Single-cell mRNA expression of *HCN1* correlates with a fast gating phenotype of hyperpolarization-activated cyclic nucleotide-gated ion channels (Ih) in central neurons

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**Abstract**

Hyperpolarization-activated currents (Ih) are key players in shaping rhythmic neuronal activity. Although candidate genes for Ih channels have been cloned (*HCN1–HCN4*), the subunit composition of different native Ih channels is unknown. We used a combined patch-clamp and qualitative single-cell reverse transcription multiplex polymerase chain reaction (RT-mPCR) approach to analyse *HCN1–4* coexpression profiles in four neuronal populations in mouse CNS. Coexpression of *HCN2, HCN3* and *HCN4* mRNA was detected in single neurons of all four neuronal cell types analysed. In contrast, *HCN1* mRNA was detected in neocortical and hippocampal pyramidal neurons but not in dopaminergic midbrain and thalamocortical neurons. *HCN1* expression was correlated with significantly faster activation kinetics on the level of individual neurons. Semi-quantitative single-cell RT-mPCR analysis demonstrated that *HCN1* mRNA expression is at least eightfold higher in cortical neurons than subcortical neurons. We show that single neurons possess complex coexpression patterns of Ih candidate genes. Alternative expression of *HCN1* is likely to be an important molecular determinant to generate the different neuronal Ih channel species adapted to tune either subcortical or cortical network activity.

**Introduction**

Hyperpolarization-activated cationic conductances (Ih) are present in many neurons and cardiac cells and are also found in other tissues (DiFrancesco, 1993; Pape, 1994). Ih channels possess small single channel conductances of ~1 pS and are permeable to K+ and Na+ ions. They are activated by hyperpolarizations beyond ~50 mV and their voltage-dependence is modulated by intracellular cyclic nucleotides. As a consequence of its mixed K+/Na+ permeability and inverse voltage-dependence, somatodendritic neuronal Ih channels are involved in several functions (Pape, 1996; Lüthi & McCormick, 1998). The classical function is contributing to a slow pacemaker depolarization after electrical discharge. In addition, Ih will tune the frequency preference and temporal summation of excitatory post-synaptic potentials (EPSPs; Magee, 1999). Thus, Ih channels might act as low-pass filters and help to determine the resonance properties of neurons (Hutcheon et al., 1996). Ih currents have also been described in axons (Takigawa et al., 1998) and presynaptic terminals (Owens et al., 1999).

At least four members of a new family of mammalian Ih candidate genes have been identified (Santoro et al., 1997; Ludwig et al., 1998; Santoro et al., 1998; Ishii et al., 1999; Ludwig et al., 1999; Seifert et al., 1999) and recently a unifying nomenclature has been adopted (Clapham, 1998): HCN1 (*mBCNG-1, HAC2*); HCN2 (*mBCNG-2, HAC1*); HCN3 (*HAC3*); and HCN4 (*mBCNG-3*). This hyperpolarization-activated, cyclic nucleotide-gated channel family is related to six transmembrane voltage-activated K+ channels as well as to cyclic nucleotide-gated nonselective channels. Heterologous expression of homomeric HCN1, HCN2 and HCN4 channels demonstrated that they all possess salient features of native Ih channels (Ludwig et al., 1998; Santoro et al., 1998; Ludwig et al., 1999). However, the role of these genes in the molecular makeup of neuronal Ih channel species is unclear.

The aim of this study was to combine patch-clamp electrophysiology with the single-cell reverse transcription multiplex polymerase chain reaction (RT-mPCR) technique developed by Rossier and coworkers (Lambez et al., 1992; Cau et al., 1997) to determine *HCN1–4* expression profiles on the level of single neurons in different populations characterized by different types of somatodendritic Ih currents. In our study, we analysed dopaminergic midbrain neurons (Mercuri et al., 1995) as well as the classical Ih locus, thalamocortical relay neurons (Pape & McCormick, 1989). We also included neocortical layer V and CA1 hippocampal pyramidal cells because they possess Ih currents with different gating properties compared to subcortical neurons (Maccabetti et al., 1993; Solomon & Nérbonne, 1993; Budde et al., 1994; Magee, 1998).

