Nicotinic acetylcholine receptor-mediated responses in medial vestibular and prepositus hypoglossi nuclei neurons showing distinct neurotransmitter phenotypes

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Abstract

Cholinergic transmission in both the medial vestibular nucleus (MVN) and prepositus hypoglossi nucleus (PHN) plays an important role in horizontal eye movements. We previously demonstrated that the current responses mediated via nicotinic acetylcholine receptors (nAChRs) were larger than those mediated via muscarinic acetylcholine receptors (mAChRs) in cholinergic MVN and PHN neurons that project to the cerebellum. In this study, to clarify the predominant nAChR responses and the expression patterns of nAChRs in MVN and PHN neurons that exhibit distinct neurotransmitter phenotypes, we identified cholinergic, inhibitory, and glutamatergic neurons using specific transgenic rats and investigated current responses to the application of acetylcholine (ACh) using whole-cell recordings in brainstem slices. ACh application induced larger nAChR-mediated currents than mAChR-mediated currents in every neuronal phenotype. In the presence of an mAChR antagonist, we found three types of nAChR-mediated currents that exhibited different rise and decay times and designated these as fast (F)-, slow (S)-, and fast and slow (FS)-type currents. F-type currents were the predominant response in inhibitory MVN neurons, whereas S-type currents were observed in the majority of glutamatergic MVN and PHN neurons. No dominant response type was observed in cholinergic neurons. Pharmacological
analyses revealed that the F-, S-, and FS-type currents were mainly mediated by $\alpha_7$, non-$\alpha_7$, and both $\alpha_7$ and non-$\alpha_7$ nAChRs, respectively. These findings suggest that cholinergic responses in the major neuronal populations of the MVN and PHN are predominantly mediated by nAChRs and that the expression of $\alpha_7$ and non-$\alpha_7$ nAChRs differ among the neuronal phenotypes.

Introduction

Evidence has accumulated regarding the expression of nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS), which play essential roles in sensory processing, learning and memory, and several cognitive functions (Albuquerque et al. 2009; Dani and Bertrand 2007; Levin and Simon 1998; Metherate and Hsieh 2004; Newhouse et al. 1997; Picciotto et al. 2008). Most nAChRs expressed in the CNS are either of the $\alpha_7$ subtype, which exhibits fast decay kinetics, low affinity for ACh, and high Ca$^{2+}$ permeability, or of the non-$\alpha_7$ (predominantly $\alpha_4\beta_2$) subtypes, which exhibit relatively slow decay kinetics and high ACh affinity (Chen and Patrick 1997; Flores et al. 1992; Holladay et al. 1997; Lindstrom et al. 1995). In addition to their distinct properties, it has been suggested that $\alpha_7$ and non-$\alpha_7$ subtypes participate in the distinct
chlorinergic transmissions, such as α7-mediated conventional synaptic transmission and
non-α7-mediated nonsynaptic transmission (volume transmission) (Bennett et al, 2012).
Therefore, elucidating which nAChRs subtypes are expressed in specific neurons can
provide important clues for understanding nAChR-mediated cholinergic transmission.
Regarding the CNS, previous studies have investigated the expression and properties of
nAChRs in neurons of the cerebral cortex and the hippocampus, where neurons
exhibiting distinct neurotransmitter phenotypes, such as glutamatergic pyramidal
neurons and GABAergic interneurons, can be easily identified based on their location
and shape, even in living slice preparations. Although the expression of nAChRs is
also found in the brainstem, the relationship between the expression patterns of nAChR
subtypes and neurons that exhibit distinct neurotransmitter phenotypes has not been
clarified, with very few exceptions, such as in midbrain dopaminergic neurons (Klink et
al. 2001; Pidoplichko et al. 1997).

The medial vestibular nucleus (MVN) and the prepositus hypoglossi nucleus (PHN) are
the brainstem centers involved in the control of horizontal gaze (Büttner and
Büttner-Ennever 2006; Fukushima et al., 1992; McCrea and Horn 2006; Moschovakis,
1997). The MVN and PHN are known to receive cholinergic input from the
pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDTg) (Higo et al. 1990; Iwasaki et al. 1999; McCrea and Horn 2006).

Autoradiographic measurements of cholinergic receptors have indicated the presence of nAChRs and muscarinic acetylcholine receptors (mAChRs) in the MVN and the PHN (Clarke et al. 1985; Schwartz 1986; Wamsley et al. 1981; Zanine tti et al. 1999).

