Layer and frequency dependencies of phase response properties of pyramidal neurons in rat motor cortex

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Abstract
It is postulated that synchronous firing of cortical neurons plays an active role in cognitive functions of the brain. An important issue is whether pyramidal neurons in different cortical layers exhibit similar tendencies to synchronise. To address this issue, we performed intracellular and whole-cell recordings of regular-spiking pyramidal neurons in slice preparations of the rat motor cortex (18–45 days old) and analysed the phase response curves of these pyramidal neurons in layers 2/3 and 5. The phase response curve represents how an external stimulus affects the timing of spikes immediately after the stimulus in repetitively firing neurons. The phase response curve can be classified into two categories, type 1 (the spike is always advanced) and type 2 (the spike is advanced or delayed depending on the stimulus phase), and are important determinants of whether or not rhythmic synchronization of neuron pairs occurs. We found that pyramidal neurons in layer 2/3 tend to display type-2 phase response curves whereas those in layer 5 tend to exhibit type-1 phase response curves. The differences were prominent particularly in the gamma-frequency range (20–45 Hz). Our results imply that the layer-2/3 pyramidal neurons, when coupled mutually through fast excitatory synapses, may exhibit a much stronger tendency for rhythmic synchronization than layer-5 neurons in the gamma-frequency range.

Introduction
The electrophysiological responses of neurons can be characterized in many different ways. For example, classification may be based on the input–output relationship, temporal firing patterns or subthreshold membrane potential behaviour (Connors et al., 1982; McCormick et al., 1985; Larkman & Mason, 1990; Kang & Kayano, 1994; Degenetais et al., 2002; Cho et al., 2004). The phase response curve (PRC) shows the responses of single neurons to a perturbative input (Reyes & Fetz, 1993a; Hansel et al., 1995; Ermentrout, 1996, 2001; Gutkin et al., 2005; Stiefel et al., 2005; Preyer & Butera, 2005; Goldberg et al., 2007). When a neuron fires periodically and thus behaves as a neural oscillator, an excitatory stimulus to the neuron should advance or delay a subsequent spike. The PRC describes how such a timing shift in the output depends on the timing (phase) of the input stimulus.

A general theory has shown that the PRCs can be classified into two categories (Hansel et al., 1995). The spike timing is always advanced in a type-1 PRC whereas it may be advanced or delayed in a type-2 PRC depending on the stimulus timing. Though the PRC describes a property of single neurons, it can be used to infer the behaviour of networks. When coupled through α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor-mediated synapses, neurons exhibiting type-2 but not type-1 PRC tend to synchronise (Hansel et al., 1995). The PRC type also has functional implications for synchronization in a population of neurons, as a neuronal population can be synchronized if every neuron pair can also be synchronized.

Theoretically, two neurons can possess qualitatively different PRCs even if their responses to a sustained injected current display similar firing patterns (Ermentrout et al., 2001; Aoyagi et al., 2003; Takekawa et al., 2004). It is therefore important to determine the PRCs through direct experimental measurements (Reyes & Fetz, 1993a,b; Oprisan et al., 2004; Galan et al., 2005; Lengyel et al., 2005; Netoff et al., 2005b; Preyer & Butera, 2005; Goldberg et al., 2007). In the previous studies (Reyes & Fetz, 1993a,b), pyramidal neurons in layer 5 of the cat motor cortex exhibited a type-1 PRC. In contrast, glutamatergic stellate cells in layer 2 of the rat entorhinal cortex displayed a type-2 PRC (Netoff et al., 2005b), which may also be the case with pyramidal neurons in layer 2/3 of the mouse visual cortex (Stiefel et al., 2003). To examine whether the type of PRC is layer-specific, we performed intracellular and whole-cell patch-clamp recordings in slice preparations of the rat motor cortex in layers 2/3 and 5, and analysed the PRCs of regular-spiking pyramidal neurons. Since the PRC type of a neuron can vary with firing rate (Gutkin et al., 2005), we measured the PRC at several different firing rates. We demonstrate that pyramidal neurons display different PRC types in layers 2/3 and 5 especially in the gamma-frequency range (20–45 Hz): the PRC was type 2 in layer 2/3 and type 1 in layer 5.

Materials and methods
All experiments were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience and the Experimental Animal Committee of the RIKEN Institute, and carried out in accordance with the Guidelines for Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience 2000).
Slice preparations

Wistar rats (postnatal days 18–45) were deeply anaesthetized with diethyl ether gas and then decapitated. Cortical slices (300–500 μm thick) were prepared with a microslicer (DTK-1500 or PRO-7; Dosaka EM, Kyoto, Japan). After a 30-min incubation at 31 °C and at least 1 h recovery at room temperature, each slice was transferred to a submerged-type recording chamber continuously circulated with normal artificial cerebrospinal fluid (aCSF; 30–32 °C), which consisted of (in mM): NaCl, 124; KCl, 2.5; KH$_2$PO$_4$, 1.2; NaHCO$_3$, 26; MgSO$_4$, 1.2; CaCl$_2$, 2.5; and d-glucose, 10; and was saturated with 95% O$_2$ and 5% CO$_2$ gas.

Electrophysiological recordings

Intracellular recordings were obtained from layer-2/3 and layer-5 pyramidal neurons in motor cortical slice preparations. The membrane potentials of neurons were recorded with a current-clamp amplifier (Axoclamp 2B; Axon Instruments, Union City, CA, USA) in a conventional bridge mode through glass electrodes filled with 1.0 M K-acetate and 10 mM biocytin (60–150 MΩ). We also performed whole-cell patch-clamp recordings using patch pipettes (7–15 MΩ) filled with (in mM) K-glucuronate, 140; NaCl, 2.0; MgCl$_2$, 1.0; Heps, 10; EGTA, 0.2; 5’-ATPNa$_2$, 2.0; GTPNa$_2$, 0.5; and biocytin, 10; pH 7.4 (Isomura et al., 2003; Fujiwara-Tsukamoto et al., 2004). Recorded signals were digitized at 40 kHz with an A/D interface (PowerLab 2/20; AD Instruments, Australia).