**Materials and methods**

**Whole-cell recordings and data analysis**

Preparation of 250 µm coronal brain slices from mice C57Bl/6J (12–16 postnatal days old killed by cervical dislocation) were performed as previously described (Liss et al., 1999). For patch-clamp recordings, slices were transferred to a chamber continuously perfused at 2–4 ml/min with artificial cerebrospinal fluid (ACSF) bubbled with a mixture of 95% O2/5% CO2 at room temperature (22–24 °C). Patch pipettes (whole-cell recordings, 1–2.5 MΩ; dendritic recordings, 8–12 MΩ) were pulled from borosilicate glass (GC150TF, Clark, Reading, UK) and filled with internal solution containing (in mM):
KCl, 140; HEPES, 5; EGTA, 5; MgCl₂, 3 at pH 7.3. Whole-cell recordings were obtained from somata and dendrites (>50μm distance from soma) of neurons visualized by infrared differential interference contrast (IR-DIC) videomicroscopy. Recordings were carried out using an EPC-9 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany). Series resistances were electronically compensated (75–85%). The program package PULSE+ PULSEFIT (HEKA Electronic, Lambrecht, Germany) was used for data

RT minus control

![Graphs and images of RT multiplex PCR and RT minus control](image)

Fig. 1. RT-multiplex PCR (RT-mPCR) of different Ih channel candidate genes. The multiplex protocol was designed to probe for the four putative Ih channel mRNAs HCN1–HCN4 in combination with the marker transcripts tyrosine hydroxylase (TH) and glutamate decarboxylase (GAD67). The products of the second, nested PCRs were run on a 2% agarose gel in parallel with a 100-bp ladder as molecular weight marker. (a) In mouse whole brain cDNA, all six PCR amplicons could be detected at the size predicted by their mRNA sequences. (b) Result of a RT-mPCR from a single dopaminergic substantia nigra (SN) neuron (single-cell PCR) where HCN2–4 and TH were detected. (c) Result of RT minus control. Without reverse transcriptase in the cDNA reaction, no PCR signal was detected in single-cell material.

Fig. 2. Slow activating Ih channels in dopaminergic substantia nigra (SN) neurons. (a) Membrane potential responses in a dopaminergic SN neuron to 1 s injections of hyperpolarizing current of increasing amplitudes (20 pA, –80 pA). Note the slow sag component that developed with increasing hyperpolarizations. (b) Somatic whole cell (wc) recordings from dopaminergic SN neurons showed Ih currents with slow activation kinetics in response to 10 s membrane hyperpolarizations from –40 mV to –120 mV in steps of 10 mV; from a holding potential of –40 mV followed by a voltage step to –120 mV to record Ih tail currents. Insert shows respective voltage-dependence of Ih activation determined from tail analysis and fitted with a Boltzmann function. (c) Voltage-dependence of mean (● ± SEM; n=8) activation time constants. Line indicates fit by monoeponential function. (d) Dendritic whole cell (wc) recording from a dopaminergic SN neuron showed Ih currents in response to 2 s membrane hyperpolarizations from –40 mV to –120 mV in steps of 10 mV from a holding potential of –40 mV followed by a voltage step to –120 mV (e) Slow activating Ih current in response to a 2 s hyperpolarization to –120 mV from a holding potential of –40 mV in a dendritic outside-out (o/o) recording from a SN neuron overlaid with a fitted monoeponential function with a time constant of τ≈1 = 518 ms.
acquisition and analysis. Recordings were digitized at 2–5 kHz and filtered with low-pass filter Bessel characteristics of 0.4–1 kHz cut-off frequency. The time constants of activation were fitted with no constraints to both mono- and biexponential functions \( e^{-x(tau)} + e^{-x(tau-1)} \) using a nonlinear least-square fitting routine (Levenberg–Marquardt algorithm) in IGOR (Wavermetrics, USA). The goodness-of-fit was analysed by computing the residuals and quantified by comparing the \( \chi^2 \) values and the standard deviation of the parameters. Significance levels were determined by Student’s t-test. Data are given as mean ± SEM. Ignoring the sigmoidal onset of Ih activation (Pape, 1996), mono- and biexponential functions were chosen to facilitate direct comparison with heterologously expressed HCN channels (Santoro et al., 1998; Ludwig et al., 1999; Seifert et al., 1999).

