (hyperpolarization-activated cation-non selective) channel gene family and the assembly of channel subunits. Recently, a number of cardiac and neurological diseases were found to be intimately associated with a dysregulation of HCN gene transcription, suggesting a critical contribution of HCN-mediated currents to the pathophysiology of excitable systems. As a starting point, we will briefly review the general characteristics of I_h and the regulatory mechanisms identified in heterologously expressed HCN channels.

2. Molecular commonalities of cellular rhythms in cardiac and nervous systems

Cardiac sinoatrial cells and some central neurons exhibit pacemaking properties, which render them capable of generating electric discharges on a defined time scale, independently of external stimuli. Rhythmicity in the heart fulfills the need to drive the periodic contractions of cardiac muscle. In the mammalian brain, rhythmic neural activity controls not only motor but also higher cognitive functions, such as the state of arousal and the encoding and retrieval of information. Interestingly, the ionic mechanisms underlying some of these rhythms, in spite of their different functions, show strong molecular commonalities. Thus, the ionic channels generating autonomous pacemaking capabilities in cardiac and nervous tissue are members of the family of voltage- and ligand-gated pacemaker channels.

Pacemaker channels belong to the superfamily of voltage-gated ion channels, yet form a distinct subgroup that is closely related to the voltage-independent, cyclic nucleotide-gated channels. The molecular structure of the four cloned channel

subunits, which are termed HCN1-4 (for hyperpolarization-activated cation non-selective channels), exhibits both voltage-sensing and ligand-binding domains (Fig. 1A; for recent reviews, see (Kaupp and Seifert, 2001; Accili et al., 2002; Biel et al., 2002; Robinson and Siegelbaum, 2003)). It is increasingly clear that the four HCN channel subtypes give rise to ionic currents involved in an unusually broad range of neural functions that goes far beyond single-cell rhythmogenesis. This wide physiological context in which HCN channels are active is based on a rich repertoire of modulatory pathways the channels can be subject to. Not only are HCN channels gated by voltage, but they also contain binding sites for intra- and extracellular ligands (for review, see (Biel et al., 2002; Robinson and Siegelbaum, 2003)). Furthermore, subunit heteromerization, glycosylation and association with auxiliary subunits are important determinants of the functional properties of expressed channels (Ulens and Tytgat, 2001a; Yu et al., 2001; Altomare et al., 2003; Decher et al., 2003; Much et al., 2003). In contrast to the rapidly expanding insight into the regulation of channel molecules in heterologous systems, the signaling pathways and the physiological context that determine the regulation of I_h in native cells is just beginning to be explored. In addition to regulatory properties reminiscent of those of expressed HCN channels, channels in native tissue appear to be regulated via activity-dependent and -independent, short- and long-term alterations in HCN mRNA and protein expression (Santoro and Baram, 2003). This latter level of regulation contributes to the developmentally controlled I_h expression, but could also account for the causes and/or consequences of some cardiac and neurological pathologies.

3. Basic properties of native I_h

Three peculiar properties of h-currents are highlighted here that make them unique amongst the family of voltage-gated ionic currents and earned them the name I_f for “funny current” in the heart (Brown et al., 1979) or I_q for “queer current” in the brain (Halliwell and Adams, 1982), when they were originally discovered. First, I_h typically activates upon membrane hyperpolarization (below ~-60 mV) rather than depolarization, just opposite to most voltage-gated ionic currents that are involved in shaping the neuronal response to excitatory input (Fig. 1B). This unusual voltage window of activation is reflected in the now widely used name I_h, where “h” stands for hyperpolarization. Upon hyperpolarization, the conductance activated is

permeable to both Na^+ and K^+ ions (permeability ratio K^+ : Na^+ = 0.2-0.3). The current is carried mainly by Na^+ ions at the membrane voltages within its activation range and produces an elevation in the intracellular Na^+ concentration (Knöpfel et al., 1998). More recently, a small permeability to Ca^{2+} ions has also been identified via imaging techniques (Yu et al., 2004b). The current is blocked by at least four distinct classes of agents: extracellularly by millimolar concentrations of Cs^+ or by capsazepine, a blocker of vanilloid receptors (Ray et al., 2003); intracellularly by the lidocaine derivative QX-314 (Perkins and Wong, 1995) or by bradycardiac agents (e.g. ZD7288, see (Pape, 1994; Harris and Constanti, 1995; Chevaleyre and Castillo, 2002; Robinson and Siegelbaum, 2003)). All these compounds, however, show non-specific effects independently of I_h: Cs^+ blocks neuronal K^+ channels (Constanti and Galvan, 1983) and interferes with K^+ uptake in glial cells (Janigro et al., 1997), whereas ZD7288 depresses synaptic transmission (Chevaleyre and Castillo, 2002). Unless more selective blockers are developed, the pharmacological identification of novel physiological roles of I_h should thus be based on the effects of several blockers belonging to different classes. Second, activation of the current is fairly slow, with activation time constants ranging between hundreds of milliseconds and seconds, even at strongly hyperpolarized voltages around -100 mV. Few exceptions include pyramidal neurons from hippocampus, cortex and cerebellum, in which activation is complete within tens of milliseconds (see section 5). Once activated, the current does not inactivate, such that a steadily activated ('standing') I_h contributes to the resting membrane potential in many neurons, often by opposing the action of tonic outward currents (Uchimura et al., 1990; Womble and Moises, 1993; Akasu and Shoji, 1994; Doan and Kunze, 1999). Third, I_h is, in most cases, exceedingly sensitive to the presence of intracellular cyclic nucleotides. The cyclic nucleotides cAMP and cGMP, the latter one probably to a weaker extent, not only accelerate the kinetics of activation, but also shift the voltage dependence of activation towards more depolarized values (Fig. 1B). In the presence of these ligands, the extent and duration of current activation at a given voltage is substantially increased.

In summary, I_h is generated by voltage-gated ionic channels that in addition are sensitive to intracellular ligands, the cyclic nucleotides. They are thus part of a small group of ionic channels that are dually gated by both ligands and voltage (Fig. 1B). The molecular correlates of I_h are phylogenetically related to the ether-à-go-go channels and plant inward rectifier currents, which are also gated by voltage and

cyclic nucleotides (Santoro and Tibbs, 1999). This dual gating imparts an unprecedented level of flexibility to channel function that has so far been illustrated most impressively by studying the function of HCN channels.

4. The multiple roles of I_h

Originally, deviations from ohmic behavior in the steady-state current-voltage relationships of electrically excitable motoneurons were described by Oshima and coworkers (Araki et al., 1961; Ito and Oshima, 1965). These were termed anomalous or inward rectification, referring to an increase in slope conductance when neuronal membranes are hyperpolarized. A physiological role for the conductance underlying this abnormal behavior was first reported in rod photoreceptor cells, in which a rebound depolarization during light-induced hyperpolarization was caused by a Cs^+-sensitive membrane conductance (Fain et al., 1978) permeable to both Na^+ and K^+ ions (Bader and Bertrand, 1984). This conductance activated at potentials below -50 mV and manifested as a slow inward current capable of depolarizing the membrane over the time course of seconds. Vertebrate rod photoreceptor cell membranes reach potentials below -50 mV upon light-induced hyperpolarization (Fain et al., 1978; Bader et al., 1979). Thus, activation of this conductance opposes the cellular response to prolonged exposure to light, and is thus involved in adaptation to visual stimuli.

The interest in slowly activating cation currents gated by hyperpolarization grew considerably when it was found that in cardiac tissue, such a current could endow cells with an intrinsic propensity to generate oscillatory activity (Fig. 1C; for review, see (DiFrancesco, 1985)). The diastolic phase of the heart beating cycle is associated with membrane hyperpolarization large enough to allow the voltage-gating of this current (Brown et al., 1979). The diastolic depolarization eventually reaches the threshold for Ca^{2+} current activation and action potential firing. Although the diastolic voltage waveform is now known to be generated by a combination of voltage-gated currents ((Zaza et al., 1997; Maier et al., 2003), for review, see (Schram et al., 2002)), I_h is essential for the generation of rhythmic cardiac output. Thus, genetic deletion of HCN channel subunits perturbs the rhythmic depolarizations in intact heart (Ludwig et al., 2003), in sinoatrial node cells (Stieber et al., 2003) and in cultured neonatal cardiomyocytes (Er et al., 2003). Moreover, zebrafish that carry a mutation in the *slo mo* gene show a slowed

heart rate, associated with a decreased amplitude of I_h in cardiomyocytes, while other currents involved in cardiac pacemaking remain unchanged (Baker et al., 1997; Warren et al., 2001).