To obtain the PRC, we repeatedly (every 16 s) injected a 3500-ms-long step current to cortical neurons to elicit periodic action potentials. While the neurons exhibited repetitive firing during each current injection, we applied trains of brief perturbative current inputs at irregular intervals of ∼300 ms. The waveform of the perturbative stimulus $i_{pt}(t)$ is given in terms of a double exponential function as

$$i_{pt}(t) = A[\exp(-t/\tau_1) - \exp(-t/\tau_2)] \quad (t > 0) \quad (1)$$

where the rise time $\tau_2 = 0.5$ ms, the decay time $\tau_1 = 2.0$ ms and $A$ is a normalization factor to adjust the peak amplitude of the input current at 0.1 nA. We fixed the peak amplitude at 0.1 nA throughout the present experiments as it is weak enough to be regarded as perturbation but strong enough to produce detectable phase responses. The shape of the perturbation was not crucial for the PRC type of the recorded neurons because the amplitude and time constant of the perturbation were very small. The responses of some neurons, especially those of cortical layer 2/3, exhibited spike-frequency adaptation in an initial transient period. The transient period typically lasted hundreds of milliseconds after the onset of a step current injection. In this study, we recorded interspike intervals (ISIs) necessary for constructing PRCs only after the adaptation period: the ISIs were collected from 1000 to 3400 ms after the current onset. In addition, some neurons recorded in this study often skipped spikes during periodic firing. In this case, we defined the PRC using only the interspike intervals that contained no spike failure.

Pharmacological application

To block the spontaneous input via AMPA receptors, N-methyl-D-aspartic acid (NMDA) receptors, and γ-aminobutyric acid receptors type A (GABA$_A$ receptors), a mixture of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 mM), DL-2-amino-5-phosphonothenoic acid (DL-AP5; 25 mM) and bicuculline methiodide (10 mM) was added to the aCSF and bath-applied to the cortical slices. All three antagonists were purchased from Sigma (St Louis, MO, USA).

Morphological analysis

For visualising biocytin-loaded recorded neurons, the slices were fixed for at least 12 h at room temperature in 0.1 M phosphate buffer (PB; pH 7.2) containing 10% (v/v) formalin and 15% (v/v) saturated picric acid. The slices were then immersed in PB containing 30% (w/v) sucrose for at least 2–3 h at 4 °C and frozen–thawed twice with a deep freezer at −20 °C. The slices were cut into 50-μm-thick sections on a freezing microtome. Each section was separately incubated with avidin–biotin–peroxidase complex (1 : 100; ABC–Elite; Vector, Burlingame, CA, USA) for at least 24 h at 4 °C in 0.1 M phosphate-buffered saline with 0.1% (v/v) Triton X-100. When washes in 0.05 M Tris–HCl buffer (TB; pH 7.6), the slices were preincubated with 0.04% NiCl$_2$ and 0.04% diaminobenzidine (Sigma) in TB for 10 min, and 0.002% H$_2$O$_2$ was added to permit peroxidase reaction for 5–15 min at room temperature. The sections were mounted on gelatin-coated glass slides, cleared in xylene and coverslipped (Cho et al., 2004; Fujiwara-Tsukamoto et al., 2004). For the purpose of cytoarchitectonic identification of the laminar pattern of the frontal agranular area, some sections were counterstained with 0.5% neutral red (Fig. 1A). The morphometric analyses were performed using NeuroLucida, and some neurons were reconstructed with Camerulica.

Simulations of neural oscillator networks

We performed numerical simulations of networks of neural oscillators which were mutually coupled through experimentally recorded PRCs. The PRC of a neural oscillator describes how a perturbative input applied at a given phase advances or delays the successive spike generation. Mathematically, the PRC can be most conveniently defined as the infinitesimal phase response curve (iPRC), $Z_{\text{iPRC}}(\theta)$, which represents the phase responses to infinitesimal delta-function-like stimuli. Then, the dynamic equation of the phase variable $\theta$ can be represented in terms of $Z_{\text{iPRC}}(\theta)$ and the frequency of neuronal firing $f$ (for the explicit form, see Eqn 8 in Results). On the other hand, what we can measure in experiments is the phase responses, $Z_{\text{exp}}(\theta)$, to finite stimuli. To define the type of the experimentally recorded PRCs, we fitted the phase response data from each neuron at each frequency using the lowest four components of the Fourier expansion (Galan et al., 2005):

$$Z_{\text{exp}}(\theta) = \sum_{n=0}^{3} (c_n \sin n\theta + d_n \cos n\theta). \quad (2)$$

We performed the data fitting by means of the least mean square. We can relate this PRC to the more fundamental iPRC through an integral equation (see Eqn 9 in Results). In the present study, we derived $Z_{\text{iPRC}}(\theta)$ by fitting this equation to experimental data by means of the least mean square. In doing so, we parametrized the iPRCs as

$$Z_{\text{iPRC}}(\theta) = \sum_{n=1}^{7} \left[ a_n \sin(n\theta/2) / n \right] \quad (3)$$

and determined the parameters $\{a_n\}$ such that the iPRC would best fit the phase response data measured in experiments. While Eqn 2 is a natural form of the series expansion, the expansion with finite $n$ often exhibited a spurious oscillatory behaviour in the vicinity of $\theta = 0$. We noted that Eqn 3 often yields a better fit by suppressing this oscillatory behaviour. Except for the overall multiplicative factor which depends on the amplitude of perturbation $i_{pt}(t)$ used in experiments, the differences between $Z_{\text{exp}}(\theta)$ and $Z_{\text{iPRC}}(\theta)$ are expected to be small if the perturbative input is sufficiently brief (see Fig. 5A).
Once the iPRC was obtained we were able to study the synchronization properties of synaptically connected pyramidal neurons in the following way. We can in general describe a pair of weakly coupled identical neural oscillators, \( \theta_1 \) and \( \theta_2 \), as follows (Kuramoto, 1984; Ermentrout, 1996):