**Multiplex and nested PCR**

Harvesting of cytoplasm and reverse transcription were carried out as previously described (Liss et al., 1999). Following reverse transcription, the cDNAs for HCN1–4, for tyrosine hydroxylase (TH) and the 67 kDa form of glutamate decarboxylase (GAD67) were simultaneously amplified in a multiplex PCR using the following set of primers (from 5’ to 3’): HCN1 (HAC2, accession No. AJ225123) sense, TCTTGGTATTACCCATT (position 985), antisense, TTTCTTCCATCCGATCG (position 1983); HCN2 (HAC1, accession No. AJ225122) sense, TACTTGGCTACGGTTCCGT (position 810), antisense, GAAATGGGCCATCCGACA (position 1775); HCN3 (HAC3, accession No. AJ225124) sense, CGCATCCAGAGTACTACGA (position 1242), antisense, CACCTCCAGGCTCCTTACGC (position 2322); HCN4 (mBCN-3, accession No. AF064874) sense, TCTGATCATCATAACCGTGG (position 295), antisense, GAAGACCTCGAAACGCAACT (position 1315); TH (accession No. M69200) sense, CACCTGGAGATCTTGTGGG (position 387), antisense, CCTGTGGTGGTGTACCTATG (position 1525); GAD67 (accession No. Z49976) sense, TGACATCCATCGAATACC (position 731), antisense, GGGTTAGATGACCATCCCG (position 1835). Multiplex and nested PCR were performed as previously described (Liss et al., 1999). Nested PCR amplifications were carried out using the following primer pairs: HCN1 sense, CTTTTTGGATACCGTG (position 1612), antisense, CATTGGAAAATCCGCG (position 1902); HCN2 sense, GTGGGAGCAGCTCTCTCGT (position 1181), antisense, GTTCACACTCTCTCACCGA (position 1550); HCN3 sense, GCAGATTTGTTACACACG (position 1808), antisense, AGCTCTACAGACGAGCT (position 2040); HCN4 sense, GACAGATCCATCGACTAC (position 1110), antisense, ACAAGACTAGTGACTGCTGT (position 1278); TH sense, TGACACAGTACGTCCTACGA (position 936), antisense TCTGACAC-GAAGTACCCCG (position 1312); GAD67 sense, CATATGAAATTTGGCCGCG (position 761), antisense, CGGTGCATGAGGGACGCTA (position 1462). The PCR products (15 µL aliquots) were separated and visualized in an ethidium bromide-stained agarose gel (2%) by electrophoresis. The predicted sizes (bp) of the PCR-generated fragments were: 702 (GAD67); 377 (TH); 370 (HCN2); 291 (HCN1); 233 (HCN3); and 169 (HCN4). All individual PCR products were verified by direct sequencing. DNA isolation and cDNA preparation for control reactions was carried out as described previously (Liss et al., 1999). All six PCR fragments were detected routinely in the positive control with the PCR protocol described above. Negative controls, where path pipettes were filled with the single-cell PCR solution, advanced into the slice preparation without harvesting single-cell material, expelled and subjected to the RT-nPCR protocol, were also carried out in parallel to all single-cell experiments. In addition, single-cell nPCR amplifications were carried out without prior reverse transcription to probe for possible amplification of genomic DNA from the harvested single nuclei (RT minus control). For semiquantitative single-cell RT-PCR of HCN1 we generated serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64) of single-cell cDNA pools. Each dilution was used as template in a nested PCR (2 × 35 cycles) with HCN1 primers as described above. Detection thresholds were analysed using agarose gel electrophoresis.