Indeed, cholinergic excitation mediated by nAChRs has been shown in MVN neurons (Phelan and Gallagher 1992). In a previous study, we investigated cholinergic responses in cholinergic MVN and PHN neurons that project to lobules IX and X (uvula and nodulus) of the cerebellar cortex (cholinergic projection neurons, ChPNs); these neurons were identified via retrograde labeling in choline acetyltransferase (ChAT)-tdTomato transgenic rat, in which cholinergic neurons express the fluorescent protein tdTomato (Zhang et al. 2014). The local application of ACh via air pressure to ChPNs induced inward currents that were mediated by both mAChRs and nAChRs. A pharmacological analysis of the current responses revealed that the inward currents mediated by the nAChRs were larger than those mediated by the mAChRs, suggesting that cholinergic responses in ChPNs are predominantly mediated by nAChRs.

However, several studies have reported cholinergic modulation of neuronal activity via mAChRs in the MVN and the PHN (Navarro-López et al. 2004, 2005; Sun et al. 2002;
Ujihara et al. 1988, 1989). Therefore, it is possible that the predominant nAChR-mediated current responses are specific to ChPNs.

In the present study, we tested this possibility by investigating the current responses to ACh application in MVN and PHN neurons other than ChPNs. The major neuronal populations in the MVN and PHN include glutamatergic, GABAergic, glycinergic, and cholinergic neurons (Barmack 2003; de Waele et al. 1995; McCrea and Horn 2006).

To identify cholinergic neurons and GABAergic and/or glycinergic inhibitory neurons, we used ChAT-tdTomato transgenic rats and vesicular GABA transporter (VGAT)-Venus transgenic rats. In the latter rat model, inhibitory neurons express the fluorescent protein Venus (Shino et al. 2011; Uematsu et al. 2008). In these rats, cholinergic neurons express tdTomato (T+), and GABAergic and/or glycinergic inhibitory neurons express Venus (V+). To identify glutamatergic neurons, we used double-transgenic rats that were generated by mating a ChAT-tdTomato rat with a VGAT-Venus rat (Saito et al., 2015). In the double transgenic rats, the majority of neurons that do not display tdTomato or Venus expression [double-negative (D-) neurons] are considered glutamatergic. Therefore, we investigated the cholinergic current responses in T+, V+, and D- neurons using whole-cell recordings in brainstem
slices obtained from these transgenic rats. In addition, we characterized the nAChR-mediated current responses while mAChR-mediated currents were blocked with atropine. We also determined which nAChR subtypes were responsible for the current responses by investigating the effects of antagonists of the different subtypes.

Materials and Methods

All experimental procedures were approved by the Animal Care and Experimentation Committee of Gunma University, and the experiments were performed in accordance with the guidelines outlined by the US National Institutes of Health regarding the care and use of animals for experimental procedures.

Transgenic rats

To identify cholinergic, inhibitory, and glutamatergic neurons in the MVN and the PHN, three types of transgenic rats were used in this study: 1) ChAT-tdTomato transgenic rats, 2) VGAT-Venus transgenic rats, and 3) double-transgenic rats. The detailed methods regarding the generation of the ChAT-tdTomato (Zhang et al. 2014) and VGAT-Venus transgenic rats (Uematsu et al. 2008) have been previously described. The transgenic
rats were genotyped via PCR using previously described primers (Shino et al. 2011; Zhang et al. 2014). A total of 50 animals of each gender were used in this study.

**Slice preparation and whole-cell recording**

The procedures for slice preparation and whole-cell patch clamp recordings were similar to those previously described (Takazawa et al. 2004; Shino et al. 2008, 2011; Saito and Yanagawa 2013; Zhang et al. 2014). Briefly, transgenic rats [16- to 21-postnatal-days-old (PND), of both genders] were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed from the skull and placed in an oxygenated ice-cold sucrose solution containing (in mM): 234 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 26 NaHCO₃, and 11 glucose. Frontal slices (250 μm thick), including the MVN and the PHN, were cut using a microslicer (Pro 7, Dosaka EM, Kyoto, Japan). After incubation in an extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 25 glucose and aeration with 95% O₂ and 5% CO₂ (pH 7.4) for more than 1 hour at room temperature, each slice was transferred to a submerged recording chamber on an upright microscope (Leica DM LFS, Leica, Wetzlar, Germany) and continuously perfused with the extracellular solution at a rate of 3 ml/min. The bath temperature was maintained at
30-32 °C using an in-line heater (SH-27A, Warner Instruments, Hamden, CT). After the neurons were identified using fluorescence optics, whole-cell recordings were performed via Nomarski microscopy using an EPC-8 patch clamp amplifier (HEKA, Darmstadt, Germany). Patch pipettes were prepared from borosilicate glass capillaries and filled with an internal solution containing (in mM): 120 K-methylsulfate, 10 KCl, 0.2 EGTA, 2 MgATP, 0.3 NaGTP, 10 HEPES, 10 Na$_2$-phosphocreatine, and 0.1 spermine, adjusted to pH 7.3 with KOH. The osmolarity of the internal solution was 280-290 mOsm/l, and the resistance of the patch electrodes was 3-7 MΩ in the bath solution. Current signals were low-pass filtered at 3 kHz and digitized at 2 kHz. Because ACh-induced currents showed slow time course even in fast-type responses, the 2 kHz-sampling could sufficiently reproduce the current signals that should be analyzed. The calculated liquid junction potential of -5 mV was corrected. The data were acquired using a pClamp9 system (Molecular Devices, Foster City, CA). Neurons that displayed a membrane potential below -50 mV immediately after patch membrane rupture and action potential peaks higher than 0 mV were used for further analyses.