\[
\begin{align*}
\frac{d\theta_1}{dt} &= \omega + Z_{\text{INF}}(\theta_1)_{\text{p}_1,2}(t), \\
\frac{d\theta_2}{dt} &= \omega + Z_{\text{INF}}(\theta_2)_{\text{p}_1,2}(t)
\end{align*}
\]

where \( \omega = 2\pi f \). Let \( \theta_\alpha = \omega t + \psi_\alpha \), with \( \psi_\alpha (\alpha = 1, 2) \) being the phase shift from the nonperturbed state. We may assume that \( \psi_\alpha \) changes sufficiently slowly compared with \( \theta_\alpha \). The perturbative inputs are given as

\[
\begin{align*}
i_{\text{p}_1,2}(t) &= \sum_{n=-\infty}^{\infty} a(t - t_s^{\text{spike}}) \\
&= \sum_{n=-\infty}^{\infty} \alpha(t - (2\pi n - \psi_\alpha))/\omega \\
&\equiv q(\omega t + \psi_\alpha)(a, b = 1, 2; a \neq b)
\end{align*}
\]

in terms of the double exponential function \( \alpha(t) = \Delta(\exp(-t/\tau_{\text{rise}}) - \exp(-t/\tau_{\text{decay}})) \) \( (t > 0) \), where the value of \( \Delta \) is as defined below Eqn 1 for a coupled neuron pair. In simulations of 30-neuron networks in Fig. 5, the value of \( \Delta \) was divided by 30. The time constants were set as \( \tau_{\text{rise}} = 0.5 \) ms and \( \tau_{\text{decay}} = 6 \) ms (AMPA) or 50 ms (NMDA). With these variables, we can perform time-averaging over the period \( T = 1/f \) to obtain

\[
\begin{align*}
\frac{d\psi_\alpha}{dt} &= 2\pi + \Gamma(\psi_\alpha - \psi_b) \\
&= 1/T \int_{0}^{T} Z_{\text{INF}}(\omega t + \psi_\alpha)q(\omega t + \psi_b)dt \\
&= 1/T \int_{0}^{T} Z_{\text{INF}}(\omega t)q(\omega t + \psi_b - \psi_\alpha)dt
\end{align*}
\]

so the phase difference \( \psi = \psi_1 - \psi_2 = \theta_1 - \theta_2 \) obeys the following equation:

\[
\frac{d\psi}{dt} = \Gamma(\psi) - \Gamma(-\psi) = 2\Gamma_{\text{odd}}(\psi)
\]

where \( \Gamma_{\text{odd}}(\psi) \) is called the ‘interaction function’ and is a \( 2\pi \)-periodic odd function for a pair of identical neural oscillators. A stable phase difference can then be given as a solution to \( \Gamma_{\text{odd}}(\psi) = 0 \) satisfying the stability condition \( d\Gamma_{\text{odd}}(\psi)/d\psi < 0 \).

A large-scale network can show a much richer variety of spatiotemporal activity patterns, such as clustering of synchronous firing, than a neuron pair. Nevertheless, studying the properties of pair-wise synchrony is often useful for exploring the dynamic
behaviour of a larger network, as demonstrated by numerical simulations in Fig. 5.

Results

To obtain the PRC, we performed intracellular and whole-cell patch-clamp recordings at the somata of layer-2/3 \((n = 65)\) and layer-5 \((n = 47)\) pyramidal neurons of the rat motor cortex (Fig. 1A).

Intracellular and whole-cell recordings typically lasted 1–2 h and were terminated if the shape of the action potentials showed any obvious change. Although cortical neurons showed a variety of responses to step current injections we only examined the neurons that displayed near-periodic firing patterns, the property prerequisite for PRC analysis. In most cases, the PRCs were determined for a fixed frequency for the individual cells and then pooled. However, in some experiments the PRCs were determined from each cell at different frequencies to see whether single neurons change their PRC types with the firing frequency.

Phase response curve

We first explain schematically how the PRC may be interpreted (Fig. 1). Neurons firing periodically can be regarded as an oscillator. We may define the phase variable \(\theta\) as \(\theta = 2\pi t / T\), where \(t\) and \(T\) denote the time from the previous spike and the period of repetitive firing, respectively. If a neuron is injected with a small perturbative current at a certain phase \(\theta\) (a certain time in the interval from one spike to the next), then the subsequent spike will be generated at an earlier or a later time (Fig. 1B and C). This advanced or delayed response to a small perturbation given at various timings or phases is defined as the PRC (Fig. 1D). For example, when a perturbative input at \(1.3\pi\) radians (rad) advances the next spike by \(0.22\pi\) rad (Fig. 1B), we plot \((1.3\pi, 0.22\pi)\) on a graph of the phase response. Similarly, when a perturbative input at \(0.59\pi\) rad delays the next spike by \(0.16\pi\) rad (Fig. 1C), we plot \((0.59\pi, -0.16\pi)\).

The PRC is of biological importance from the viewpoint of the network dynamics as it has crucial information about rhythmic synchronization (Kuramoto, 1984; Hansel et al., 1995; Ermentrout, 1996; 2001; Galan et al., 2005; Gutkin et al., 2005; Netoff et al., 2005a,b). In general, neurons with a type-2 PRC can easily be synchronized when they are mutually coupled via fast excitatory synaptic connections, while those with a type-1 PRC may not lock in phase (Hansel et al., 1995). Note that the relation between the time constant of the excitatory postsynaptic current and spiking frequency is closely related to the dynamics of oscillators. As the spiking frequency drops, the phase difference between coupled oscillators generally becomes smaller. Indeed, the stable phase difference calculated with the type-1 PRC can be close to zero at low firing rates (Fig. 5).