H-currents with properties similar to the originally described cardiac I_f were later identified in a large number of electrically excitable cells, ranging from uterine smooth muscle cells (Satoh, 1995) and enteric neurons (Galligan et al., 1990) to the pyramidal neurons of hippocampus (Halliwell and Adams, 1982) and cortex (Solomon et al., 1993). However, convincing evidence for a role of I_h as a pacemaker current, in particular the determination of its active pacemaker role independently of effects on resting membrane potential, is so far available for a fairly small number of neural rhythms. The most prominent among these include sleep-related rhythms generated by thalamocortical neurons (McCormick and Pape, 1990a) and thalamic networks (Bal and McCormick, 1996; Lüthi and McCormick, 1998b), γ-oscillations in hippocampus (Fisahn et al., 2002), synchronized oscillations in inferior olive (Bal and McCormick, 1997), and subthreshold oscillations in entorhinal cortex (Dickson et al., 2000). Furthermore, in slice preparations, spontaneous firing of hippocampal interneurons (Maccaferri and McBain, 1996), neostriatal interneurons (Bennett et al., 2000), substantia nigra (Neuhoff et al., 2002) and area postrema neurons (Funahashi et al., 2003) depends on activation of I_h in between individual action potentials. In a number of rhythmically active systems, however, other currents are rhythmogenic with a minor role of I_h, such as in respiratory rhythms (Thoby-Brisson et al., 2000), in supraoptic neurons (Ghamari-Langroudi and Bourque, 2000) or in paroxysmal discharges in neonatal hippocampus (Agmon and Wells, 2003).

Besides being involved in rhythmicities and control of membrane potential, I_h is now known to contribute to additional central neuronal functions, such as dendritic integration, synaptic release and two types of primary sensory reception (Fig. 2, for further review, see (Robinson and Siegelbaum, 2003)). The novel roles of I_h rely predominantly on its partial steady activation at the resting membrane potential and its modulation by intra- and extracellular signaling molecules. In dendrites of hippocampal CA1 (Magee, 1999) and neocortical layer V pyramidal (Williams and Stuart, 2000a; Berger et al., 2001) neurons, a standing I_h contributes to the resting membrane potential of dendrites by up to 11 mV (Williams and Stuart, 2000a). The rapid deactivation of I_h during excitatory inputs produces a hyperpolarization that

accelerates the decay of synaptic potentials. During repetitive presynaptic discharges at intervals up to less than 5 ms, the temporal summation of postsynaptic responses will be further attenuated by the accruing deactivation of I_h. Interestingly, the density of I_h augments by severalfold along the somatodendritic axis of apical dendrites (Lörincz et al., 2002). Therefore, its effects on the temporal summation of distal synaptic inputs will be increasingly pronounced. Indeed, the density of I_h appears to be tuned such that it compensates exactly the incrementing filtering effects of the dendritic cables, indicating that I_h is a major factor in normalizing temporal summation in CA1 and cortical pyramidal cells. As a physiological consequence, temporal summation of subthreshold excitatory inputs in principal hippocampal and cortical neurons, and hence the eventual timing of action potential generation as well, are independent of the location of synaptic input (for review, see (Magee, 2000; Desjardins et al., 2003)). The subcellular expression of I_h therefore helps excitatory inputs into the dendritic trees to convey the same temporal information independently of where they were generated.

The h-current, by virtue of its voltage dependence, also dampens cellular responses to inhibitory synaptic input and allows a rapid resumption of tonic firing, as shown via recording the response of Purkinje cells to ramp- or pulse-like injections of currents when I_h was either blocked (Williams et al., 2002) or the HCN1 gene was knocked-out (Nolan et al., 2003). HCN1-deficient Purkinje cells completely lack an h-current and show a retarded generation of action potentials during the transition from sub- to suprathreshold current injections (Nolan et al., 2003). Furthermore, sinusoidal current injections into the dendrites of cortical and hippocampal principal cells revealed that I_h controls the temporal relationship between the phase of the current injection and the timing of action potentials (Magee, 2001; Ulrich, 2002). The involvement of I_h in the control of the phase relationship between a periodic stimulus and repetitive action potential generation may have direct consequences at the behavioral level. Thus, HCN1-knock-out mice are selectively compromised in learning repetitive motor tasks that involve the phasic excitation and inhibition of cerebellar Purkinje cells. To explain this deficit, it has been proposed that I_h of Purkinje cells may be involved in the plastic events leading to motor learning, perhaps by facilitating the coincidence of pre- and postsynaptic activity in the time window required for synaptic plasticity of afferents to Purkinje fibers (Nolan et al., 2003).

Interestingly, HCN1 protein is also expressed in presynaptic terminals of cerebellar basket cells (Santoro et al., 1997), as well as, together with HCN2 and HCN4, in the presynaptic zones of retinal bipolar cells (Müller et al., 2003). Clustering of HCN3 protein was found at the base of the pedicles generated by cone photoreceptor cells, which form synapses to a number of retinal cells (Müller et al., 2003). Electrophysiologically, the presence of I_h has been verified in the terminals of inhibitory cerebellar basket cells and in those of excitatory brainstem afferents, but the role it plays in neurotransmitter release appears minor (Southan et al., 2000; Cuttle et al., 2001). If I_h is activated for prolonged periods of time (>10 s), the Ca^{2+} ions permeating through the channels can, however, facilitate neurotransmitter release in response to repetitive action potential discharge in dorsal root ganglia neurons (Yu et al., 2004b). This suggests that the elevation of basal Ca^{2+} levels during periods of hyperpolarization can modulate synaptic short-term plasticity (Yu et al., 2004b). It has also been proposed that presynaptically expressed I_h may contribute to modulate synaptic transmission and underlie presynaptic forms of long-term potentiation via sensing the cAMP generated via either receptors for neuromodulatory transmitters or via Ca^{2+}-sensitive adenylyl cyclases (ACs) ((Beaumont and Zucker, 2000; Beaumont et al., 2002; Mellor et al., 2002), see, however, (Chevaleyre and Castillo, 2002)).

Finally, primary sensory pathways exploit I_h for stimulus detection. Upon application of a low pH solution, a subset of cells sensitive to sour taste generate an inward current which is blocked by Cs^+ ions (Stevens et al., 2001). The expression of HCN1 and HCN4 protein in these cells implicates I_h as a major component in the detection of sour stimuli (Stevens et al., 2001). Reduction of a standing I_h by lowering temperature also contributes to control the thermosensation of a subgroup of trigeminal neurons (Viana et al., 2002).

5. Basic properties and regulation of cloned HCN channels

Three groups succeeded independently of each other in identifying the genes encoding HCN channel subunits (Clapham, 1998; Gauss et al., 1998; Ludwig et al., 1998; Santoro et al., 1998). These display the overall membrane topology of voltage-gated K^+ channels, with six transmembrane domains S1-S6 (Fig. 1A). The sequence motifs typical for voltage-gated ion channels are present in these domains, including the amino acid sequence GYG which is characteristic for the

narrow portion of the selectivity filter in voltage-gated K^+ channels between S5 and S6 and the voltage-sensor motif with regularly spaced, positively charged amino acids in S4 ((Doyle et al., 1998), for review, see (Gauss and Seifert, 2000)). So far, HCN1, HCN2 and HCN4 genes transcribed in heterologous expression systems give rise to currents, with the properties typical for I_h: activation upon hyperpolarization and modulation by intracellular cAMP. Currents mediated by HCN3 subunits have not been described (see (Much et al., 2003)). The detailed characteristics of these properties are strikingly different between channels generated by homomeric assembly of HCN1, 2 or 4. Whereas channels composed of HCN1 subunits activate rapidly (within tens of milliseconds at voltages below -100 mV) and are weakly sensitive to cAMP, HCN2 and especially HCN4 subunits give rise to currents that activate slowly (hundreds of milliseconds to seconds below -100 mV) and are highly sensitive to cAMP. Besides generating hyperpolarization-activated inward currents, HCN1 and HCN2 homomers also give rise to an instantaneous current component that is Cs^+-insensitive and voltage-independent (Proenza et al., 2002; Macri and Accili, 2004).

The expression of HCN genes at the level of the mRNA distribution in the brain reveals complementary, yet partially overlapping expression profiles, which correlate reasonably well with the characteristics of native I_h in diverse neuronal cell types. Thus, HCN1 is predominantly expressed in cortical, hippocampal and cerebellar regions, whereas HCN2 expression is widespread and, concomitantly with HCN4, found in regions in which I_h functions as a pacemaker (Moosmang et al., 1999; Franz et al., 2000; Monteggia et al., 2000; Santoro et al., 2000). To a first approximation, the heterogeneous properties of native I_h are thought to arise from this differential expression of HCN1, 2 and 4.