*Local application of acetylcholine*
The application of ACh was performed via pressurized air (30 psi, 5 ms) using a pneumatic PicoPump (PV820, World Precision Instruments, Sarasota, FL). A glass pipette was filled with 1 mM ACh, diluted with the extracellular solution. The current responses to ACh increased as the tip of the pipette was moved toward the soma of a recorded neuron. However, the responses changed minimally as the tip was moved closer once the tip reached a certain distance from the soma. The tip was maintained at the position where the responses changed minimally, and ACh was applied to the recorded neuron at 15 s intervals. ACh-induced currents were recorded as the slices were perfused with the extracellular solution containing 0.25 μM tetrodotoxin (TTX).

The neurons were routinely held at -70 mV during the recordings. To monitor the series resistance during the recordings, a short voltage pulse (-10 mV, 100 ms) was applied prior to the application of ACh.

**Drugs**

Acetylcholine chloride, mecamylamine, atropine sulfate and methyllycaconitine citrate salt hydrate were purchased from Sigma-Aldrich Japan (Tokyo). Dihydro-β-erythroidine hydrobromide was purchased from R&D Systems/Tocris Bioscience. Other drugs including TTX were purchased from Wako Pure Chemical
Inst. (Osaka, Japan). The antagonists were dissolved in water (1,000 times the final concentration) and stored as stock solutions at –20 °C before being diluted in the extracellular solution.

**Data analysis**

Off-line analysis was performed using Axograph software (Molecular Devices). Data were used for analysis only if the change in the series resistance during the recording was within 10% of the initial value. We analyzed both the current amplitude and the charge transfer, because the kinetics of each current response were not identical in individual neurons. ACh-induced currents returned to baseline within 2.3 s after the application of ACh even if they showed a slow decay time. Therefore, the charge transfer was estimated as the current area for 2.3 s following the application of ACh. Three current traces were used to measure the current amplitude and charge transfer; these data were then averaged. All values are reported as the mean ± SD. Student’s paired t-test, Wilcoxon-matched-pairs tests and a one-way ANOVA followed by post hoc Tukey-Kramer tests were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Pearson’s chi-square test was performed to compare the distributions of the cholinergic response types using R version 3.1.0 (The R Foundation
for Statistical Computing, Vienna, Austria). Statistical significance was determined at the level of $p < 0.05$.

Results

**Cholinergic responses in the three types of neurons**

To determine whether cholinergic neurons including ChPNs exhibit nAChR-dominant current responses, we recorded ACh-induced currents in randomly sampled T+ neurons in the MVN and the PHN. Figure 1A shows an example current response of a T+ MVN neuron to a puff application of 1 mM ACh in the absence and presence of either 5 μM atropine or 10 μM mecamylamine, which are mAChR and nAChR antagonists, respectively. The inward current induced by ACh was partially blocked by atropine, and the residual current was almost completely abolished by the addition of mecamylamine. Compared to the mAChR-mediated current amplitude a larger nAChR-mediated current amplitude current was observed in all of the T+ neurons in the MVN (n = 16) and the PHN (n = 21). The statistical analysis revealed that the nAChR-mediated inward currents were significantly larger than the mAChR-mediated inward currents in the MVN and the PHN (Fig. 1B1). The nAChR-mediated charge transfer was also larger than the mAChR-mediated charge transfer in the MVN and
PHN. Statistically, the nAChR-mediated current amplitude and charge transfer were significantly larger than the mAChR-mediated current amplitude and charge transfer in the V+ (Fig. 1B2, MVN, n = 17; PHN, n = 15) and D- neurons (Fig. 1B3, MVN, n = 15; PHN, n = 14), although some V+ neurons in the MVN (n = 2) and PHN (n = 2) and some D- neurons in the MVN (n = 2) and PHN (n = 1) exhibited larger mAChR-mediated currents. These results indicate that nAChR-dominant current responses are common in the major neuronal population of the MVN and PHN. When the nAChR-mediated currents and charge transfers were compared in the three types of neurons, a significant difference between the T+ MVN neurons and the V+ MVN neurons was observed only for charge transfer (Fig. 1C1). The current amplitude and charge transfer did not significantly differ among the three types of neurons in the MVN (Fig. 1C1) and PHN (Fig. 1C2). These results suggest that nAChRs are expressed at similar levels on the soma-dendritic membrane in the major neuronal populations.

