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Differences in Response to Muscarinic Activation Between First and Higher Order Thalamic Relays

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Varela C, Sherman SM. Differences in response to muscarinic activation between first and higher order thalamic relays. *J Neurophysiol* 98: 3538–3547, 2007. First published October 17, 2007; doi:10.1152/jn.00578.2007. The mammalian thalamus is composed of two types of thalamocortical relay. First order relays receive information from subcortical sources and relay it to cortex, whereas higher order relays receive information from layer 5 of one cortical area and relay it to another. Recent reports suggest that modulatory inputs to first and higher order relays may differ. We used rat thalamic brain slices and whole cell recordings from relay cells in various first order (the lateral geniculate nucleus, the ventral posterior nucleus, and the ventral portion of the medial geniculate body) and higher order (the lateral posterior, the posterior medial nucleus, and the dorsal portion of the medial geniculate body) relays to explore their responses to activation of muscarinic receptors. We found that, whereas all first order relay cells show a depolarizing response to muscarinic activation, ~20% of higher order relay cells respond with hyperpolarization. The depolarization is accompanied by an overall increase in input resistance, whereas the hyperpolarization correlates with a decrease in resistance. Because activation of cholinergic brain stem afferents to thalamus increases with increasing behavioral vigilance, the findings suggest that increased vigilance will depolarize all first order and most higher order relay cells but will hyperpolarize a significant subset of higher order relay cells. Such hyperpolarization is expected to bias these relay cells to the burst firing mode, and so these results are consistent with evidence of more bursting among higher order than first order relay cells.

INTRODUCTION

It is almost 200 years ago that Karl F. Burdach (Burdach 1822) made the first clear descriptions of thalamic nuclei. Since then, there have been many attempts to classify thalamic nuclei (e.g., Luys 1865; Macchi et al. 1996; Rose and Woolsey 1849), a particularly compelling one recently suggested by Guillery (1995) calls for relay nuclei to be divided into first and higher order (Fig. 1*A*). Briefly, first order relays are nuclei that receive primary afferents, or drivers (Sherman and Guillery 1998), from ascending, noncortical, afferent pathways, whereas higher order relays receive their driver input from layer V of cortex and relay this to a different cortical area and therefore could provide a cortico-thalamo-cortical route of information transfer. Examples of first and higher order relays, respectively, are the lateral geniculate nucleus and the lateral posterior-pulvinar complex (vision), the ventral posterior and posterior medial nuclei (somatosensory), and the ventral versus dorsal divisions of the medial geniculate body (auditory) (for details, see Sherman and Guillery 2006).

In addition to driver afferents, the origin of which determines the order (first or higher) of the thalamic relay, relay cells receive a large number of modulator afferents (Sherman and Guillery 1998). Modulators originate in a number of different brain regions, primarily in various brain stem nuclei including cholinergic centers, and in layer 6 of the cortex (Bourassa et al. 1995; Hallanger et al. 1987; reviewed in Sherman and Guillery 2006). Evidence of modulatory differences between first and higher order relays is accumulating. For instance, afferents to visual, auditory, and somatosensory higher order relay cells consist of a larger proportion of modulatory inputs with respect to driver inputs (Van Horn and Sherman 2007; Wang et al. 2002), the zona incerta and anterior pretectal nucleus provide GABAergic inputs to higher order relay cells but little or no innervation to first order relays (Barthó et al. 2002; Bokor et al. 2005), and dopaminergic inputs appear to target higher order relays fairly selectively in the monkey (Sánchez-González et al. 2005).

Modulatory inputs also may function differently in first and higher order relays. Mooney et al. (2004) have found, in the rat, that a great proportion of cells in the higher order portion of the medial geniculate body are hyperpolarized by muscarinic receptor activation, whereas the effect on the first order portion of the medial geniculate body is depolarization. We sought to determine if this is part of a general difference between first and higher order relays.

METHODS

Intracellular recordings

The data have been gathered from current-clamp and continuous single electrode voltage-clamp recordings obtained in the whole cell configuration from thalamic relay cells in rat coronal brain slices. No subtraction of liquid junction potential (which is estimated to be ~10 mV with the solutions used in our experiments) has been made from membrane potential values. Relay cells have been recorded in first order (the lateral geniculate nucleus, the ventral posterior nucleus, and the ventral portion of the medial geniculate body) and higher order (the lateral posterior, the posterior medial nucleus, and the dorsal portion of the medial geniculate body) thalamic nuclei.