With the exception of HCN2+HCN3, all dual combinations of channel subunits can give rise to heteromeric channel complexes inserted into membranes (Much et al., 2003). Electrophysiologically, the currents generated by at least some of the heteromeric channels show properties that are intermediate, but distinct from those predicted via interpolating between the characteristics of the homomeric channels (Chen S. et al., 2001; Altomare et al., 2003). The demonstration that heteromerization generates unique forms of I_h, together with the overlapping expression patterns of mRNA for HCN subunits in cardiac cells and in neurons

(Ludwig et al., 1998; Santoro et al., 1998; Moosmang et al., 1999; Franz et al., 2000; Monteggia et al., 2000; Santoro et al., 2000), indicates that formation of heteromers may contribute to the specification of native I_h. This issue was addressed by comparing the properties of native currents and of heteromers consisting of the subunits expressed in the cells. In sinoatrial node, heteromers generated by HCN1/HCN4 reproduce the kinetics but not cAMP sensitivity of the native current (Altomare et al., 2003). In contrast, a linear superposition of the currents generated by HCN1 and HCN4 homomers accounts well for the kinetics of I_h found in subtypes of retinal bipolar cells (Müller et al., 2003).

Heterologous expression experiments have additionally reported that accessory proteins may further determine native current properties. Thus, currents generated by HCN1, HCN2 and HCN4 homomers are substantially larger in amplitude and modulated in activation kinetics when co-expressed with the protein MinK-related peptide 1, an accessory protein of a number of K^+ channels (for a review, see (Abbott et al., 2001)). These single transmembrane-spanning proteins could functionally interact with the C-terminal domain of HCN channels and contribute to the diversity in the whole-cell current ((Yu et al., 2001; Decher et al., 2003), see, however, (Altomare et al., 2003)).

The molecular correlate of the observed cAMP sensitivity of I_h resides in a cytosolic C-terminal cyclic nucleotide binding domain (CNBD) that is highly homologous to the cyclic nucleotide-binding domain of kinases, and to catabolite gene activator protein, a metabolic protein from *E. coli* (Santoro and Tibbs, 1999; Kaupp and Seifert, 2001). Removal of the CNBD or mutations of single amino acids abolishes the cyclic nucleotide-sensitivity of the expressed channels (Chen S. et al., 2001; Wainger et al., 2001; Ulens and Siegelbaum, 2003). The CNBDs of each subunit must be bound to cAMP to achieve a maximal effect on the voltage dependence (Ulens and Siegelbaum, 2003). An exposed C-terminal domain, likely containing the CNBD, confers cAMP sensitivity in native currents, as well. Thus, in cardiac cells, infusion of C-terminal specific proteases into the intracellular compartment abolishes the cyclic nucleotide sensitivity of I_h (Barbuti et al., 1999), while leaving voltage sensitivity intact. The dual gating of both cloned and native I_h appears thus to be based on the modular composition of channel subunits by sequentially arranged voltage- and ligand-sensing domains.

A complicating aspect of the dual gating of HCN channels is the fact that voltage- and ligand sensing portion do not act independently of each other. This is particularly evident when recording currents generated by channels devoid of a CNBD. Such truncated channels show a dramatically accelerated activation that is comparable to that induced by exposure of the cytosolic face to maximal concentrations of cAMP (Wainger et al., 2001). This suggests that, in the intact channel, the ligand-free CNBD influences the voltage-sensing transmembrane channel portions in a manner that retards the opening of the pore (Barbuti et al., 1999; Wainger et al., 2001), while binding of the cyclic nucleotides has an effect on activation kinetics equivalent to that of physically removing the CNBD from the protein. The interaction between ligand- and voltage-sensing domains not only accounts for the current acceleration in the presence of cAMP, but also for the shift in the voltage dependence of I_h by cAMP (DiFrancesco, 1999; Wainger et al., 2001; Wang et al., 2001). In the case of HCN1 and HCN2, the differences in the kinetics and the variable cAMP response of the homomers (see above) arise, at least in part, by sequence differences within the CNBD and the C-linker domains connecting the CNBD to S6 (Wang et al., 2001).

Dual gating by voltage and ligand has additional important implications for the dynamics of current activation: it allows for a prolonged activation of I_h that outlasts the presence of free ligands. To understand the generation of such persistently activated I_h, two computational studies approximated the gating of channels in cyclic allosteric gating models (Fig. 3A) (DiFrancesco, 1999; Wang et al., 2002a). In a Monod-Wyman-Changeux model, four distinct states of the entire channel were arranged in a cycle (Wang et al., 2002a): the closed and open unliganded states, and the closed and open liganded states. The stabilized activation of I_h is explained by an 80-fold increase in the cAMP binding affinity to the open compared to the closed channel, such that voltage-gating of the unliganded channel facilitates binding of cAMP. In a Hodgkin-Huxley model on cardiac I_h incorporating two allosterically gated channel subunits, a 6-fold decrease in the dissociation constant was obtained, thus yielding a 36-fold decrease for the dimer (DiFrancesco, 1999). Both models demonstrate that the dually gated channels represent the channel configuration with the greatest free energy decrease (DiFrancesco, 1999). These are therefore most reluctant to closure via an imposed

depolarizing voltage and lead to a persistence of channels in the dually gated mode. These allosteric models therefore show that, via becoming 'trapped' in the dually gated mode, channels can remain activated for prolonged periods of time even if they have been only transiently exposed to both stimuli (Wang et al., 2002a), resulting in the appearance of persistent, very slowly decaying current components.

In addition to being gated by voltage and cyclic nucleotides, HCN2 channel subunits are also sensitive to pH changes. Decreases in pH from 7.4 to 6.4 in the intracellular compartment result in a down-regulation of the current and in a slow-down of the speed of activation. Conversely, alkalinization enhances current amplitude and activation rate. The sensitivity to pH_i allows shifts in voltage dependence of up to 20 mV and is mediated by a single His residue located within the linker between domains S4 and S5 (Zong et al., 2001). While HCN2 is sensitive to changes in internal pH, the channel subunits HCN1 and HCN4 sense extracellular pH alterations, albeit with comparatively weak sensitivity. HCN1-mediated currents show a positive shift in the voltage dependence of up to 35 mV when pH_e is decreased from 7.4 to 3.9, associated with a strong acceleration in the activation time course.

The primary sequence of HCN channels shows at least one potential consensus phosphorylation site for protein kinase A (PKA) which resides within the CNBD (Santoro et al., 1998). Furthermore, one of the successful approaches to clone the HCN channels was based on a yeast-two-hybrid screen searching for proteins interacting with the SH3 domain of the neural specific form of the protein tyrosine kinase Src (Santoro et al., 1997). This suggests that some HCN channels may molecularly interact with protein kinases, analogously to the association of invertebrate cation channels (Magoski et al., 2002) or human K^+ channels (Holmes et al., 1996) with their regulatory kinases via Src homology 3-domains. However, direct evidence for a functional consequence of phosphorylation of HCN channels is currently lacking, although preliminary reports suggest a role for PKA and protein tyrosine kinases (PTKs) in controlling the maximal conductance and voltage dependence of HCN2 and HCN4 (Proenza and Accili, 2001; Yu et al., 2003). In further support for potentially interesting roles of HCN channel phosphorylation is the finding that close relatives of HCN channels, the cyclic

nucleotide-gated channels, can be phosphorylated by both Serine/Threonine as well as Tyrosine kinase activity (for review, see (Kaupp and Seifert, 2002)). These phosphorylations control channel apparent affinity for cyclic nucleotides and could be important for circadian modulation of ligand sensitivity in cone photoreceptor cells (Ko et al., 2001).

In summary, the cloning and functional expression of HCN channel subunits has revealed an array of modulatory capacities of the corresponding currents. We will now discuss which of these are likely functionally exploited in native cells, and how channel expression is regulated under pathological cardiac and neural conditions.

6. Regulation of native I_h

6.1. Regulation by ligands and phosphorylation

Direct regulation by cAMP. The recognition of I_h regulation by cyclic nucleotides was intimately associated with the identification of the currents themselves. Thus, the quest for the ionic mechanism underlying the acceleration of the heartbeat by adrenaline was found to be associated with an enhancement of I_h amplitude upon exposure to adrenaline (Brown et al., 1979). Noradrenaline had long been known to be associated with increases in the concentration of intracellular cyclic nucleotides in cardiac cells (Brooker, 1973; Hartzell, 1988). It was then demonstrated that the amplitude of I_h in sinoatrial node cells could be enhanced by cAMP applied directly to the cytosolic portion of cell-free patches, and neither constitutively active PKA nor PKA blockade interfered with this modulation (DiFrancesco and Tortora, 1991). Furthermore, a β-adrenergically mediated membrane depolarization in CA1 pyramidal cells was not affected by PKA inhibitors, but blocked by Cs^+ ions (Pedarzani and Storm, 1995). This suggested that I_h could be regulated by a direct effect of cAMP rather than via a cAMP-dependent activation of protein kinase A (PKA), the principal intracellular receptor for cAMP. A modulation of I_h by neurotransmitter receptors with experimental evidence implicating a direct action of cAMP has since been found in a number of neurons in slice preparations. Prominent among these are β-adrenergic receptors in thalamocortical neurons (McCormick and

Pape, 1990b; Pape, 1992; Frère and Lüthi, 2004), 5-HT receptors in hypoglossal neurons (Bobker and Williams, 1989) and neonatal rat motoneurons (Larkman et al., 1995; Larkman and Kelly, 1997). Furthermore, endogenous neuropeptides such as vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide potentiate I_h via a mechanism involving cAMP (Lee and Cox, 2003; Sun et al., 2003), whereas substance P-mediated activation of neurokinin 1 receptors inhibits I_h in sensory neurons through a messenger pathway yet to be determined (Jafri and Weinreich, 1998). The increase of cAMP produced by prostaglandins in sensory neurons is also detected by I_h (Ingram and Williams, 1996). The application of PKA inhibitors did not affect the action of the agonists for at least some G_s-coupled neurotransmitter receptors (Pedarzani and Storm, 1995; Ingram and Williams, 1996; Larkman and Kelly, 1997), further supporting the idea that the effects of cAMP were direct also in preserved cellular preparations.