**Three types of nAChR-mediated current responses**

To analyze the nAChR-mediated currents individually, we isolated the currents via the continuous application of 5 μM atropine. Figure 2A shows a typical example of a nAChR-mediated current isolated in a T+ neuron. Based on the rise and decay times,
the nAChR-mediated currents were characterized into the three types; current responses showing a fast rise and a fast decay (F type) (Fig. 2A1), current responses showing a slow rise and a slow decay (S type) (Fig. 2A2), and current responses showing a fast rise and a slow decay (FS type) (Fig. 2A3). To quantitatively separate these current responses, we analyzed the rise time from 10% to 90% of the peak current amplitude (10%-90% rise time) and the half width of the current. Figure 2B shows the 10%-90% rise times plotted against the half widths of the nAChR-mediated currents for all of the T+ neurons recorded in the MVN and the PHN (n = 51). These plots revealed that the three response types were separated according to a rise-time boundary at approximately 20 ms and a half width boundary at approximately 100 ms (the dotted lines in Fig. 2B): data from neurons exhibiting F-type currents exhibited a rise time less than 20 ms and a half width less than 100 ms (n = 18), data from the neurons exhibiting S-type currents exhibited a rise time larger than 20 ms and a half width larger than 100 ms (n = 21), and data from the neurons exhibiting the FS-type currents exhibited a rise time less than 20 ms and a half width larger than 100 ms (n = 12). Therefore, we adopted these criteria based on the rise time and half width to classify the nAChR-mediated currents. Figure 2C shows the distribution of the three response types of the T+ neurons in the MVN (n = 23) and PHN (n = 28). A dominant type was not observed in the T+ neurons in the
MVN and the PHN, although FS-type currents appeared to be a minor type in the PHN neurons. The nAChR-mediated currents in the V+ (n = 60) and D- (n = 57) neurons were also separated into the three types. Figure 2D shows the distribution of the three response types in the V+ neurons in the MVN (n = 28) and the PHN (n = 32). The proportion of MVN neurons exhibiting F-type currents was markedly larger than the proportion exhibiting the other two types. In contrast, a dominant current type was not observed in the PHN neurons, although the largest proportion of PHN neurons exhibited the F type. Figure 2E shows the distribution of the three response types in the D-neurons in the MVN (n = 28) and the PHN (n = 29). The proportion of D- MVN and PHN neurons that exhibited S-type currents was much larger than the proportion that exhibited the other two types (Fig. 2E). The distributions of the F-, S-, and FS-types were significantly different among the T+, V+, and D- neurons in the MVN (p < 0.0001). In the PHN, the distributions were not significantly different between the T+ and V+ neurons in the PHN (p = 0.41) but were significantly different between T+ and D- neurons and between V+ and D- neurons (p < 0.0001). The distributions of the cholinergic response types of T+ (p = 0.11), V+ (p = 0.17), and D- (p = 0.16) neurons were not significantly different between MVN and PHN. These results suggest that the distribution of the F-, S-, and FS-types were not different between the MVN and
PHN but different among the T+, V+, and D- neurons. Furthermore, the F-type and S-type may be the dominant current type in V+ MVN neurons and D- neurons, respectively.

The amplitude of the currents of each response type was compared among the three types of neurons (Fig. 2F-H). Although many V+ MVN neurons exhibited F-type currents, the currents of the V+ neurons were smaller than the currents of the other neuron types in the MVN (Fig. 2F1). Smaller F-type responses were also observed in the V+ PHN neurons, although no significant difference was observed between the V+ and D- neurons (Fig. 2F2). The S-type and FS-type responses did not differ significantly among the three types of neurons (Fig. 2G, H).