Brain slices were prepared from Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) of 11 to 18 days postnatal age; these ages were chosen to allow better visualization of cells for the patch-clamp recordings and to ensure functional properties as close as possible to the adult rat (Ramoa and McCormick 1994). Animals were quickly anesthetized by inhalation of isofluorane (AErrane, from Baxter Pharmaceutical, Deerfield, IL). Respiration depth and rate (as

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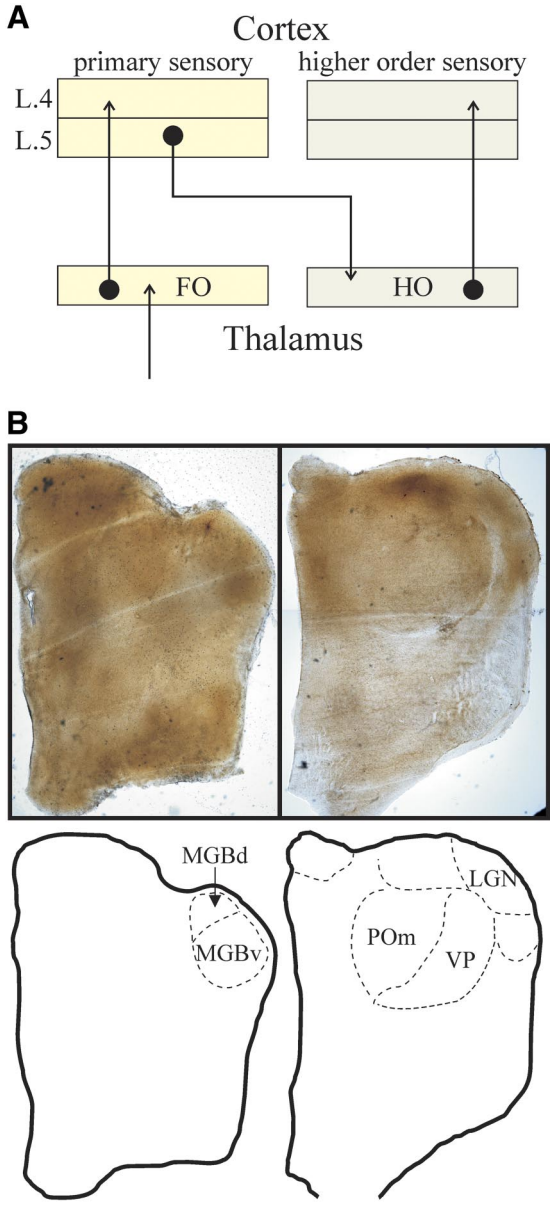


FIG. 1. *A*: schematic representation of the origin of driver afferents and main projections for first order (FO) and higher order (HO) thalamic relays. L4 and L5 refer to layer 4 and layer 5, respectively. *B, top*: typical coronal slices used in our experiments as seen after processing for biocytin. Only 1 hemisphere was used; cortex has been removed. *Bottom*: tracings of sections showing outlines of the 6 nuclei from which cells have been recorded. LGN, (dorsal) lateral geniculate nucleus; LP, lateral posterior nucleus; MGBd, dorsal portion of the medial geniculate body; MGBv, ventral portion of the medial geniculate body; POm, posterior medial nucleus; VP, ventral posterior nucleus.

observed by experimenter) and the hind limb withdrawal reflex were used to verify depth of anesthesia. Once the withdrawal reflex was absent, the animal was decapitated, and the head was immediately submerged into an icy solution of artificial cerebrospinal fluid (ACSF, composed of, in mM: 125 NaCl, 25 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, and 25 glucose). The brain was removed in <1 min, blocked into a small cube containing the thalamus, and glued (with instant Krazy Glue) onto the platform of a motorized vibratome (from WPI, Sarasota, FL); the platform was submerged into icy ACSF (continuously bubbled with a mixture of 95% O_2-5% CO_2), and 300- to 500-μm-thick (typically 400 μm) coronal slices including the nuclei of interest were obtained. Four to eight slices were prepared in this way and placed in a beaker containing bubbled (95% O_2-5% CO_2) ACSF at 30°C for ~30 min, then kept at room temperature for the duration of the experiment. Individual slices were transferred to the recording chamber when needed.