In contrast to the G_s-dependent stimulatory effects, G_i-dependent inhibitory actions on cAMP-dependent regulation of I_h have been documented in a few cases only, and a complete demonstration that these are mediated via inhibition of endogenous AC activity has remained more difficult. In nodose ganglion neurons, an inhibitory effect of opioids on cAMP synthesis was demonstrated for forskolin-stimulated AC activity, whereas no effect was observed on basal current amplitude (Ingram and Williams, 1993). In contrast, in thalamocortical and cholinergic mesopontine neurons, activation of adenosine A1 receptors inhibited I_h in a manner consistent with inhibition of basal AC activity (Pape, 1992; Rainnie et al., 1994), although a decrease in cAMP sensitivity of the channels was not excluded in these studies. Using a combined assessment of both basal AC activity and channel sensitivity, we have recently provided more complete evidence that, in the case of the $G_{i/o}$-coupled $GABA_B$ receptors, a substantial portion of the reduction in I_h amplitude is attributable to the inhibition of a comparatively high basal AC activity in thalamocortical neurons (Frère and Lüthi, 2004).

An additional pathway of cAMP-dependent regulation was uncovered upon addressing the question of how positive and negative stimuli on cAMP synthesis summated when activated simultaneously. Contrary to a linear summation of the effects induced by agonists for β-adrenergic and $GABA_B$ receptors, and thus to a cancellation of these two stimuli, a marked potentiation of I_h amplitude was revealed

that appeared to be induced by a distinct, powerful synthesis of cAMP (Frère and Lüthi, 2004). Furthermore, I_h can also be regulated by cAMP following increases in intracellular Ca^{2+} levels (Lüthi and McCormick, 1999), or by cGMP via the nitric oxide pathway (Pape and Mager, 1992). Thus, HCN channels are targeted by multiple pathways of cyclic nucleotide synthesis, suggesting that the channels may be surrounded by several, perhaps molecularly distinct, ACs (see below).

Downstream from the neurotransmitter receptors, little is known about the molecular organization and the subtypes of ACs and associated regulatory enzymes that target I_h. To date, at least nine different subtypes of ACs are characterized molecularly (for review, see (Hanoune and Defer, 2001; Cooper, 2003)), many of them with distributions overlapping the areas in which HCN channels are expressed (Matsuoka et al., 1997; Ihnatovych et al., 2002). Based on the findings of native current regulation, it is conceivable that several molecularly distinct types of ACs generate cAMP that is detected by HCN channels. An exemplary case is the regulation of I_h in thalamocortical neurons. In these cells, the influence of Ca^{2+} in the regulation of I_h points to an involvement of the Ca^{2+}-sensitive ACs type I and/or type VIII, both of which are expressed in thalamocortical neurons (Cali et al., 1994; Matsuoka et al., 1997). Furthermore, the synergistic effect found by co-activation of G_s- and G_i–coupled neurotransmitter receptors strongly suggests a functional association of ACs type II or IV with I_h, which require binding of both G_s- and $G_{\beta\gamma}$-subunits for activation (for review, see (Cooper, 2003)). Finally, guanylyl cyclase also modulates I_h in thalamocortical cells (Pape and Mager, 1992). The fact that an ionic current is regulated by multiple enzymes producing the same second messenger suggests that, in native membranes, 1) channels giving rise to I_h are localized in subpopulations or clusters, each of which is associated with a distinct cAMP synthesis pathway, similar perhaps to the association of Ca^{2+}-dependent K^+ channels with specific sources of Ca^{2+} (for review, see e.g. (Sah and Davies, 2000)) or 2) channels underlying I_h are colocalized with several types of ACs. A future goal in elucidating the regulation of I_h may thus focus on the characterization of the subcellular organization of channels with associated regulatory systems, in a manner similar to that achieved for other ionic channels involved in cardiac (Marx et al., 2002) or neuronal (Sah and Davies, 2000) rhythmicity.

The extensive characterization of the multiple pathways of cyclic nucleotide-dependent regulation of I_h stands in opposition to a relative lack of understanding of the physiological conditions during which these types of regulation are induced. For example, it has remained unclear whether the G-protein-coupled neurotransmitter receptors (GPCRs) leading to regulation of I_h can be activated synaptically. Alternatively, extrasynaptically located receptors may set a background level of on-going G-protein activity that determines a tonic exposure of I_h to cyclic nucleotides. We recently studied the effects of $GABA_B$ receptor-mediated modulation of I_h, and we found that synaptically activated receptors can contribute to potentiate β-adrenergically mediated augmentation of the current (Frère and Lüthi, 2004). In contrast, synaptic activation of $GABA_B$ receptors alone did not result in a modulation, although addition of agonists for these receptors to the bath downregulated I_h (Frère and Lüthi, 2004). Thus, at least some pathways of cAMP synthesis targeting I_h are coupled to GPCRs that can be activated following synaptic stimulation. In addition, synaptically activated ionotropic glutamate receptors can also contribute to the sources of Ca^{2+} leading to acute regulation of I_h (Van Welie et al., 2002). Currently available data therefore clearly show that channels generating I_h belong to the family of ion channels regulated by synaptically activated neurotransmitter receptors.

Allosteric regulation. A few years before the characterization of the dual allosteric gating of HCN channels, experiments addressing the dynamics of I_h activation by cAMP were strongly suggestive of a preferred interaction of cAMP with open as opposed to closed ion channels. Thus, the duration of cAMP-mediated effects was found to depend upon the voltage protocol that was used to activate the current (Lüthi and McCormick, 1999). If a transient cAMP stimulus was applied to a cell whose membrane potential was held constantly within the activation range of I_h, current upregulation was prolonged by severalfold compared to when the current was gated with brief hyperpolarizing steps from a holding potential outside the activation range, revealing a slowly developing, persistently activated current component (Lüthi and McCormick, 1999). Such prolonged activation of I_h is associated with a number of rhythmic network activities (Bal and McCormick, 1996; Bal and McCormick, 1997; Strata et al., 1997). One illustrative example is found in the spindle waves, which arise predominantly during early periods of slow-wave sleep and are generated from a reciprocal synaptic interaction between thalamocortical neurons and nucleus

reticularis neurons (for review, see (McCormick and Bal, 1997)). *In vitro*, spindle waves appear as 1-3 s periods of 6-14 Hz synchronized oscillatory activity interspersed with silent periods of 5-20 s (Fig. 3B). These silent periods are associated with a slowly decaying, I_h-dependent membrane depolarization that is maximal after the end of a phase of synchrony and has fully disappeared before the occurrence of the next spindle wave. It was initially proposed that this slow form of I_h enhancement can be explained by the slow kinetics of voltage-dependent deactivation of I_h (Bal and McCormick, 1996). However, closer inspection of the factors inducing the upregulation revealed a critical role for increases in intracellular Ca^{2+}, primarily triggered by the low-threshold Ca^{2+} bursts occurring during spindling (Lüthi and McCormick, 1998a). The Ca^{2+} ions are detected by a Ca^{2+}–sensitive AC, producing an increase in cAMP synthesis that enhances I_h (Lüthi and McCormick, 1999). The dual exposure of I_h to cAMP and to the repetitive inhibitory input during a spindle wave facilitates persistently activated I_h, which, in turn, prevents the next spindle wave until I_h is slowly decayed (Fig. 3B). Persistent activation of I_h, based on allosterically stabilized ion channel configurations, is thus the electrophysiological consequence of activity-induced synaptic and biochemical events associated with synchronized network rhythms.