The nAChR subtypes involved in the three response types

The kinetics of nAChR-mediated currents depend on the nAChR subtypes that comprise the receptor (Albuquerque et al. 2009; McGehee and Role 1995). Therefore, we determined the nAChR subtypes responsible for the three current response types via pharmacological analyses using methyllycaconitine (MLA), an antagonist of the α7 subtype (Drasdo et al. 1992; Palma et al. 1996), and dihydro-β-erythroidine (DHβE), an
The analyses were performed on a total of 18, 21, and 12 T+ neurons that exhibited F-type, S-type and FS-type currents, respectively. Figure 3A1 shows the effect of 10 nM MLA and 1 μM DHβE on the F-type response. MLA blocked most of the current, and the small amount of residual current was abolished by DHβE. To clarify which of the MLA-sensitive or DHβE-sensitive currents predominated in the F-type response, we analyzed the proportion of each current included. In the F-type responses, the MLA-sensitive currents and charges were significantly larger than the DHβE-sensitive currents and charges (Fig. 3A2, 3, n = 18). Figure 3B1 shows the effect of the antagonists on the S-type response. The current was slightly reduced by MLA and largely blocked by DHβE. For this type of current, the DHβE-sensitive currents and charges were significantly larger than the MLA-sensitive currents and charges (Fig. 3B2, 3, n = 21). Figure 3C1 shows the effect of the antagonists on the FS-type response, which was composed of both a fast and slow component. Because the peaks of the fast and slow components, which were identified by comparing the traces before and after the application of the antagonists, were 23.0 ± 5.1 ms and 142.4 ± 48.8 ms, respectively, the effects of the antagonists on the currents were analyzed separately (Fig. 3C2, n = 12). The MLA-sensitive fast currents were larger than the DHβE-sensitive fast currents,
whereas the MLA-sensitive slow currents were smaller than the DHβE-sensitive slow currents. A comparison of the charge transfer revealed that the DHβE-sensitive charge was larger than the MLA-sensitive charge (Fig. 3C3, n = 12). These characteristics of the MLA-sensitive and DHβE-sensitive components of the F-, S-, and FS-types currents were also confirmed in the V+ neurons (4 F-type, 4 S-type, and 2 FS-type responses) and the D- neurons (5 F-type, 11 S-type, and 2 FS-type responses) (data not shown). These results indicate that the F-type and S-type currents are primarily mediated by α7 and non-α7 nAChRs, respectively. The FS type is a mixture of the F and S types and is mediated by α7 and non-α7 nAChRs.

Discussion

Cholinergic responses in MVN and PHN neurons

In the present study, MVN and PHN neurons exhibited inward current responses to the local application of ACh. Because the inward currents were induced in the presence of TTX, they were directly mediated by AChRs expressed by the recorded neurons. Pharmacological analyses revealed that these current responses were mediated by mAChRs and nAChRs. This finding supports a previous intracellular recording study,
in which some MVN neurons expressed both mAChRs and nAChRs (Phelan and Gallagher 1992). Our study further demonstrates that the nAChR-mediated currents are larger than the mAChR-mediated currents in the cholinergic, inhibitory, and glutamatergic neurons that constitute the major neuronal populations of the MVN and PHN (Barmack 2003; de Waele et al. 1995; McCrea and Horn 2006). The nAChR-dominant current responses in MVN and PHN neurons suggest that nAChRs are more highly expressed than mAChRs or that nAChRs are located closer to the soma than mAChRs. It should be noted that in our experimental condition using whole-cell recordings, it may be difficult to clarify mAChR-dependent events completely. In addition, several mAChRs-dependent modulations have been reported in the MVN and PHN (Navarro-López et al. 2004, 2005; Sun et al. 2002; Ujihara et al. 1988, 1989). Therefore, we do not rule out the significant effects of mAChRs on MVN and PHN functions.

In this study, we used 5 μM atropine to completely block mAChR-mediated currents because, in our preliminary study, the application of atropine at a concentration less than 5 μM did not completely block the currents in some cases. However, a high concentration of atropine partially blocks nAChR-mediated currents in addition to
mAChR-mediated currents (Zwart and Vijverberg 1997). Indeed, when the amplitude of the ACh-induced currents at approximately 20 ms, a majority of which were composed of nAChR-mediated currents, were compared before and after the application of atropine, approximately 20% of the currents were reduced (before, 264.8 ± 153.3 pA; after, 217.3 ± 128.3, n = 30).