Recordings were made in a standard visualized patch-clamp recording rig. A slice was kept in a chamber located in the light path of an Olympus microscope (Olympus America, Center Valley, PA) equipped with DIC-IR (model BX51-WI); in a few experiments a similarly equipped Zeiss microscope (Carl Zeiss, Thornwood, NY) was used (model Axioskop FS). Cells were selected with the aid of the microscope. All drugs were bath applied; the chamber had a volume of ~700 μl and the inflow rate of ACSF (warmed to 30 ± 2°C before entering the chamber with a temperature controller from Warner Instruments, Hamden, CT) was kept at ~2 ml/min.

The whole cell configuration was achieved using glass micropipettes (pulled from borosilicate glass from Garner Glass, Claremont, CA) with tip resistances of 4–8 MΩ. The micropipette solution contained (in mM) 135 KGluconate, 7 NaCl, 10 HEPES, 2 Na_2ATP, 0.3 Na_3GTP, and 2 $MgCl_2$. In 75 of the cells, the micropipette solution was (in mM) 117 KGluconate, 13 KCl, 10 HEPES, 2 Na_2ATP, 0.4 Na_3GTP, 1 $MgCl_2$, 0.07 $CaCl_2$, and 0.1 EGTA. No difference was found in the results related to micropipette solutions and the cells have been pooled together. Biocytin (0.1–0.5%) was included in some of the micropipettes to allow anatomical reconstruction of the recorded cells. To avoid any confusion identifying cells, as well as any possible modification in the response due to multiple applications of agonists to metabotropic receptors (Maloteaux and Hermans 1994), only one cell was recorded in each slice.

Once a cell was in whole cell configuration, it was injected with a set of negative and positive square current pulses of ≥400-ms duration and at different intensities; the pulses were intended to verify the viability of the cell by driving it through a wide range of physiological voltages (from about −100 mV to an over-threshold voltage) to test for normal voltage responses. Unstable cells (e.g., with shifting resting membrane potential) were discarded, as were cells with input resistance <100 MΩ and cells with access resistance >30 MΩ.

The recorded signals were amplified and filtered (30 kHz) with an Axoclamp 2A amplifier (Axon Instruments, Union City, CA) in "bridge" or continuous single-electrode voltage-clamp configuration. Data were digitized using the AD converter Digidata 1200B from Axon Instruments. Data were acquired at 10 kHz and recorded with software purchased from Axon Instruments (Clampex 8.2). Clampfit 8.2 (Axon Instruments) and Matlab (7.1 R14, The Mathworks) were used for quantification and statistical analysis. Sample size plus mean ± standard deviation (SD) were used to characterize each group of data. Because the underlying distributions of the parameters quantified are not known in most of the thalamic relays under study, nonparametric tests were chosen for population comparisons: the χ^2 test was used to compare the frequencies of observed effects among nuclei (see following text); the Wilcoxon-Mann-Whitney test, to compare the distributions of two samples; the Kruskal-Wallis test was used when more than two groups were compared; and the Brown-Forsythe, to test the homogeneity of group variances.

Pharmacological agents

Acetyl-β-methylcholine (MCh, usually at 250 μM) was bath applied for ~0.5 min, and the response was recorded for 5–10 min (with a so-called gap-free protocol in Clampex). When used, antagonists were bath applied during ≥8 min previous to the application of MCh to ensure effective antagonism. Drugs were purchased from Sigma-Aldrich (St. Louis, MO): tetrodotoxin (TTX), MCh, and atropine from Tocris (Ellisville, MO): TTX, SR95531.