Regulation by phosphorylation. In parallel to the identification of direct actions of cAMP on I_h, several studies reported that, in some preparations, cAMP-dependent actions on I_h could indeed be blocked completely when inhibitors of protein kinases were present, including PKA (Tokimasa and Akasu, 1990; Chang et al., 1991). Support for a role of Ser/Thr protein kinase activity in tonically controlling the properties and regulation of cardiac I_h was found in studies using selective Ser/Thr phosphatase inhibitors, which induced a positive shift in the activation curve and, at least in one preparation, an increase in the maximal conductance of the current (Yu et al., 1993a; Accili et al., 1997). In dorsal root ganglia and olfactory receptor neurons, the voltage dependence of basal I_h is subject to PKA-dependent phosphorylation, as assessed by specific inhibitors of this enzyme (Raes et al., 1997; Vargas and Lucero, 2003). Stimulation of PKA can lead to a shift in the activation curve that is superimposed on that induced by a maximal dose of cAMP (Raes et al., 1997). In addition, activated PKA alters the dose-response curve of current activation to cAMP, rendering the channels preferentially sensitive to larger changes in the concentration of this cyclic nucleotide (Accili et al., 1997; Raes et al., 1997). Altogether, the

presently available data indicate that, at least for certain types of I_h, PKA activity is an additional parameter that determines the functionality of the ionic channels and the associated regulated systems. The level of action of the phosphorylation process could occur as a covalent modification of the channel subunits or of auxiliary subunits, and regulation of channel protein recycling to alter the maximal conductance could also be a potential target of kinases.

The phosphorylation of channel proteins has repeatedly been reported in playing a pivotal role in the maintenance of current properties over time (for review, see (Levitan, 1999; Kramer and Molokanova, 2001)), and phosphorylation-dependent processes could conceivably contribute to stabilize I_h. Curiously, I_h generated by either expressed or native channels shows a pronounced hyperpolarizing shift in voltage dependence ranging up to 40-60 mV when maintained in cell-free patches, while cAMP sensitivity remains relatively unaltered (DiFrancesco and Mangoni, 1994; Chen S. et al., 2001). This indicates the presence of essential regulatory factors besides cAMP that maintain the voltage dependence of the channels within a physiological range, and ATP could be at least partially responsible (Raes et al., 1997). In addition to PKA-mediated regulation of I_h, protein kinase C and PTKs may contribute to the control of current amplitude (Wu and Cohen, 1997; Shibata et al., 1999). Such regulation can be initiated by growth factors (Thoby-Brisson et al., 2003) and neurotransmitters (Cathala and Paupardin-Tritsch, 1997), but may also contribute to the basal properties of the current.

Regulation by pH. By virtue of its sensitivity to strong extracellular pH changes, I_h may serve as a transducer for sour stimuli (pH 3-5) in a subset of taste cells by generating a depolarizing inward current in response to low pH (Stevens et al., 2001). More moderate changes in extracellular pH by up to one unit, such as those occurring during transient ischemia in the brain (Silver and Erecinska, 1992) may not be sensed by the native currents (Munsch and Pape, 1999a). Conversely, the high sensitivity of I_h to intracellular pH changes has been proposed to underlie the protective action of carbonic anhydrase inhibitors in generalized seizures (Munsch and Pape, 1999b). Carbonic anhydrases catalyse the hydration of carbon dioxide, and their inhibition causes an increase in steady-state pH, presumably through an accumulation of intracellular hydrogen carbonate. In thalamocortical neurons, the resulting augmentation of I_h depolarizes neurons and prevents the generation of

rebound calcium spikes, thus reducing their engagement in synchronized paroxysmal discharges typical for some types of generalized seizures.

6.2. Regulation at the level of mRNA and protein expression

In addition to the regulation of I_h and/or associated regulatory systems by voltage and ligands, differential up- and downregulation of individual HCN channel subunits in defined cell types occurs during development. These maturational processes at the mRNA and protein level correspond to a remarkable extent to the developmental changes in current density and properties. Moreover, their temporal profile matches that of rhythmic synchronized electrical discharges occurring during circuit maturation, suggesting that age-specific network activity patterns may be promoted by regulated HCN channel transcription. Strikingly, it was recently observed that abnormal electrical activity, whether occurring in cardiac or neuronal cells, can profoundly disturb the properties of I_h in both immature and adult systems (see (Santoro and Baram, 2003)), and that this pathological modulation often is associated with an altered transcription of HCN channel subunits. In general, an enhancement of neuronal or cardiac electric activity beyond normal seems to be a major cause in triggering changes in HCN gene expression, although the changes in each subunit HCN1-4 take place seemingly independently of each other (see Table 1). The alterations in the level of transcripts develop on both short- and long-term time scales and often parallel those in the properties of functional channels at least in a qualitative manner. Transcriptional regulation of HCN channels may thus be implicated not only in developmental processes and homeostasis of neuronal excitability, but also in mechanisms of neurologic and cardiac disease.

Developmental regulation. The developmental regulation of HCN expression has so far been studied most extensively in developing mouse ventricular myocytes and in rodent hippocampus. Early embryonic myocytes show prominent regular beating and express a large I_h, which is probably carried primarily by HCN1 and HCN4 channels (Yasui et al., 2001). The amplitude of the current decreases >80% perinatally as spontaneous activity ceases, and involves a strongly decreased expression of HCN1 and HCN4, whereas HCN2 is now the most predominant. The predominance of HCN2 over HCN4 increments even further during aging (Shi et al., 1999).

In pyramidal cells of the mouse CA1 and CA3 region, the densities of I_h conductance undergo a transient increase in the course of the first five to ten postnatal days, before smaller adult values are reached around postnatal day 20 (Vasilyev and Barish, 2002). During this time, I_h activation rates increase up to 10-fold. The expression of HCN1 protein increases strongly in CA1 and CA3 regions and includes both somatic and dendritic layers, with a particularly strong signal in *stratum lacunosum*. HCN2 and HCN4 show a much weaker, but progressive and uniform increase. RNA transcripts encoding HCN genes are also detectable in developing interneurons, in which a differential expression arises around the fifth postnatal day (Bender et al., 2001). Transcripts for HCN1 are found predominantly in parvalbumin-reactive interneurons within the pyramidal cell layer and the *stratum radiatum*. Transcripts for HCN2 and HCN4, however, appear within *stratum oriens* and co-express frequently with the neuropeptide somatostatin. These developmental expression patterns, which are specific for each HCN subunit, may relate to an age-specific role of I_h in the generation of slow network oscillations during the first postnatal weeks.

Cardiopathies. Cardiac myocytes undergo substantial electrical and structural remodeling to adapt to external stressful factors such as pressure overload (e.g. hypertension), inflammation (myocarditis), and infarction (for review, see (Tomaselli and Marban, 1999; Armoundas et al., 2001)). These adaptations are beneficial to maintain cardiac function initially, but can eventually give rise to contractile abnormalities and sudden cardiac death. Myocardial hypertrophy is a complication commonly associated with cardiovascular pathology. Hypertrophied ventricular myocytes from animal models of pressure overload and from the failing human heart show a prolonged duration of the action potential associated with a decrease in repolarizing outward currents, thus principally enabling them 1) to increase Ca^{2+} entry, impair Ca^{2+} uptake and retard relaxation and 2) to contribute to the arrhythmias observed in cardiac disease (for review, see (Tomaselli and Marban, 1999)). Interestingly, in a more advanced stage of hypertrophy, recordings from ventricular myocytes of spontaneously hypertensive rats or from failing human heart revealed the increased appearance of a diastolic depolarization in between the prolonged action potentials, which was associated with the presence of I_h activation at physiological voltages (Cerbai et al., 1994; Cerbai et al., 1997; Hoppe et al., 1998). Notably, in

normal ventricular cells, I_h does not activate until hyperpolarizations well below – 100 mV in these non-pacing regions of the heart (Yu et al., 1993b), suggesting that sustained hypertrophy led to an alteration in I_h properties. In the failing heart, remodeling of I_h was also found in sinoatrial node cells (Verkerk et al., 2003). The degree of myocardial hypertrophy was positively correlated with an increase in the density of I_h (Cerbai et al., 1996), while current voltage dependence, kinetics and modulation by sympathetic stimulation remained unaltered (Cerbai et al., 1996; Fernandez-Velasco et al., 2003). These electrophysiological changes were paralleled by an upregulation of the HCN2 and HCN4 mRNA levels (Hiramatsu et al., 2002; Fernandez-Velasco et al., 2003), which are the predominant isoforms underlying ventricular I_h (Shi et al., 1999). The changes in expression levels were most pronounced in those cardiac regions with highest pressure overload (Fernandez-Velasco et al., 2003), indicating that the processes leading to hypertrophy directly affected the level of HCN expression. The sequence of events leading from hypertrophy to enhancement of I_h in non-pacing regions of the heart appears to involve the activation of the type I angiotensin receptor, as its blockage not only prevents myocyte hypertrophy, but also reverses I_h upregulation and overexpression of HCN2 and HCN4 mRNA (Cerbai et al., 2000; Hiramatsu et al., 2002). Moreover, given the similarities in the expression profile of fetal and hypertrophied myocytes, it has been speculated that cardiac hypertrophy provokes a re-entry of cells into a fetal program and the re-initation of the corresponding gene expression patterns (Cerbai et al., 1996; Yasui et al., 2001). In support of this idea, the density of I_h is higher in rat neonatal ventricular myocytes and progressively decreases postnatally (see Section 6.2.) (Cerbai et al., 1999; Yasui et al., 2001). The studies on the consequences of myocardial hypertrophy presented the first evidence in favor of an altered I_h due to cellular mechanical stress and consequent abnormal electrical activity.