When the nAChR-mediated currents were isolated due to the application of atropine (5 μM), three response types, namely the F, S, and FS types, were identified based on the rise time and the half width of their current responses. However, this classification may be significantly affected due to the partial block of nAChR-mediated currents by the high concentration of atropine. To validate this possibility, we analyzed the 10%-90% rise times and the half widths of the ACh-induced currents, which were used for the analyses in Fig. 2, both before and after the application of the atropine, and classified the currents into the three types based on the parameters. Although not all traces before the atropine application were recorded, comparisons of the response types before and after the atropine application were possible in 30 of the 51 T+ neurons, 46 of the 60 V+ neurons, and 36 of the 57 D- neurons. All of the F-type responses (10 T+, 23 V+, and 4 D- neurons) before the atropine application did not change after the
application. In the S-type responses (13 T+, 17 V+, and 26 D- neurons) before the atropine application, one T+ neuron, three V+ neurons, and one D- neuron were determined to exhibit FS-type responses after the application. Among the FS-type responses (7 T+, 6 V+, and 6 D- neurons) before the atropine application, the responses of two V+ neurons and one D- neuron were determined as F-type responses after the application. All changes that occurred in the S-type and FS-type responses were caused by the decrease in the slow component of the currents, which were composed largely of mAChR-mediated currents. Therefore, the effect of a partial block of the nAChR-mediated currents due to atropine on the overall distributions of the F-, S-type, and FS-type responses in the T+, V+, and D- neurons is likely to be small.

The present study clarified the different distributions of the cholinergic response types among the T+, V+, and D- neurons. Our previous study reported that up to 30% of ChAT-expressing neurons expressed VGAT in the PHN but only a few neurons co-expressed ChAT and VGAT in the MVN (Saito et al., 2015). Because the neurons that co-expressed VGAT and ChAT were not checked in the present study, we do not know how many co-expressing neurons were included in the present data. One of the reasons why the distributions of the cholinergic response types were not significantly
different between the T+ and V+ neurons in the PHN may be that the co-expressing neurons were partly included in the data obtained from PHN neurons.

Pharmacological analyses indicated that the F-type and S-type responses are mediated predominantly by α7 and non-α7 nAChR subtypes, respectively, and that the FS type is a mixed response mediated by both α7 and non-α7 subtypes. In previous studies of hippocampal neurons (Alkondon and Albuquerque 1993; Albuquerque et al. 1995; Alkondon et al. 1997b; 1999), four types of nAChR-mediated currents have been reported: type IA currents exhibit fast decay and are mediated by the α7 subtype, type II currents exhibit slow decay and are mediated by the α4β2 subtype, and type IB currents are a combination of type IA and II currents. These currents may correspond to the F, S, and FS types described in our study. Although no more than 2% of the hippocampal neurons exhibit type III currents, which are presumably mediated by the α3β4 subtype, we did not encounter this type of current in any of the neurons recorded from the MVN and PHN. Because similar nAChR-mediated currents are observed in other brain regions such as dopamine neurons in the ventral tegmental area (Yang et al. 2009), the three or four types of nAChR-mediated currents may be a common feature of the CNS cholinergic system (Zoli et al. 1998). Among the distinct types, type IA (F type) is the
predominant nAChR-mediated response in hippocampal neurons (Albuquerque et al. 1995). However, in the MVN and PHN, the F type was predominant only in the inhibitory neurons. The majority of the glutamatergic neurons exhibited predominantly S-type (type II) responses. Therefore, neuron type-specific responses may be characteristic of MVN and PHN neurons.

Although different nAChR-mediated currents were identified in the present study, whether the nAChRs that give rise to the current responses are located on the synaptic membrane remains an open question. In addition to conventional cholinergic synaptic transmission, ACh is also used for nonsynaptic transmission known as “volume transmission” (Bennett et al. 2012; Lendvai and Vizi 2008; Sarter et al. 2009). Because volume transmission occurs at the extrasynaptic membrane, the time course of the transmission is extremely slow. In addition, volume transmission is known to be mediated primarily via α4β2 nAChRs (Bennett et al. 2012; Ren et al. 2011). In the present study, the plots of the 10%-90% rise times against the half widths revealed a wide distribution of S- and FS-type responses. Furthermore, the S-type responses and the slow component of the FS-type currents were predominantly mediated by DHβE-sensitive, putatively α4β2, nAChRs. These findings suggest the intriguing
possibility that the S- and FS-type current responses may be induced, at least in part, by
the activation of the \( \alpha 4 \beta 2 \) nAChRs that are involved in volume transmission.