Typical phase response curves of layer-2/3 pyramidal neurons

The reconsticted image of a typical neuron in layer 5 is shown in Fig. 2A. This neuron belonged to the major class comprising two-thirds of the reconstructed neurons from layer 5, with large cell bodies and apical dendrites extending to layer 1 \((n = 19/29)\). The remaining one-third of the neurons had apical dendrites that terminated in layer 2/3 or 5, although we could not exclude the possibility that the different dendritic arborization was due to the truncation of apical dendrites in the slicing process or by imperfect transport of biocytin to the distal part. A more thorough morphological analysis of all recorded neurons is detailed below.

All of the data presented in Fig. 2 were recorded from the same neuron. The neuron exhibited regular periodic firing up to \(~30\) Hz (Fig. 2B). For this neuron, frequency adaptation reached a steady state within \(100\) ms after the stimulus onset (Fig. 2C and D). Such frequency adaptation is a characteristic of RS1 neurons under the classification scheme of Agmon & Connors (1992) and Degenetakis et al. (2002). Figure 2E shows the PRCs that were constructed at frequencies of \(~9\) Hz (alpha-frequency range; blue) and \(~21\) Hz (gamma-frequency range; red). Each dot represents a phase shift induced by an input current and the blue and red curves were the PRCs fitted by Eqn 2.

As mentioned previously, the PRC of a neuron can be roughly classified into type 1 or type 2 (Hansel et al., 1995), depending on whether the curve almost always takes positive values (type 1) or takes both positive and negative values (type 2). The PRCs displayed in Fig. 2E did not show significantly negative values at any part of the phase range; we may thus categorise these PRCs as type 1. However, the recorded PRCs were noisy and exhibited large fluctuations around the average values. In fact, we could fit the distributions of these fluctuations in the phase responses at \(\theta < \pi\) with Gaussian distributions in both the alpha- and the gamma-frequency ranges (Fig. 2F). It should be noted here that the amplitude of the variances around the mean remained almost constant in the range \(\theta < \pi\) while it decreased in the range \(\theta > \pi\). To determine the type of PRC more convincingly, we analysed the PRCs in a population of neurons (see below).

Cortical layer and frequency dependence of the phase response types

To quantify the criteria for defining the PRC types, we approximated the phase response data recorded from each neuron at each frequency using \(Z_{FNT}(\theta)\) given in Eqn 2 (Galan et al., 2005). We then defined the PRC as type 1 if the area surrounded by the negative portion of \(Z_{FNT}(\theta)\) and the abscissa was <\% of the entire area between \(0\) and \(2\pi\) rad. Otherwise, the PRC was defined as type 2.
In layer 5, the type-1 PRC was the majority in the frequency ranges of alpha (8–13 Hz), beta (13–20 Hz) and gamma (20–45 Hz; Table 1). This was particularly true of the gamma-frequency range, in which 88% of the phase responses recorded were of type 1. Thus, we may conclude that the phase response of layer-5 pyramidal neurons is almost always the type 1 in the gamma-frequency range (\(n = 23/26\); binomial test, \(P < 0.0001\)).

In contrast, the phase responses recorded from layer-2/3 pyramidal neurons constituted a heterogeneous mixture of the two types, and the dominant PRC type was different at different frequencies of firing. The left column of Table 1 shows that type 2 was the dominant phase response type of layer-5 pyramidal neurons in the gamma-frequency range (\(n = 10/12\); binomial test, \(P < 0.02\)). The type-2 neurons also tended to be dominant in the theta-frequency range (\(n = 8/12\)), while more than half of the recorded neurons displayed type 1 in the alpha- and beta-frequency ranges (\(n = 15/26\) and 15/28, respectively). However, the distributions of the two types observed in these frequency ranges were not statistically significant. These results have shown that the PRC type of the majority of layer 2/3 neurons varied from type 1 to type 2 with changes in firing frequency from the alpha and beta to the gamma range. In most of the remaining neurons, the PRC type remained type 2.

The above results derived from the population data suggest that the PRC type of a single neuron may vary with the frequency of firing. To examine this, we recorded the phase responses of layer-2/3 and layer-5 neurons in more than one frequency range. To ensure a stationary phase response, we recorded the PRC from each neuron only in two, or at most three, different frequency ranges. In 46% (\(n = 6/13\)) of the layer-2/3 neurons and 30% of the layer-5 neurons (\(n = 7/23\)), the PRC types were different in the different frequency ranges tested. An example is shown in Fig. 3E.

Electrophysiological and morphometric properties of type-1 and type-2 neurons

The cortical pyramidal neurons can be classified into several subclasses according to the electrophysiological properties (Connors et al., 1982; McCormick et al., 1985; Larkman & Mason, 1990; Agmon & Connors, 1992; Degenetais et al., 2002; Cho et al., 2004). In this study, we classified the recorded neurons based on the following three criteria: (i) types of frequency adaptation; (ii) the presence or absence of subthreshold oscillations; and (iii) the presence or absence of intrinsic bursting. The results are summarised in Table 2. Cortical neurons were previously classified into two subtypes, RS1 (steep adaptation) and RS2 (moderate adaptation), according to the length of the transient period for spike frequency adaptations (Agmon & Connors, 1992; Degenetais et al., 2002). Consistent with the previous...
results, the RS2 neurons were the minor subtype in layer 5 \((n = 10/47)\). In the present results, this subtype also constituted the minor class in layer 2/3. We could find no obvious correlation between the adaptation types and the PRC types in the present study.

Some of the neurons recorded in the present study skipped spikes in some cycles of periodic firing, especially after the frequency adaptation was over. The phenomenon has been reported previously (Llinas et al., 1991; Yang et al., 1996) and was termed ‘subthreshold oscillation’. The spike failure occurred more frequently at lower firing rates in both layer-2/3 and layer-5 neurons, probably due to weaker intensity of the injected current. In the present experiments, 55% of the layer-2/3 pyramidal neurons and 19% of the layer-5 pyramidal neurons showed subthreshold oscillations (Table 2). About two-thirds of the layer-2/3 neurons showing subthreshold oscillations had the type-2 PRCs \((n = 15/23)\). This tendency to the type-2 nature, however, was not statistically significant.