Epilepsies. Currently available data firmly establish that the expression of HCN channels at the molecular level is sensitively controlled by aberrant neuronal activity. Even brief periods of seizures can be sufficient to persistently modify I_h function. These alterations can be neuroprotective or facilitate hyperexcitability, depending on the system in which they occur. For example, in febrile seizures occurring during development, changes in the properties of I_h appear to facilitate rather than to counteract hyperexcitability. Seizures induced by fever are the most common type of

seizure in the developing brain and affect up to 5% of small children (< 5 years old) during periods of high fever. Febrile-like seizures can be induced in a rat model, in which 10-day-old rats are exposed to hyperthermia for a single period of ~30 min. Such animals reliably (>98% of animals) develop epileptic convulsions in the hippocampus that can be prevented by anti-epileptic drugs (Tóth et al., 1998). Moreover, these rats show an increased susceptibility to develop seizures in adulthood (Dube et al., 2000), suggesting that this type of early life seizures may predispose to later epileptic susceptibility (Walker and Kullmann, 1999; Baram et al., 2002). Three persistent modifications of neuronal excitability were determined in hippocampal neurons from such rats. In addition to a long-lasting increase in the release of GABA (Chen et al., 1999) and an increased retrograde signaling via endocannabinoids (Chen et al., 2003), I_h was increased persistently in CA1 cells, even if animals had experienced only a single seizure lasting, on average, 23 minutes (Chen et al., 2001). Other intrinsic currents important for hippocampal cell firing, as well as passive cell properties, remained unaffected. The enhanced expression of I_h induced an augmentation of a rebound sag potential and an increased probability of action potential generation. Interestingly, while the enhanced vesicular release of GABA was dependent upon activation of PKA (Chen et al., 1999), the augmentation of current amplitude was independent of this kinase (Chen et al., 2001), suggesting that multiple independent mechanisms controlling homeostasis of excitability were affected during seizure activity. The functional changes in I_h were paralleled by an altered expression of HCN channel subunits (Brewster et al., 2002), that could qualitatively explain the changes in current properties. These changes were not observed when seizures were prevented by antiepileptics. Thus, hyperthermia-induced brief hyperexcitability led to a persistent functional modification of I_h, likely mediated to a large extent via modifications at the level of channel subunit transcription.

Functional changes in I_h have also recently been reported for animal models of generalized epilepsies, in which the thalamocortical system is primarily involved. In the stargazer mouse, cortical hyperexcitability was found to be associated with a three-fold enhanced amplitude of I_h in cortical layer V neurons (Di Pasquale et al., 1997). In the WAG/Rij rat, an established model for human absence epilepsy, I_h activation was shifted negatively and cAMP sensitivity reduced in thalamocortical cells (Budde et al., 2003). Although comparatively modest, these modifications hyperpolarize the membrane of thalamocortical cells and further their involvement in

the burst discharges typical for spike-and-wave activity. Again, current changes could be largely explained by the observed enhanced expression of HCN1, the channel subunit with the lowest cAMP sensitivity. Both animal models show that, in these generalized seizures, alterations in HCN channel expression appear to be maladaptive by exacerbating the capacity of neurons to integrate in synchronized oscillations associated with absence seizures.

In contrast, in tissue from chronically epileptic human patients, a strong upregulation of HCN1 channel transcripts was found in the dentate gyrus (Bender et al., 2003). The survival of these granule cells in sclerotic hippocampus (Isokawa et al., 1997) suggested that the augmented expression of HCN channels could act in a neuroprotective manner. Indeed, the degree of upregulation in surviving cells was proportional to the extent of cell death in the granule cell layer. Further support for a neuroprotective role of enhanced I_h expression comes from the observation that the anti-epileptic agents lamotrigine and gabapentin upregulate dendritic I_h in hippocampal pyramidal neurons (Poolos et al., 2002; Surges et al., 2003). The dampening effect on neuronal excitability in the hippocampus likely arises via an I_h–mediated decrease of neuronal input resistance and/or a reduction of the temporal summation of repetitive synaptic inputs (see Section 4). Consequently, the enhanced dendritic expression of HCN1 subunits in sclerotic tissue could indeed represent an endogenous neuroprotective process developing during prolonged hyperexcitability. This unique role of I_h shows that in designing anti-epileptic drugs to selectively target molecular subtypes of channel subunits, the family of HCN channels should also be considered (Cosford et al., 2002; Wickenden, 2002).

In view of these various studies on animal models of epilepsy, it is clear that the control of HCN subunit expression in neurons is determined not only by the type of seizures, but also by the cell type and the developmental stage. This latter factor appears to play a particularly important role, because the effects of single febrile seizures in young animals are persistent, whereas those of the stronger kainate seizures in adult animals show no consequence on HCN expression (Bräuer et al., 2001; Brewster et al., 2002). In general, the alterations in the expression of channel message largely explain the functional alterations in current properties, although there are some disagreements with respect to the persistence of the effects at these two levels (see e.g. (Brewster et al., 2002)). The mechanisms translating seizures into altered HCN channel expression remain, so far, unexplored, but could range from

acute influences, such as synaptic activity, (see e.g. (Van Welie et al., 2002)) to long-term, chronic modulation of channel expression, for example via hormones and inflammatory processes (see e.g. (Pachucki et al., 1999)). Detailing the mechanisms controlling transcription and expression of HCN channel genes will certainly be facilitated via cultured preparations which allow the induction of defined types of hyperexcitability for defined periods of time (see (Brewster et al., 2003)).

Nerve injuries. Abnormal spontaneous action potential discharge is a frequent consequence of peripheral nerve injury and is believed to be critical in the initiation and persistence of neuropathic pain syndromes, such as tactile allodynia (strong sensation evoked by light mechanical stimuli) and spontaneous painful sensations (Rappaport and Devor, 1990). Acute injury to the spinal cord can result in a hyperexcitability of not only nociceptive pathways, but also to dorsal root ganglia cell bodies that give rise to large, myelinated Aβ/δ-fibers not normally involved in the transmission of pain (Shir and Seltzer, 1990). A resulting hypersensitivity of sensory pathways and a misrepresentation of sensory information is thought to contribute to the clinical symptoms of neuropathic pain, although central pain processing mechanisms are likely involved as well (Rappaport and Devor, 1990). Several rat models of spinal cord injury, such as axotomy (Black et al., 1999), chronic constriction (Dib-Hajj et al., 1999) or ligation (Kim et al., 2001a) of spinal nerves, show that an altered expression of several voltage-gated Na^+ channel subunits contributes to the persistence of neuronal firing in injured cells (for review, see (Waxman, 2001)). Interestingly, however, neuropathic pain behavior was reversed in a spinal nerve ligation model by the I_h blocker ZD7288, which also reversed the spontaneous discharges in injured large myelinated fibers (Chaplan et al., 2003). In this, as well as in a chronic compression model (Yao et al., 2003), the maximal current density was 1.5-2.5-fold enhanced compared to control, with variable effects on voltage dependence and kinetics. These findings establish I_h upregulation, resulting from nerve injury, as an essential factor leading to the sensitization of spinal cord neurons and to neuropathic pain. The molecular identity of the HCN channels contributing to these changes remains to be determined, but appears to involve a decrease in the amount of HCN1 and HCN2 mRNA and protein in the case of nerve ligation (Chaplan et al., 2003).

Besides neuronal injury, lesions in excitatory input can also cause an altered expression of HCN channels. In the case of deafferentiation of the hippocampus by lesions to the entorhinal cortex, the downregulation of mRNA for HCN1 appears prominently in a differential display of total mRNA from isolated hippocampi, and a strong reduction in HCN1 protein expression in several neuronal cell types of the hippocampus (Bräuer et al., 2001). This decreased expression was paralleled by a strong hyperpolarizing shift (up to 19 mV) in current voltage dependence. The strong changes were partly reversed following reactive sprouting and replacement of entorhinal input by septal and associational afferents.