**Results**

**Single-cell RT-nPCR for Ih candidate genes**

An RT-nPCR protocol was designed to simultaneously detect, at the single-cell level in mouse brain slices, the mRNAs of the putative Ih channel subunits HCN1 (BCNG-1, HAC2), HCN2 (BCNG-2, HAC1), HCN3 (BCNG-4, HAC3), and HCN4 (BCNG-3). In addition, the protocol probed for the expression of the marker genes TH for dopaminergic neurons and GAD67 for GABAergic neurons. Figure 1a shows that six specific PCR products were detected from whole brain cDNA diluted to the picogram range. The size of each of the PCR

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amplicons was predicted by its respective mRNA sequence. Consistent with previous findings, all mRNAs for the four Ih channel subunits are expressed in brain as well as TH and GAD67. The established single-cell RT-mPCR protocol was then used to analyse Ih channel subunit expression in several neuronal cell types, e.g. dopaminergic substantia nigra (SN) neurons (Fig.1b). As the genomic structures of the HCN genes were unknown at the time of primer design, single-cell mPCR amplifications were carried out without prior reverse transcription to probe for possible amplification of genomic DNA from the harvested single nuclei (RT minus control). For all analysed neurons (n=10) no PCR products were detectable (Fig.1c). Thus, our protocol was suited to study genuine HCN mRNA expression profiles of single neurons.

Fast and slow activating somatodendritic Ih channels in central mouse neurons

In previous studies, large amplitude, slow activating Ih currents were described in dopaminergic SN neurons (Mercuri et al., 1995) and thalamic relay neurons in rat and other species (Pape, 1996). As evident from their slow sag components in response to hyperpolarizing currents (Fig. 2a) mouse dopaminergic SN neurons also displayed significant Ih conductances. We characterized Ih properties in the standard whole-cell configuration in the presence of 500 nM tetrodotoxin, 50 μM picrotoxin and 10 μM DNQX to block sodium channels and fast synaptic transmission. Slow activating Ih currents were elicited by 2–20 s membrane hyperpolarizations of increasing amplitude followed by a voltage step to −120 mV to analyse Ih tail currents (Fig. 2b). The tail currents were used to analyse the voltage dependence of Ih in dopaminergic SN neurons. As previously observed (Seifert et al., 1999), half-maximal activation of Ih critically depends on pulse duration. With 2 s voltage steps, the mean half-maximal activation (V 50) of Ih was −89.2 ± 1.1 mV and the mean slope was 9.6 ± 0.4 mV (n = 13). Using 10 s voltage steps (Fig. 2b), the mean V 50 was more positive at −82.9 ± 1.0 mV and the mean slope was 6.5 ± 0.3 mV (n = 8). Further increase of the duration of the hyperpolarizing voltage step to 20 s gave very similar results (V 50 = −81.3 ± 1.2 mV, slope = 6.7 ± 0.3 mV, n = 4) indicating a saturation of the pulse-length-dependent shift of V 50. The time course of Ih activation during 10 s hyperpolarizing voltage steps was best described by double-exponential functions. In the voltage range between −80 mV and −120 mV the dominant component of Ih activated with time constants ranging between 5 s and 500 ms in dopaminergic SN neurons (Fig. 2c). Close to full activation, the mean time constant of the dominant faster component was τ1 (at −120 mV) = 482 ± 70 ms (n = 8) and that of the slower one was τ2 = 120 mV = 2.09 ± 0.42 s (n = 8). Similar time constants were

Fig. 4. Fast activating Ih channels in neocortical layer V neurons. (a) Membrane potential responses in a neocortical layer V neuron to 1 s injections of hyperpolarizing current of increasing amplitudes (20 pA, −80 pA). Note the fast sag component. (b) Somatic whole cell (wc) recording from a neocortical layer V neuron demonstrated Ih currents with fast activation kinetics in response to 2 s membrane depolarizations from −40 mV to −120 mV in steps of 10 mV from a holding potential of −40 mV followed by a voltage step to −120 mV to record Ih tail currents. Insert shows representative voltage-dependency of Ih activation fitted with a Boltzmann function. (c) Voltage-dependence of mean (± SEM, n = 8) activation time constants. Line indicates fit by monoexponential function. (d) Dendritic whole cell (wc) recording from a neocortical layer V neuron demonstrated Ih currents with a fast activation. Voltage protocol as in (b). (e) Ih current with fast activating component in response to a 2 s membrane hyperpolarization to −120 mV in a dendritic outside-out (o/o) recording from a neocortical layer V neuron overlaid with a fitted biexponential function with τ1 = 63 ms and τ2 = 420 ms.
obtained from analysis of Ih activation elicited with shorter (2 s) hyperpolarizing voltage steps (\( \tau_{1} @ -120 \text{mV} = 387 \pm 42 \text{ ms}, \tau_{2} @ -120 \text{mV} = 1.82 \pm 0.33 \text{ ms}; n = 29 \)).