Functional implications of nAChRs in the MVN and PHN

In contrast to the findings regarding mAChR-mediated modulation of neuronal firing in
the MVN (Sun et al. 2002; Ujihara et al. 1988, 1989) and the sustained excitation that
may be involved in gaze holding in the PHN (Navarro-López et al. 2004, 2005), the
functional significance of nAChRs has not been investigated in the MVN and PHN.
The fact that nAChRs are ionotopic receptors indicates that the excitatory effects of
these receptors, as shown by depolarization of the membrane potential in MVN neurons,
result from ACh application (Phelan and Gallagher 1992). In addition, high Ca\(^{2+}\)
permeability is characteristic of nAChRs. Although the high Ca\(^{2+}\) permeability of the
\( \alpha 7 \) subtype is well known, non-\( \alpha 7 \) subtypes, represented by \( \alpha 4 \beta 2 \)-containing receptors,
show higher Ca\(^{2+}\) permeability than conventional glutamatergic AMPA receptors,
including the GluA2 subtype, which are widely distributed in the brain (Fucile 2004;
McGehee and Role 1995; Pankratov and Lalo 2014). Therefore, Ca\(^{2+}\) influx into
neurons via nAChRs can participate in several Ca\(^{2+}\)-dependent cellular events (Rathouz
et al. 1996; Role and Berg 1996; Shen and Yakel 2009). We previously demonstrated
that the increase in intracellular Ca$^{2+}$ that occurs via repetitive activation of Ca$^{2+}$-permeable AMPA receptors (CP-AMPARs) activates Ca$^{2+}$-activated nonselective cation channels and induces sustained activation of the glutamatergic excitatory networks in the PHN, which may be involved in gaze holding (Saito and Yanagawa 2010). However, the CP-AMPARs-dependent mechanism alone is not sufficient because the duration of the sustained activity was shorter than the time constant of a centripetal drift of the rodent (Saito and Yanagawa 2010), and therefore, additional mechanisms are needed to reach the time constant. The present finding that PHN neurons exhibit significant nAChR-mediated current responses suggests the possibility that the activation of nAChRs is one of the mechanisms that participate in the sustained activity of PHN networks. A moderate number of PHN neurons exhibit the predominant expression of CP-AMPARs (Saito and Yanagawa 2010), whereas most PHN neurons predominantly express nAChRs. Therefore, the activation of nAChRs of widely distributed neurons may contribute to sustaining the activation of the local excitatory network that originates from some activated neurons that express CP-AMPARs by premotor burst inputs.

We recently demonstrated another Ca$^{2+}$-dependent event in PHN neurons—spontaneous
miniature outward currents (SMOCs), which are induced by the increase in intracellular 
Ca\(^{2+}\) that occurs through Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the endoplasmic reticulum via ryanodine receptors (Saito and Yanagawa 2013). However, the observation that the application of mecamylamine does not affect the induction of SMOCs (Saito and Yanagawa 2013) suggests that Ca\(^{2+}\) influx via nAChRs does not necessarily contribute to SMOCs.

The present study demonstrated that the proportion of neurons exhibiting F-type responses was larger in inhibitory neurons than in the other types of neurons. This result suggests that many inhibitory neurons in the MVN and PHN preferentially express \(\alpha 7\) nAChRs. The functional significance of the expression of \(\alpha 7\) nAChRs in GABAergic neurons has been shown for local GABAergic circuits in the hippocampus. The activation of the \(\alpha 7\) nAChRs of hippocampal GABAergic interneurons regulates the activity of pyramidal neurons via direct inhibition or indirect disinhibition (Alkondon et al. 1999; Ji and Dani 2000). Furthermore, a study of local networks in the auditory cortex demonstrated that the disinhibition of pyramidal neurons induced by the activation of GABAergic interneurons via nAChRs is needed for the acquisition of associative fear memory (Letzkus et al. 2011). Therefore, inhibitory neurons that
express α7 nAChRs appear to regulate the activation of local networks in the PHN and
MVN. However, some inhibitory neurons project to other brain areas, such as the
abducens nucleus and inferior olive (McCrea and Horn 2006; McEligott and Spencer
2000). Therefore, it is necessary to determine whether α7 nAChR-mediated regulation
via inhibitory neurons extends to other projection areas to elucidate the functional roles
of the α7 nAChRs in MVN and PHN neurons.

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Figure Legends

Figure 1. Cholinergic responses of the three neurotransmitter phenotypes of MVN and PHN neurons to a puff application of ACh.