7. Conclusions.

The past five years have seen an explosion of information on the molecular basis of I_h and its role in normal and pathological processes. The crucial impetus arose with the identification of the molecular subunits constituting the channels and is currently progressing with the arrival of the knock-out animals, which now also allow insight into possible behavioral roles of this current. In addition, the misexpression of mRNA in diseased or injured tissue shows that HCN channels can promote channelopathies arising at the transcriptional level (Waxman, 2001). Although such pathogenic properties are well-known for other channel types (for review, see (Waxman, 2001)), they appear to be particularly dramatic for the HCN channels since these often occupy a unique physiological role in a cell's channel repertoire that can not be easily complemented or substituted by other ionic channels. Dysregulated expression of HCN channels is further complicated by its variable appearance. It can be reversible or persistent in time, and appears to be dependent on the precise cellular and developmental context in which it occurs. Furthermore, inflammation or ischemia occurring under pathological situations may influence the current (Erdemli and Crunelli, 1998; Linden et al., 2003).

A few recent studies have highlighted that understanding the regulation of I_h functionality in intact systems will also require considerable attention in the near future. For example, in cardiac cells, the properties of I_h are determined by sympathetic innervation (Qu et al., 2000) and appear to be co-regulated by β_2-adrenergic receptors (Graf et al., 2001). In lobster stomatogastric neurons, I_h expression is co-regulated with the expression of channels giving rise to transient K^+

currents. This regulation occurs in an activity-independent manner and appears to take place at the translational level, as it is not influenced by blockers of transcription (MacLean et al., 2003). Altogether, these data strongly suggest that many aspects of channel expression, including activation of promoter regions of HCN genes, channel synthesis and trafficking, as well as on-site regulation of the channels carefully control both density and properties of I_h. Understanding these pathways, in a manner as elaborated perhaps as that on the trafficking and homeostasis of synaptic glutamate receptors, may prove crucial in designing novel therapeutic targets for cardiac and neuronal pathologies. Moreover, they may contribute in the development of a major conceptually novel therapeutic approach emerging in the HCN channel field: the *de novo* creation of biological pacemakers capable of driving the heart when the sinus node signal fails (Plotnikov et al., 2004) and, perhaps, also for diseases associated with rhythmicity in the brain.

Figure legends.

Fig. 1. **Summary of basic functional and structural characteristics of I_h and the underlying HCN channels**. **A**. Transmembrane topology of the cloned HCN channels. S1-S6 symbolize the 6 transmembrane-spanning domains of the channels, N and C the N- and C-terminus, respectively. The box at the C-terminus represents the cyclic nucleotide-binding domain, which is connected to the channel via a C-linker domain (Wang et al., 2001) important in coupling the binding of cyclic nucleotide to alterations in voltage-gating of the channel. The number of amino acids at both termini vary for the four HCN subunits. **B**. *Left*, activation curves of I_h recorded in ferret thalamocortical cells in the presence of incrementing concentrations of 8Br-cAMP, a weakly hydrolyzable analog of cAMP, in the whole-cell recording pipette. Note the progressive rightward shift of the activation curve with increasing levels of the cyclic nucleotide. *Inset* shows a family of current responses generated by steps to the voltages indicated. Vertical and horizontal scale bars indicate 400 pA and 400 ms, respectively. *Right*, concentration-response curve of the shift in the half-activation voltage induced by 8Br-cAMP. The maximal shift corresponds to 10 mV. **C**. Identification of the 'funny' current, activating at voltage ranges

covered by the diastolic potential. Reproduced from Reference 11 (copyright permission from Nature).

Fig. 2. **Summary of old and novel physiological roles of I_h in neurons**. A single cell is drawn schematically with an axonal (A), somatic (S) and dendritic (D) compartment. The axonal compartment can also represent a presynaptic specialization in retinal neurons. The roles of I_h are described with key words assigned to these compartments. The different regulatory pathways involved in these roles are symbolized by ionic channels with various colours, as indicated in the legend.

Fig. 3. **The dual gating of I_h by voltage and cyclic nucleotides: model and its physiological consequences**. **A**. Model of a channel occupying four possible states following concerted voltage-induced transitions between the four closed (squares) and the four open (circles) subunits, as well as via ligand binding and unbinding. Binding sites for cyclic nucleotides are shown as half-squares or half-circles attached to the closed and open subunits by a line. Cyclic nucleotides are symbolized by filled circles. Thick arrows mark the preferred directions of transitions. For further details, see (Wang et al., 2002a). **B**. Dual gating of I_h by voltage and ligands results in a persistent activation of the channels that contributes to the timing of slow network rhythms. An intracellular recording from a ferret thalamocortical cell participating in spindle waves *in vitro* is shown, indicating the coincidental occurrence of repetitive hyperpolarizing inputs (inhibitory postsynaptic potentials, IPSPs) and the rebound Ca^{2+} spikes, which lead to the synthesis of cAMP (filled circles). Note the small depolarization (I_h-mediated) following each spindle wave. Presumed channel states occupied preferentially during the different phases of the network rhythms are shown at the bottom. Note the persistence of channels in the dually gated states even after the cAMP transient has mostly dissipated. For further details, see references in Chapter 6.1.

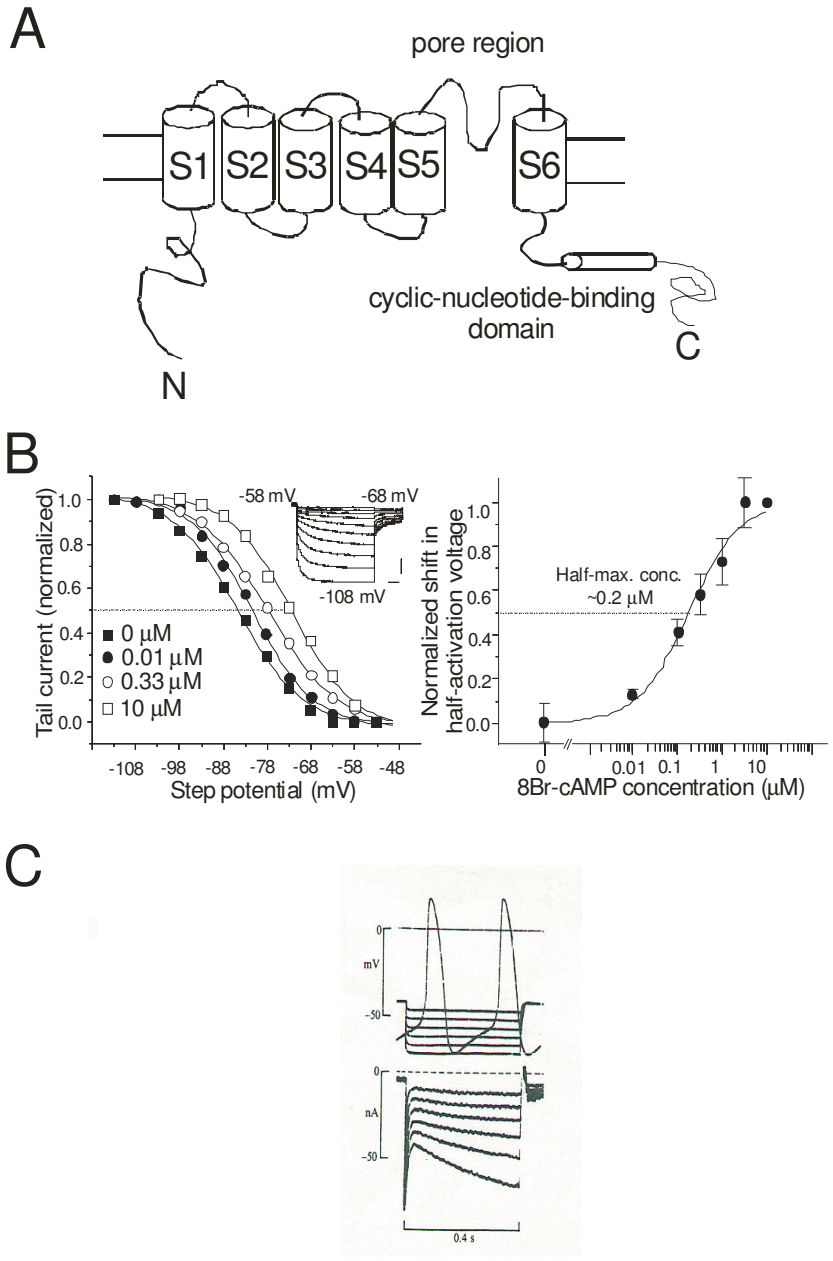
A
pore region
S1
S2
S3
S4
S5
S6
cyclic-nucleotide-binding
domain
C
N
B
Tail current (normalized)
1.0
0.8
0.6
0.4
0.2
0.0
-58 mV
-68 mV
-108 mV
0 μM
0.01 μM
0.33 μM
10 μM
-108 -98 -88 -78 -68 -58 -48
Step potential (mV)
Normalized shift in
half-activation voltage
Half-max. conc.
~0.2 μM
0 0.01 0.1 1 10
8Br-cAMP concentration (μM)
C
0
mV
-50
0
nA
-50
0.4 s

Figure 1.