The inclusion of 1 mM 8-Bromo-cAMP in the pipette solution induced a positive voltage shift of 11 mV to a mean half-activation voltage of \(-78.7 \pm 1.1 \text{ mV} \) (2 s voltage-steps) without significant changes in the slope of the activation curve \((9.6 \pm 0.6 \text{ mV}, n = 6)\). Ih currents were not detectable in large (nucleated) outside-out patches from dopaminergic SN neurons (not shown) which indicated that Ih channels are preferentially expressed in dendritic compartments. Indeed large Ih currents were observed in dendritic whole-cell recordings (Fig. 2d) and Ih channels were also present in dendritic outside-out recordings (Fig. 2e) from dopaminergic SN neurons. Although differences in series resistances and cable properties in the dendritic tree are expected to affect the results, the comparison of the voltage-dependencies in somatic and dendritic whole-cell recordings revealed no significant differences (2 s voltage-steps: dendritic whole-cell \( V_{50} = -87.0 \pm 0.6 \text{ mV}, \text{slope} = 8.1 \pm 0.6 \text{ mV}, n = 7 \)). In contrast to somatic recordings, the kinetics of Ih activation in dendritic recordings were best described by monoeponential functions with time constants similar to the fast component in somatic recordings (dendritic whole cell \( \tau_{1} @ -120 \text{mV} = 497 \pm 27 \text{ ms}, n = 7 \); dendritic outside-out \( \tau_{1} @ -120 \text{mV} = 542 \pm 22 \text{ ms}, n = 5 \)).

For comparison, we also studied the properties of Ih currents in thalamocortical relay neurons. As shown in Fig. 3, activation kinetics and voltage dependence of Ih in this neuronal population were similar to those obtained in dopaminergic SN neurons and a similar pulse-length dependent shift of \( V_{50} \) was observed \( V_{50} = -90.9 \pm 1.2 \text{ mV}, \text{slope} = 8.7 \pm 0.5, n = 11; V_{50} = -90.5 \pm 0.8 \text{ mV}, \text{slope} = 6.6 \pm 0.8, n = 6; V_{50} = -83.1 \pm 1.8 \text{ mV}, \text{slope} = 7.1 \pm 0.6, n = 4 \). Also for thalamocortical neurons, the time course of Ih activation during 10 s hyperpolarizing voltage steps (Fig. 3a) was best described by double-exponential functions. In the voltage range between \(-80 \text{ mV} \) and \(-120 \text{ mV} \) the dominant component of Ih activated with time constants ranging between 7.5 s and 600 s in thalamocortical neurons (Fig. 3b). Close to full activation, the mean time constant of the dominant faster component was \( \tau_{1} @ -120 \text{mV} = 602 \pm 80 \text{ ms} (n = 8) \) and that of the slower one was \( \tau_{2} @ -120 \text{mV} = 2.70 \pm 0.56 \text{ s}, n = 8 \). Similar time constants were obtained from analysis of thalamocortical Ih activation elicited by shorter, 2 s hyperpolarizing voltage steps (\( \tau_{1} @ -120 \text{mV} = 445 \pm 25 \text{ ms}, \tau_{2} @ -120 \text{mV} = 2.53 \pm 0.36 \text{ ms}, n = 20 \)). The ratios between the amplitudes of the dominant fast (\( \tau_{1} \)) and the slow (\( \tau_{2} \)) components (\( R_{\text{tau1/2}} \)) were also similar in dopaminergic SN neurons (\( R_{\text{tau1/2}} @ -120 \text{mV} = 2.8 \pm 0.6, n = 8 \)) and thalamocortical neurons (\( R_{\text{tau1/2}} @ -120 \text{mV} = 2.6 \pm 0.3, n = 8 \)).