(A) Current responses of a T+ MVN neuron to a puff application of 1 mM acetylcholine (ACh). The ACh-induced currents were blocked by 5 μM atropine (muscarinic receptor antagonist) and 10 μM mecamylamine (nicotinic receptor antagonist). (B) Comparisons of the muscarinic (Mus)- and nicotinic (Nic)-sensitive currents and charges of T+ (1), V+ (2), and D- neurons (3) in the MVN and PHN. (B1) The Nic-sensitive currents (MVN, 4.1 ± 2.6 pA/pF, n = 16; PHN, 4.9 ± 3.8 pA/pF, n = 21) and charge (MVN, 1.0 ± 0.5 pC/pF, n = 16; PHN, 1.0 ± 0.7 pC/pF, n = 21) were significantly larger than the Mus-sensitive currents (MVN, 2.1 ± 1.4 pA/pF, p = 0.0013; PHN, 1.0 ± 0.7 pA/pF, p < 0.001) and charges (MVN, 0.4 ± 0.2 pC/pF, p < 0.0001; PHN, 0.3 ± 0.2 pC/pF, p < 0.0001) in the T+ neurons. (B2) The Nic-sensitive currents (MVN, 2.4 ± 1.2 pA/pF, n = 17; PHN, 2.9 ± 2.0 pA/pF, n = 15) and charges (MVN, 0.5
Mus-sensitive currents (MVN, $1.3 \pm 1.2 \, \text{pA/pF, } p = 0.0005$; PHN, $1.1 \pm 0.8 \, \text{pA/pF, } p = 0.0022$) and charges (MVN, $0.4 \pm 0.4 \, \text{pC/pF, } p = 0.008$; PHN, $0.3 \pm 0.3 \, \text{pC/pF, } p = 0.0035$) in the V+ neurons. (B3) The Nic-sensitive currents (MVN, $3.4 \pm 2.0 \, \text{pA/pF, } n = 15$; PHN, $3.4 \pm 2.0 \, \text{pA/pF, } n = 14$) and charges (MVN, $0.9 \pm 0.5 \, \text{pC/pF, } n = 15$; PHN, $0.9 \pm 0.6 \, \text{pC/pF, } n = 14$) were significantly larger than the Mus-sensitive currents (MVN, $2.0 \pm 2.1 \, \text{pA/pF, } p = 0.0047$; PHN, $1.9 \pm 1.2 \, \text{pA/pF, } p = 0.0132$) and charges (MVN, $0.6 \pm 0.6 \, \text{pC/pF, } p = 0.002$; PHN, $0.6 \pm 0.4 \, \text{pC/pF, } p = 0.0025$) in the D- neurons. The gray plots connected by a line were obtained from individual neurons. (C) Comparisons of the Nic currents and charges among the T+, V+, and D- neurons in the MVN (1) and PHN (2). * $p < 0.05$; ** $p < 0.01$.

Figure 2. Characterization of nAChR-mediated current responses in MVN and PHN neurons.

(A) The three types of nAChR-mediated current responses exhibited distinct rise and decay times. A response showing fast rise and fast decay (F-type) (A1), a response showing slow rise and slow decay (S type) (A2), and a response showing fast rise and slow decay (FS type) (A3). (B) Plots of the 10%-90% rise times against the half
widths of nAChR-mediated current responses obtained from 51 T+ neurons. Each plot represents the data obtained from an individual neuron. (C-E) Percentages of T+ (C), V+ (D), and D- (E) neurons exhibiting the F-, S-, and FS-type responses in the MVN (C1, n = 23; D1, n = 29; E1, n = 28) and PHN (C2, n = 28; D2, n = 31; E2, n = 29). (F-H) Comparison of the amplitudes of nAChR-mediated F- (F), S- (G) and FS-type (H) current responses in T+, V+, and D- neurons in the MVN (1) and PHN (2). The amplitudes of F-type current responses in the V+ neurons (2.6 ± 1.0 pA/pF) were significantly smaller than those in the T+ (5.5 ± 2.9 pA/pF) and D- neurons (6.8 ± 6.2 pA/pF) in the MVN (F1). The amplitudes of F-type current responses in the V+ neurons (5.3 ± 4.1 pA/pF) were also significantly smaller than those in the T+ neurons (11.0 ± 4.8 pA/pF) in the PHN (F2). * \( p < 0.05 \).

Figure 3. nAChR subtypes involved in the three response types in MVN and PHN neurons. (A1) F-type nicotinic responses induced by a puff application of 1 mM ACh in the presence of 5 μM atropine. The current was almost completely abolished by 10 nM methyllycaconitine (MLA). (A2, A3) Comparisons of the current amplitude and charge transfer between the MLA-sensitive and dihydro-β-erythroidine
(DHβE)-sensitive responses, respectively (7 MVN and 11 PHN neurons). (B1) S-type nicotinic responses were only slightly blocked by 10 nM MLA but were largely suppressed by 1 μM DHβE. (B2, B3) Comparisons of the current amplitude and charge transfer between the MLA-sensitive and DHβE-sensitive responses, respectively (9 MVN and 12 PHN neurons). (C1) FS-type nicotinic response showing that the fast component was almost completely blocked by 10 nM MLA, the slow component was only partially suppressed by MLA, and the remaining slow current was abolished by 1 μM DHβE. (C2, C3) Comparisons of the amplitudes of the fast and slow currents and of the charge transfer between the MLA-sensitive and DHβE-sensitive responses (8 MVN and 5 PHN neurons). **p < 0.01.