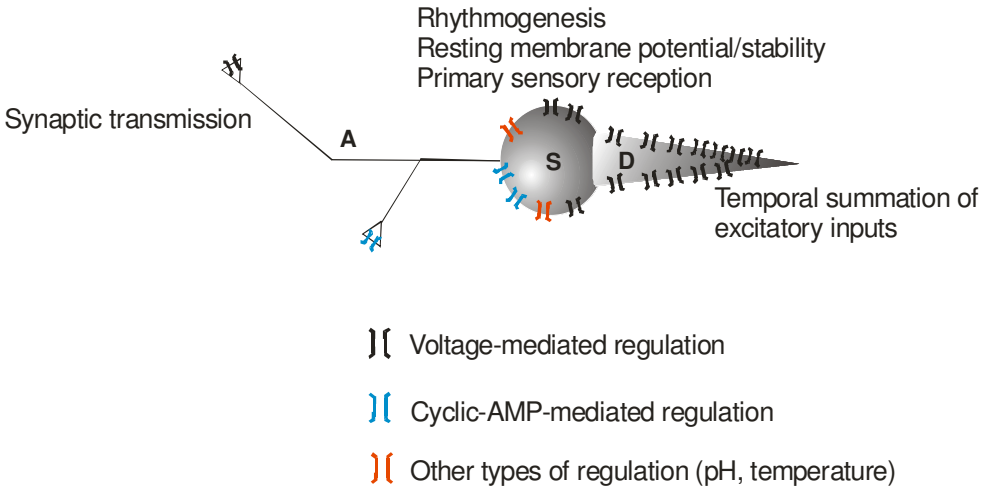
Rhythmogenesis
Resting membrane potential/stability
Primary sensory reception
Synaptic transmission
A
S
D
Temporal summation of
excitatory inputs
Voltage-mediated regulation
Cyclic-AMP-mediated regulation
Other types of regulation (pH, temperature)

Figure 2.

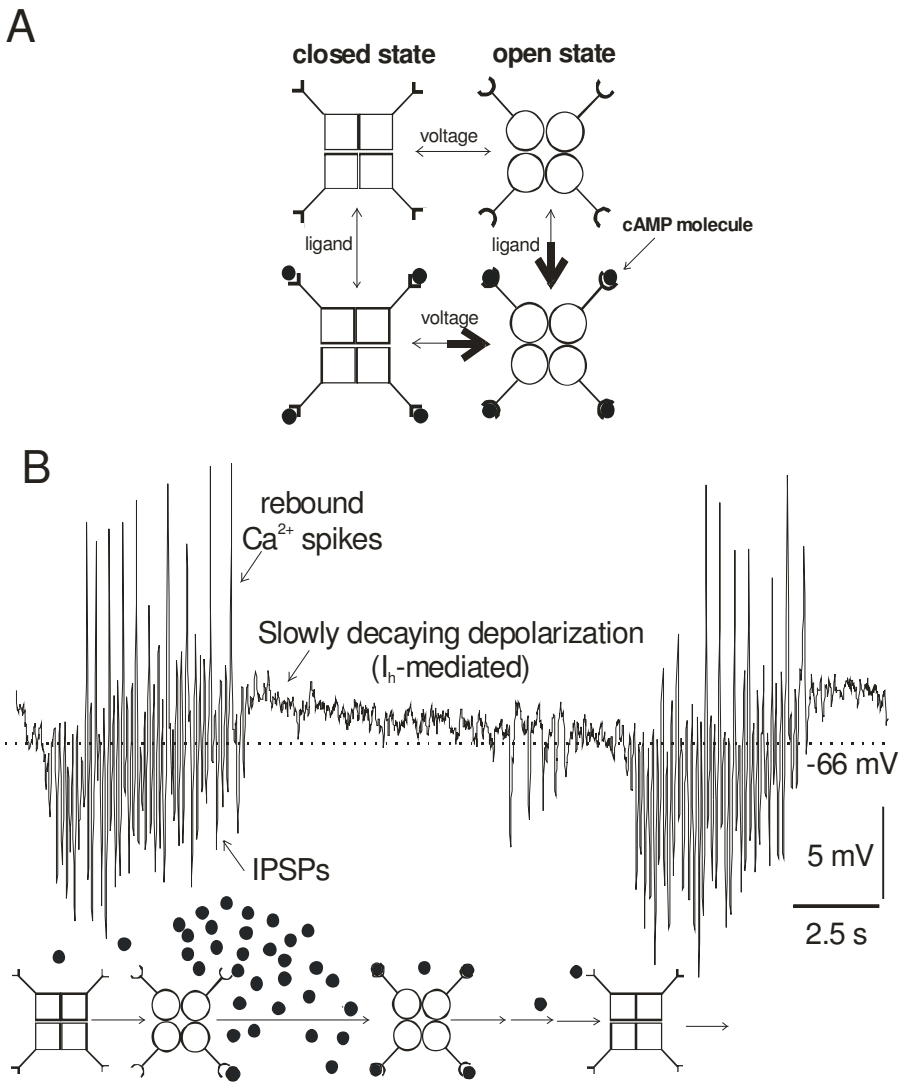
A
closed state
open state
voltage
ligand
ligand
cAMP molecule
voltage
B
rebound
Ca2+ spikes
Slowly decaying depolarization
(Ih-mediated)
-66 mV
IPSPs
5 mV
2.5 s

Figure 3.

HCN subunit	Transcription enhanced	Transcription diminished
HCN1	• Chronic temporal lobe epilepsy (174) • WAG/Rij rat, thalamocortical neurons (173)	• Febrile seizures, CA1 (171) • Kainate seizures in young animals, CA1+CA3 (171) • Entorhinal cortex lesion, hilar neurons • Spinal nerve ligation, dorsal root gangl cells (189)
HCN2	• Febrile seizures, CA1 (171) • Kainate seizures in young animals, CA1+CA3 (171) • Hypertrophy of cardiac ventricle (159-160)	• Spinal nerve ligation, dorsal root gangli cells (189)
HCN4	• Hypertrophy of cardiac ventricle (159-160)	

Table 1. Changes in mRNA expression for HCN subunits found in tissues producing abnormal cardiac or neural activity. Numbers in parentheses indicate the reference. The correspondence between altered mRNA expression and functional current alterations are often incomplete and not presented in the table. In the following studies, the changes in mRNA expression were paralleled by similar alterations in protein expression: 174, 180, 189

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List of abbreviations

$[cAMP]_i$	intracellular concentration of cAMP
4-AP	4-aminopyridine
AC	Adenylyl cyclase
ACPD	(±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid
AMPA	S-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid
AP	Action potential
APT	Anterior pretectum
Bac	Baclofen
BC	Basket cells
BZ	Benzodiazepine
cAMP	Cyclic 3', 5'-adenosine monophosphate
CAT	Chloramphenicol acetyl transferase
CB	Calbindin
CCK	Cholecystokinin
CI	Calcium impermeable
CP	Calcium permeable
CR	Calretinin
CRE	cAMP responsive element
CRH	Corticotrophin-releasing hormon
CT	Corticothalamic
dLGN	dorsal lateral geniculate
EEG	Electroencephalo gramm/graphic
EPS P/C	Excitatory postsynaptic potential/current
FeedB	Feedback
FeedF	Feedforward
GAD	Glutamic acid decarboxylase
GPCR	G-protein-coupled receptors
FS	Fast-spiking
GABA	γ-aminobutyric acid
$GABA_A$ receptor	γ-aminobutyric acid receptor type A

$GABA_B$ receptor	γ-aminobutyric acid receptor type B
$GABA_C$ receptor	γ-aminobutyric acid receptor type C
HCN	Hyperpolarization-activated cationic non-selective
HVA	High-voltage activated
HOn	Higher-order nuclei
I_{AHP}	Afterhyperpolarization or calcium-dependent potassium current
I_h	Hyperpolarization-activated cationic current
IPS P/C	Inhibitory postsynaptic potential/current
I_T	Low-threshold calcium current
Iso	Isoproterenol
LD	Thalamic latero-dorsal nucleus
LTS	Low-threshold spike
LVA	Low-voltage activated
LP	Thalamic latero-posterior nucleus
MF	Mossy fiber
NEM	N-ethylmaleimide
NMDA	*N*-methyl-D-aspartate
NPY	Neuropeptide Y
PKA	Protein kinase A
Po	Thalamic posterior nucleus
nRt	Nucleus reticularis
PC	Pyramidal or principal cell
PV	Parvalbumin
SST	Somatostatin
SWD	Spike-and-wave discharge
TC	Thalamocortical
TEA	Tetraethylamonium
VB	Ventrobasal
VIP	Vasoactive intestinal peptide
ZI	Zona incerta
ZIv	Ventral domain of the zona incerta

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