Analysis of the functional properties of Ih currents both in neocortical layer V and hippocampal CA1 neurons suggested that these pyramidal cells possess a different, significantly faster activating Ih species (Figs 4 and 5). This was already evident from comparing the kinetics of the sag component in response to hyperpolarizing current injections in cortical (Fig. 4a) vs subcortical neurons (Fig. 2a). The voltage-dependence of cortical and hippocampal Ih determined with 2 s voltage steps was very similar, with mean half-maximal activation voltages at \(-83.3 \pm 1.1 \text{ mV} \) (cortex, \( n = 14 \)) and \(-84.3 \pm 0.5 \text{ mV} \) (hippocampus, \( n = 15 \)) and mean slopes of \( 9.8 \pm 0.5 \text{ mV} \) (cortex, \( n = 14 \)) and \( 10.1 \pm 0.3 \text{ mV} \) (hippocampus, \( n = 15 \)), respectively. Also in these neurons, the time course of Ih activation in response to hyperpolarizing voltage steps was best described by double-exponential functions with a dominant faster component that had time constants ranging between 50 and 500 ms in the voltage range between \(-80 \text{ mV} \) and \(-120 \text{ mV} \) (Figs 4c and 5b). The minor slow component had time constants in the order of 500–700 ms (cortex \( \tau_{1} = 84.1 \pm 4.6 \text{ ms}, \tau_{2} = 660 \pm 81 \text{ ms}, n = 19 \); hippocampus \( \tau_{1} = 64.4 \pm 2.9 \text{ ms}, \tau_{2} = 537 \pm 47 \text{ ms}, n = 18 \)). The ratios between the amplitudes of the fast (\( \tau_{1} \)) and the slow (\( \tau_{2} \)) components (\( R_{\text{tau1/2}} \)) were also very similar in cortical (\( R_{\text{tau1/2}} @ -120 \text{mV} = 1.5 \pm 0.1, n = 19 \)) and hippocampal pyramidal neurons (\( R_{\text{tau1/2}} @ -120 \text{mV} = 1.6 \pm 0.1, n = 18 \)).

The effect of cyclic nucleotides on this Ih species was smaller compared to that of subcortical neurons. In hippocampal CA1 neurons, inclusion of 1 mM 8-Bromo-cAMP in the pipette solution only induced a positive shift of 7 mV to a half-maximal activation voltage of \(-77.4 \pm 2.5 \text{ mV} (n = 5)\). Similar to subcortical neurons, Ih channels in hippocampal CA1 were shown to be preferentially expressed in dendritic compartments (Magee, 1998). This was also the case for neocortical layer V neurons where Ih currents were detected in dendritic whole-cell and dendritic outside-out recordings.
Fig. 6. Qualitative single-cell RT-mPCR expression profiles of HCN1–4 mRNAs in four neuronal populations. (a–c) Left panels show current responses to 2 s membrane hyperpolarizations from −50 mV to −120 mV in steps of 10 mV from a holding potential of −40 mV followed by a voltage step to −120 mV from different neuronal cell types as indicated. After electrophysiological analysis, cytoplasm of the cells was harvested for single-cell RT-mPCR. Middle panels show the respective agarose gel analyses of HCN1–4 expression profiles in these cells. Marker transcripts are not shown. Right panels show the detection (in percentage) of the different HCN transcripts by single-cell PCR in the respective neuronal populations.
(Fig. 4de). The voltage-dependence and activation kinetics of Ih currents obtained from dendritic recordings in cortical layer V cells were similar to those of somatic recordings (dendritic whole-cell V<sub>30</sub> = −78.9 ± 0.8 mV, slope = 6.8 ± 0.8 mV; tau-1 (@ −120 mV) = 82.7 ± 10.0 ms, tau-2 (@ −120 mV) = 417 ± 83 ms, n = 6; dendritic outside-out tau-1 (@ −120 mV) = 65.0 ± 5.3 ms, tau-2 (@ −120 mV) = 449 ± 31 ms n = 4).