We examined bursting in 22 type-1 and nine type-2 neurons in layer 5, and found that 45% of the type-1 neurons \((n = 10)\) and none of the type-2 neurons exhibited burst firing. In total, 32% of the layer-5 neurons were bursting neurons. In contrast, none of the 18 type-1 and 24 type-2 layer-2/3 neurons \((n = 42)\) exhibited intrinsic bursting. Therefore, all of the bursting neurons found in this study were layer-5 neurons and had the type-1 phase response property. In particular, eight layer-2/3 and 18 layer-5 neurons were tested for the bursting property in the gamma-frequency range. All of the seven bursting neurons were obtained from layer 5 and displayed the type-1 PRCs.

We investigated whether the PRC type of a neuron might correlate with the electrophysiological or morphometric properties listed in Table 2 for the full range of frequencies used in this study. The resting potential and input resistance showed significant differences between layers 2/3 and 5 \((t\)-test, \(P < 0.01\) and \(P < 0.0001\), respectively). However, these two properties and the membrane time constant exhibited no significant differences between type 1 and type 2. In this study, we measured four morphometric parameters with Neurolucida. The cell body area, the cell body perimeter \((P < 0.0001)\) and the

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**Table 1. Proportion of the PRC types in different frequency ranges**

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Layer 2/3</th>
<th>Layer 5</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Type 1</td>
<td>Type 2</td>
</tr>
<tr>
<td>Theta 4–8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Alpha 8–13</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Beta 13–20</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Gamma 20–45</td>
<td>2</td>
<td>10</td>
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</table>

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**Fig. 3.** Morphometric and electrophysiological properties and phase response curves of a typical layer-5 pyramidal neuron. (A) Reconstruction of the morphological structure of the neuron. (B) Response to weak (0.1 nA; blue) and strong (0.3 nA; red) injected current. (C) The relationship between firing frequency and the time from the onset of current injection is shown to quantify the frequency adaptation. The amplitude of current input was varied within 0.2–0.5 nA. (D) Frequency–current curves are given as the inverse of the first, second, third and steady-state interspike intervals. (E) Phase response curves obtained in the alpha (blue; 9 Hz) and gamma (red; 24 Hz) frequency ranges. The abscissa represents the phase at which the neuron was stimulated by a perturbative input and the ordinate the phase response to it. Each dot represents a noisy phase response to a stimulus. Blue and red solid curves display the average PRCs obtained by the least-mean-square method. (F) Gaussian distributions fit well the fluctuations in the phase responses recorded at \(\theta < \pi\) rad.
Table 2. Summary of the electrophysiological and morphometric properties of the recorded neurons in all four ranges and in the gamma-frequency range

<table>
<thead>
<tr>
<th></th>
<th>Layer 2/3</th>
<th></th>
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<th>Layer 5</th>
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<tbody>
<tr>
<td></td>
<td>Type 1</td>
<td>(n)</td>
<td>Type 2</td>
<td>(n)</td>
<td>Total</td>
<td>(n)</td>
<td>Type 1</td>
<td>(n)</td>
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<td>All four ranges</td>
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<td>Electrophysiological properties</td>
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<tr>
<td>Resting potential (mV)</td>
<td>-71.0 ± 10.4 (27)</td>
<td>-69.5 ± 7.8 (28)</td>
<td>-70.2 ± 9.1* (55)</td>
<td>-64.5 ± 6.8 (35)</td>
<td>-66.7 ± 6.3 (11)</td>
<td>-65.0 ± 6.7* (46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>90.2 ± 35.7 (27)</td>
<td>88.8 ± 35.2 (28)</td>
<td>89.5 ± 35.1*** (55)</td>
<td>52.2 ± 23.6 (35)</td>
<td>54.8 ± 27.6 (11)</td>
<td>52.8 ± 24.3*** (46)</td>
<td></td>
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</tr>
<tr>
<td>Time constant (ms)</td>
<td>13.3 ± 5.0 (28)</td>
<td>15.6 ± 5.7 (32)</td>
<td>14.5 ± 5.5 (60)</td>
<td>15.1 ± 6.3 (36)</td>
<td>18.8 ± 7.7 (11)</td>
<td>16.0 ± 6.7 (47)</td>
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<tr>
<td>Subthreshold oscillation (%)</td>
<td>44 (8/18)</td>
<td>63 (15/24)</td>
<td>55 (23/42)</td>
<td>14 (3/22)</td>
<td>33 (3/9)</td>
<td>19 (6/31)</td>
<td></td>
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<tr>
<td>Bursting (%)</td>
<td>0 (0/18)</td>
<td>0 (0/24)</td>
<td>0 (0/42)</td>
<td>45 (10/22)</td>
<td>0 (0/9)</td>
<td>32 (10/21)</td>
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<tr>
<td>Morphometric measurements</td>
<td></td>
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<td>161.4 ± 50.9*** (56)</td>
<td>282.2 ± 88.5 (22)</td>
<td>216.4 ± 56.0 (8)</td>
<td>264.6 ± 85.4*** (30)</td>
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<td>Cell body perimeter (µm)</td>
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<td>52.3 ± 9.9 (31)</td>
<td>51.3 ± 9.7*** (56)</td>
<td>65.8 ± 11.3 (22)</td>
<td>58.7 ± 7.4 (8)</td>
<td>63.9 ± 10.7*** (30)</td>
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<td>1.54 ± 0.25 (56)</td>
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<td>1.54 ± 0.33 (30)</td>
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<tr>
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<td>5.1 ± 1.2 (30)</td>
<td>5.0 ± 1.2* (53)</td>
<td>6.1 ± 1.5 (21)</td>
<td>5.6 ± 1.2 (8)</td>
<td>6.0 ± 1.4* (29)</td>
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<td>Electrophysiological properties</td>
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<td>Resting potential (mV)</td>
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<td>-72.2 ± 5.5 (9)</td>
<td>-70.6 ± 7.5 (11)</td>
<td>-66.4 ± 7.7 (18)</td>
<td>-61.7 ± 7.8 (3)</td>
<td>-65.8 ± 7.7 (21)</td>
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<td>Input resistance (MΩ)</td>
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<td>110.4 ± 40.7 (9)</td>
<td>107.6 ± 44.6* (11)</td>
<td>58.4 ± 23.2 (18)</td>
<td>65.8 ± 48.1 (3)</td>
<td>59.5 ± 26.4* (21)</td>
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<td>Time constant (ms)</td>
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<td>17.5 ± 6.2 (9)</td>
<td>17.0 ± 6.7 (11)</td>
<td>15.0 ± 7.8 (18)</td>
<td>18.1 ± 6.5 (3)</td>
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<td>7 : 3</td>
<td>-</td>
<td>8 : 4</td>
<td>-</td>
<td>16 : 1</td>
<td>-</td>
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<td>- (5/6)</td>
<td>- (6/8)</td>
<td>- (3/16)</td>
<td>- (0/2)</td>
<td>- (3/18)</td>
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<td>Bursting (%)</td>
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<td>- (0/6)</td>
<td>- (6/8)</td>
<td>- (7/16)</td>
<td>- (0/2)</td>
<td>- (7/18)</td>
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<td>Morphometric measurements</td>
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<td>Cell body area (µm²)</td>
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<td>Cell body perimeter (µm)</td>
<td>52.2 ± 0.4 (2)</td>
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<td>65.1 ± 13.2 (14)</td>
<td>56.8 ± 4.3 (3)</td>
<td>64.0 ± 12.2*** (17)</td>
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<tr>
<td>Cell body aspect ratio</td>
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<td>1.56 ± 0.33 (14)</td>
<td>1.35 ± 0.02 (3)</td>
<td>1.52 ± 0.31 (17)</td>
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<tr>
<td>No. of primary basal dendrites</td>
<td>4.0 ± 1.4 (2)</td>
<td>4.1 ± 0.8 (8)</td>
<td>4.1 ± 0.9** (10)</td>
<td>6.3 ± 1.8 (13)</td>
<td>6.0 ± 1.7 (3)</td>
<td>6.3 ± 1.7** (16)</td>
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</table>

All data but the last three rows in the ‘Electrophysiological properties’ category represent the mean number ± SD. *P < 0.01, **P < 0.001 and ***P < 0.0001 (t-test).
number of primary basal dendrites ($P < 0.01$) indicated significant differences between the two cortical layers. However, these three properties and the aspect ratio of the cell body exhibited no significant differences between the two PRC types. We obtained similar results from the analysis of cortical neurons only within the gamma-frequency range (Table 2). In summary, we could find no obvious correlations between the phase response type and the electrophysiological or morphometric properties, irrespective of the cortical layers from which the recorded neurons were obtained.

**Frequency-dependent population phase response curves**

To investigate how the PRC type of cortical neurons may depend on the firing rate, we obtained the PRCs of layer-2/3 ($n = 65$) and layer 5 ($n = 47$) pyramidal neurons in theta (4.8–13 Hz), alpha (8–13 Hz), and gamma (20–45 Hz) frequency ranges. Figure 4 displays the PRC averaged over all recorded neurons in each of these frequency ranges: theta ($n = 12$ and 6 in layers 2/3 and 5, respectively), alpha ($n = 26$ and 15), and gamma ($n = 12$ and 26). The thick black curves display the PRC averaged in each bin of width $2\pi/15$ rad. The error bars represent the SDs of the estimated mean PRC. Since negative values of these PRCs are crucial for determining its type, we tested whether the negative values of each mean PRC could be regarded as statistically significant ($P < 0.01$). The dashed curves represent the 99% confidence interval for the mean, and asterisks represent such negative portions as are significantly different. The results revealed that cortical layer-2/3 pyramidal neurons have the type-2 properties in the gamma-frequency range and presumably also in the theta-frequency range. The PRC exhibited a weak type-2 feature in the alpha- and beta-frequency ranges. As shown previously, however, single pyramidal neurons in layer 2/3 can exhibit the type-1 PRC in these frequency ranges, and the PRCs displayed here emerged from a heterogeneous mixture of the two neuronal groups with different PRC types (this explains why the negative portions are smaller in these PRCs). In contrast, the PRCs displayed in Fig. 5A, lower panels: the negative portions are smaller in these PRCs. In contrast, the population PRCs constructed from layer-5 pyramidal neurons displayed no significantly negative phase in any frequency range. These results seem to indicate that the PRC type of the layer-5 pyramidal neurons was primarily type 1.

**Simulation tests for the validity of noisy phase response curves**

The results shown in the preceding section revealed that layer-2/3 and layer-5 pyramidal neurons exhibit different types of PRC in the gamma-frequency oscillations. Reciprocal fast excitatory synaptic connections may promote in-phase synchronous firing between a pair of layer-2/3 pyramidal neurons but not between a pair of layer-5 pyramidal neurons at gamma frequencies. However, the PRCs recorded from the pyramidal neurons were rather noisy. Large fluctuations in the phase shift of the PRC were also seen in the results of other recording studies (Gutkin et al., 2005; Netoff et al., 2005b). Although our statistical test shows that the negative phase responses of layer-2/3 pyramidal neurons in the gamma-frequency range are significant, the large fluctuations question whether the small negative portion has any practical effect on the synchronization of neurons. To address this issue, we performed numerical simulations of neuronal oscillators that are all-to-all coupled via the experimentally determined PRCs.