**Alternative coexpression of HCN subunits correlates with different Ih phenotypes in central neurons**

Figure 6 summarizes the results of our single-cell RT-qPCR study for all four neuronal populations analysed. The left panels show current responses to 2 s membrane hyperpolarizations of increasing amplitudes followed by a voltage step to −120 mV. The middle panels depict the respective agarose gel analyses of the HCN1–4 expression profiles for the same cells. The right panels give the relative percentage of detection of all HCN mRNAs probed in the respective cell populations. Both subcortical cell types, dopaminergic SN (n = 17, GAD67<sup>−</sup> TH<sup>+</sup>) and thalamic relay neurons (n = 10; GAD67<sup>−</sup> TH<sup>+</sup>) not only displayed very similar Ih phenotypes but also identical HCN2–4 coexpression profiles while HCN1 was not detected (Fig. 4a and b). In contrast, neocortical layer V pyramidal neurons (n = 10; GAD67<sup>−</sup> TH<sup>+</sup>) and hippocampal CA1 neurons (n = 12; GAD67<sup>−</sup> TH<sup>−</sup>), which both possessed a fast activating Ih subtype, displayed HCN1 expression in addition to HCN2–4 (Fig. 6c and d). Figure 7 plots single-cell genotype/phenotype correlations for HCN1–4 comparing the expres-
We discovered a strong single-cell correlation between the presence of a fast activating Ih current with time constants <100 ms and the detection of HCN1 mRNA. In contrast to HCN1 mRNA, which was detected only in hippocampal and neocortical neurons, HCN2–4 mRNAs were coexpressed in both cortical and subcortical neurons. Also, HCN2–4 detection was not correlated with neither the fast cortical nor the slow subcortical Ih gating phenotype. To further characterize the qualitative differences in HCN1 expression revealed by the single-cell RT-multiplex PCR, we applied a semiquantitative single-cell RT-PCR protocol based on serial dilutions of single-cell cDNA pools. Assuming comparable reverse transcription efficiencies in different cell types, the semiquantitative protocol compares relative expression levels of mRNA species by determining their single-cell detection thresholds (Tkatch et al., 1998, 2000). We did not detect HCN1 in serial dilutions of dopaminergic SN neurons. In comparison, the mean HCN1 detection threshold was about 1/8 of the single-cell cDNA pool in neocortical neurons. This indicates that the abundance of HCN1 mRNA is at least eightfold higher in neocortical compared to dopaminergic SN neurons.

Our results suggest that differential expression of HCN1 subunits might be an important mechanism in the generation of functional diversity of neuronal Ih channels. Our findings are consistent with the properties of heterologously expressed HCN species. HCN1 channels possess time constants <100 ms, while HCN2 channels have time constants in the range of 200–400 ms (Santoro et al., 1998; Ludwig et al., 1999). HCN3, as well as HCN4, channels activate even slower with time constants ranging from 400 ms to several seconds (Jegla et al., 1999; Seifert et al., 1999). The immunocytochemical localization of HCN1 protein in dendrites of pyramidal neurons

Discussion

We have determined the single-cell mRNA coexpression profiles of Ih candidate genes HCN1–4 in four different neuronal populations.
(Santoro et al., 1997) further supports its proposed role as a key subunit in cortical and hippocampal neurons. In addition, recombina-
tant HCN1 channels possess a smaller cAMP sensitivity compared to
HCN2 and HCN4 (Clapham, 1998; Seifert et al., 1999), which is
also consistent with our observation of smaller cAMP sensitivities of
native Ih channels in hippocampal neurons compared to those in SN
neurons.
In contrast to fast gating Ih currents, our qualitative single-cell RT-
multiplex study did not identify a correlation between qualitative
detection of a single HCN subtype and the slow activating subcortical
Ih phenotype. HCN2, HCN3, and HCN4 were coexpressed in most
dopaminergic and thalamic neurons. Recent in situ hybridization
studies might indicate a prominent role for HCN4 in the generation of
subcortical Ih channels (Moosmang et al., 1999; Seifert et al., 1999).
However, our current knowledge about native HCN channel
complexes is limited. The functional stoichiometry, the degree of
heteromerization, and subcellular targeting of native HCN channel
complexes in vivo is completely unknown. Our results present the
first evidence that alternative coexpression of HCN mRNAs does
occur on the level of single neurons and might be involved in the
generation of different types of somatodendritic Ih channels. These
molecular differences are likely to have important functional
consequences which could help to establish the different frequency
preferences of postsynaptic computation between cortical and
subcortical neurons (Connors & Amitai, 1997; Lüthi & McCormick, 1998; Steriade, 1998).

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Abbreviations
GAD, glutamate decarboxylase; RT-mPCR, reverse transcription multiplex
polymerase chain reaction; SN, substantia nigra; TH, tyrosine hydroxylase,
wC, whole-cell.

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