To begin with, we derived the theoretical phase response, $Z_{\text{INF}}(\theta)$, to an infinitesimal delta-function-like stimulus given at phase $\theta$ (see Materials and methods for mathematical details). Then, the dynamics of a phase oscillator with a natural frequency $f$ can be derived in terms of the iPRC (Kuramoto, 1984):

$$df/dt = 2\pi f + \left( Z_{\text{INF}}(\theta) + D_{\text{PRC}}(\theta)\xi_{\text{PRC}}(t) - i_p(t) + fD_{\text{NSI}}\xi_{\text{NSI}}(t) \right).$$

Here, $i_p(t)$ represents a perturbative current input, which may be replaced with the excitatory synaptic input from surrounding neurons, $\xi_{\text{PRC}}$ and $\xi_{\text{NSI}}$ stand for two independent normalized Gaussian white noises, and $D_{\text{PRC}}$ and $D_{\text{NSI}}$ for their amplitudes, respectively. The terms $D_{\text{PRC}}\xi_{\text{PRC}}$ and $D_{\text{NSI}}\xi_{\text{NSI}}$ describe the noise component induced by the external input and the fluctuations in the natural oscillator frequency, respectively. The iPRC is $2\pi$-periodic. Experimentally, we can obtain phase responses to finite stimuli, $Z_{\text{FNT}}(\theta)$, as shown in Figs 2 and 3. The relationship between the two PRCs is given as follows:

$$Z_{\text{FNT}}(\theta) = \int_0^{2\pi} i_p[(\phi - \theta)/2\pi f]Z_{\text{INF}}(\phi)d\phi.$$  

We used this equation in fitting the experimental data with the least mean square. We parametrized the iPRC as in Eqn 2 and adjusted the values such that the left-hand side of Eqn 9 best fitted the averaged PRCs measured in experiments (Table 3). We applied the procedures to the noisy type-1 and type-2 PRCs of the gamma-frequency range shown in Figs 2E and 3E, and obtained their iPRCs which we then used for simulating Eqn 8. We determined the phase-dependence of $D_{\text{PRC}}$ by fitting the SDs of the phase responses obtained in the gamma-frequency range with the following function (Fig. 5A, lower panels):

$$D_{\text{PRC}}(\theta) = \begin{cases} h_1 & (\theta < 2\pi - b_1/b_2) \\
-b_2(\theta - 2\pi) & (\theta \geq 2\pi - b_1/b_2). \end{cases}$$

The values of the parameters are listed in Table 3. For simplicity of analysis, we set the value of $D_{\text{NSI}}$ equal to that of $D_{\text{PRC}}$. We note that in general $\xi_{\text{NSI}}(\theta)$ exerted much stronger influences on the phase dynamics than $\xi_{\text{NSI}}(\theta)$. The iPRCs thus obtained are shown in Fig. 5A (upper) after an appropriate rescaling of the amplitudes. As expected, the estimated iPRCs (dashed curve) were essentially unchanged from $Z_{\text{FNT}}(\theta)$ (solid curve), as the perturbative input current was sufficiently brief compared with the period of the gamma oscillations and might be regarded as a delta function at this time scale.

Using the above iPRCs we can study what stable phase differences may appear in the stationary state of a coupled neural oscillator pair. For this purpose, we derived the so-called interaction function for the phase difference between the oscillator pair following the mathematical procedure demonstrated by Eqns 3–6 in Materials and methods. The steady-state analysis was performed in two different situations, in which a pair of neurons was coupled through either fast AMPA or slow NMDA receptor-mediated synapses. With the fast synaptic transmissions, the neuron pair coupled through the present type-1 PRCs generally did not exhibit in-phase synchrony at any gamma frequency (20–45 Hz in Fig. 5B, left, solid curve). This tendency was further enhanced if the type-1 neuron pair was coupled through the slow synaptic transmissions (Fig. 5B, left, dotted curve). In contrast, the steady state of the type-2 neuron pair coupled through the fast synapses maintained in-phase synchrony up to a frequency of 45 Hz (Fig. 5B, right, solid curve). With the slow synapses, the upper bound for the frequency allowing in-phase synchrony was much lower (Fig. 5B, right, dotted curve).
The time evolution of synchrony in these networks was determined using the order parameter defined as
\[ \theta = \frac{1}{30} \exp(i \theta_j) \] (Fig. 5D). Now, \( i \rho(t) \) in Eqn 8 describes the sum of recurrent excitatory inputs mediated by AMPA synapses of uniform strength. The stable phase differences between the two noisy phase oscillators approximately coincided with those predicted by the iPRCs without noise. The results seem to be reasonable as the multiplicative noise component in the PRC should influence the dynamics of the phase difference only in a

\[ \theta = \frac{1}{30} \exp(i \theta_j) \]

Fig. 4. The PRCs averaged over all layer-2/3 (left) and layer-5 (right) pyramidal neurons recorded in this study (n = 65 and 47 in layers 2/3 and 5, respectively) are shown in theta (A; 4–8 Hz), alpha (B; 8–13 Hz), beta (C; 13–20 Hz) and gamma (D; 20–45 Hz) frequency ranges. The abscissa represents the phase at which each neuron was perturbed and the ordinate shows the phase responses. Black solid lines represent the averaged PRC. Vertical bars are SDs and dashed curves show the 99% confidence interval of the estimated mean PRC. The negative phase responses marked with asterisks are statistically significant (t-test; \( P < 0.01 \)).
brief period for which $i_d(t)$ can be regarded as nonvanishing. The contributions of the additive noise term to the integration of $\theta$ vanish on average. The fluctuations around the mean could have grown in proportion to the square root of time in the absence of the interactions between the neuronal oscillators, i.e. the second term in the right-hand side of Eqn 8. However, the interaction term inhibits a free random walk and fluctuations of the oscillators when noise is sufficiently weak. Therefore, the noise components created only jitter around the stable phase difference determined by the iPRCs.

We finally noted that neuronal oscillators coupled through the type-1 PRCs exhibited near-synchronous oscillations in the steady state at a frequency of 10 Hz (see Fig. 5, Cc and D). The stable phase difference between the neuronal oscillators was generally close to zero at relatively low frequencies, although it may not vanish exactly as in the case of type-2 PRCs (see Fig. 5B). This implies that classifying the theoretical types of PRCs is less meaningful in theta-, alpha- and beta-frequency ranges than in the gamma-frequency range from the viewpoint of synchrony.

### Discussion

Oscillatory synchronous activities in several frequency ranges have been thought to play active roles in various cognitive functions (Farmer, 1998; Maldonado et al., 2000; Fries et al., 2002; Woelbern et al., 2002; Lee, 2004; Spencer et al., 2004; Samonds & Bonds, 2005). Theta- and gamma-frequency oscillations have been shown to be induced by a brief stimulation to cortical slices (Plenz & Kitai, 1996), suggesting that local cortical networks may engage in the generation of these oscillations. In this study, we constructed the PRCs of regular-spiking pyramidal neurons in layers 2/3 and 5 in slice preparations of the rat motor cortex. The PRC is of particular interest as it gives some information on whether neurons may be synchronized or desynchronized with a given recurrent synaptic input. We have found that the intrinsic response property of pyramidal neurons in oscillatory synchronization depends on the range of firing rates and the cortical layers they belong to.

Both layer-2/3 and layer-5 pyramidal neurons exhibit type-2 PRCs in the alpha-frequency range (see Fig. 4B), implying that a recurrent network of these neurons interconnected through AMPA excitatory synapses tends to produce synchronous firing in this frequency range. By contrast, in the theta- and gamma-frequency range layer-2/3 and layer-5 pyramidal neurons tend to possess type-2 and type-1 PRCs, respectively. These imply that recurrent AMPA synaptic connections possibly promote synchronous neuronal firing of layer-2/3 pyramidal neurons, but such recurrent connections are not likely to enhance synchronization of layer-5 pyramidal neurons in the gamma-frequency range, which is of particular cognitive importance (Ward, 2003; Herrmann et al., 2004). In the beta-frequency ranges, the PRCs display type-1 properties in both layer-2/3 and layer-5 pyramidal neurons. However, we have shown that the stable phase difference is close to zero at such low frequencies even if the PRC belongs to type 1 (Fig. 5, Cc). Therefore, the differences in the PRC type seem to be less important for synchronous firing of pyramidal neurons in the theta-, alpha- and beta-frequency ranges than in the gamma-frequency range.

The phase advances or delays recorded from cortical neurons showed large fluctuations around the average values in the present and previous studies (Gutkin et al., 2005; Netoff et al., 2005b). The noise in PRCs, however, did not significantly affect the synchronization of coupled neural oscillators in the present numerical simulations with fast excitatory synapses. Thus, the fast synchronization dynamics of neurons is primarily governed by the mean values of the PRC. If, however, neurons receive strong noise that cannot be treated as a perturbation, the dynamic behaviour of the oscillator system may be qualitatively different from the predictions by the PRC (Ermentrout & Saunders, 2006). Slow excitatory synapses between pyramidal neurons, such as NMDA receptor-mediated ones, lead the neurons to asynchronous firing that shows time-varying relative phases irrespective of the PRC type (Fig. 5B).

We may compare the present results with those of previous studies. It has been suggested that pyramidal neurons in layer 5 of the cat motor cortex possess a type-1 PRC at a firing rate of 40 Hz (Reyes & Fetz, 1993a,b; Gutkin et al., 2005). Another experimental study has reported that the PRC belongs to type 2 for glutamatergic stellate cells firing at 6–7 Hz in layer 2 of the rat entorhinal cortex (Netoff et al., 2005b). These results seem to be consistent with the present findings that layer-2/3 and layer-5 neurons in the rat motor cortex exhibit type-2 and type-1 PRCs, respectively, at frequencies $> 20$ Hz (the gamma range), and that the PRC of pyramidal neurons in layer 2/3 are of type 2 presumably at frequencies $< 10$ Hz. The results of these studies suggest that the AMPA receptor-mediated recurrent synaptic input promotes gamma-oscillatory synchronization of pyramidal neurons in the superficial layers but not in the deep layers. In addition, the synaptic input seems to enhance synchrony in the theta-frequency range in the superficial layers. In fact, Netoff et al. (2005b) have confirmed that pyramidal neurons in layer 2 of the entorhinal cortex exhibit synchronous firing in this frequency range by using the dynamic clamp technique (Robinson & Kawai, 1993; Sharp et al., 1993). The present study, however, focused on dynamics of pyramidal neuron networks without interneurons, which exert strong influences on the synchronization properties of excitatory networks (Lytton & Sejnowski, 1991; Ermentrout et al., 2001). In particular, fast-spiking interneurons form networks with chemical and electrical synapses, displaying both synchronous and asynchronous firing modes (Lewis & Rinzel, 2003; Nomura et al., 2003; Merriam et al., 2005). The pyramidal-to-pyramidal interactions described by the PRCs are part of the complex mechanism that governs synchronous activity of cortical neurons.

The spike generating dynamics of cortical neurons are complex and have very many degrees of freedom. The PRC type of a neuron probably depends on a specific combination of the ionic currents expressed in that neuron, and hence should be measured directly by experiments. The majority of layer-2/3 pyramidal neurons changed their PRC type depending on the firing frequency. Although we have not clarified the mechanism of this change in the present study, we suggest that M-current may underlie the frequency dependence of the PRC type (Gutkin et al., 2005). Indeed, Stiefel et al. (2003) demonstrated that carbachol switches the two PRC types. Further pharmacological experiments are required to clarify the mechanism of the changes in the PRC type.

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Based on the knowledge of nonlinear systems, we may speculate about possible implications of the present findings for layer-specific cortical computations. The majority of excitatory synapses on layer-2/3 pyramidal neurons originate from the surrounding layer-2/3 pyramidal neurons (Thomson & Bannister, 2003; Binzegger et al., 2004). The layer-2/3 pyramidal neurons might thus serve as 'resonant oscillators' (Izhikevich, 2000, 2004) through the synergistic effects of the rich recurrent synapses and the type-2 PRC. By contrast, layer-5 pyramidal neurons might operate as 'integrators' as the type-1 neuron exhibits a continuous spectrum of firing rate from very low to high frequencies (Hodgkin, 1948; Tateno et al., 2004). However, the layer-5 pyramidal neurons projecting to other cortical regions and those forming local circuits with neighbouring layer-5 neurons may belong to different electrophysiological subtypes (Akemann et al., 2004). It would be intriguing to study whether these subtypes may have different PRC types.

Acknowledgements

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References