Histology

Biocytin (0.1–0.5%) dissolved in the intracellular solution was included in many of the recording electrodes. At the end of the

TABLE 1. *Sample sizes in individual nuclei*

	First Order (n = 76)			Higher Order (n = 197)		
Total = 273	VP	MGBv	LGN	POm	MGBd	LP
n	23	15	38	74	65	58
Hyperpolarization	0	0	0	16	8	15
Depolarization	22	12	35	55	34	29
Mixed	1	2	0	2	0	3
No effect	0	1	3	1	23	11

Distribution of the observed effects (rows) of muscarinic agonist in the thalamic nuclei (columns) used for this study; cell total was 273. *VP*, ventral posterior; *MGBv*, medial geniculate body (ventral portion); *LGN*, lateral geniculate nucleus (dorsal); *POm*, posterior medial; *MGBd*, medial geniculate body (dorsal portion); *LP*, lateral posterior.

experiment, those slices were preserved in 4% paraformaldehyde for ≥1 wk and then processed to reveal the morphology of the recorded cell. The tissue was reacted with 1:100 avidin/biotin complex (ABC reaction, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) and then with diaminobenzidine. The location of cells in the target nuclei was confirmed and certain morphological parameters (i.e., soma area and number of primary dendrites) examined using a Leitz Wetzlar light microscope, an Axiocam digital color camera (Carl Zeiss) and AxioVision software (Rel. 4.5, Carl Zeiss).

RESULTS

From the rat thalamus, we recorded 273 relay cells that fulfilled the criteria of stability and normal voltage-dependent properties. Of these, 76 were first order relay cells and 197 were higher order. Table 1 summarizes the complete data set. Appropriate sample sizes are indicated for those particular experiments or analyses for which not all cells were tested, and these data are summarized in Table 2. Cells in first order relays were recorded in the ventral portion of the medial geniculate body (n = 15), in the lateral geniculate nucleus (n = 38) and in the ventral posterior nucleus (n = 23). For the higher order sample, recordings were made in the dorsal portion of the medial geniculate body (n = 65), in the lateral posterior nucleus (n = 58), and in the posterior medial nucleus (n = 74). Figure 1*B* shows the location of these thalamic nuclei in the coronal slices used in our recordings.

Overview of effects on membrane potential

Bath application of the general muscarinic agonist MCh (used at 250 μM unless otherwise indicated) evoked an effect in 234 (85.7%) of the cells. Figure 2, *A–D,* displays examples of the responses to MCh application. Figure 2*A*, which shows a current-clamp recording of 10-min duration, illustrates the response to MCh application of a representative first order relay cell from the ventral posterior nucleus. The solution perfusing the slice was switched to ACSF containing MCh for 30 s (indicated by the short red horizontal bar above the trace); after a delay of a minute or so, the cell depolarized and then slowly returned to baseline levels. In some cases, as in the example of Fig. 2*A*, the depolarization was sufficient to evoke action potentials, and these have been truncated in the figure for presentation purposes. The downward deflections correspond to current pulses used to estimate input resistance. The upward deflections in the last minutes of the response are due to partial activation of I_T; the negative pulses used for testing input resistance de-inactivated I_T, and the return to baseline after switching off the testing pulse activated I_T (see following text). For first order relay cells, the average depolarization at the peak in response to application of MCh (250 μM) for 30 s was 14.6 ± 6.5 mV (mean ± SD reported here and in the following text; n = 49). The evoked response was significantly larger in lateral geniculate cells (17.3 ± 5.9 mV) when compared with ventral posterior (12 ± 5.9 mV) or the ventral portion of the medial geniculate body (12 ± 7 mV; P = 0.02; Kruskal-Wallis). In most (n = 204) of the cells, both first and higher order and independent of their response to MCh, DC current injection was used to keep the cells at a similar initial resting potential (around −60 to −65 mV) to make the results comparable.

Figure 2*B* shows an example of a higher order relay cell recorded in the lateral posterior nucleus and depolarized by MCh application. The higher order relay cells depolarized by MCh showed a response amplitude, time course, and input resistance pattern (see following text) indistinguishable from that seen in first order relay cells. The average depolarization for the higher order relay cells in this group was 12 ± 6.5 mV (n = 85), measured under comparable conditions to those of the first order relay cells. No significant difference was found across the three higher order nuclei (P > 0.05; Kruskal-Wallis). Figure 2*C* shows an example of a higher order relay cell from the posterior medial nucleus hyperpolarized by MCh application. This hyperpolarization followed a time course similar to the depolarizing effects in other cells, and the average hyperpolarization for 34 higher order relay cells showing this effect was −7.3 ± 3.3 mV. Again, the amplitude of the

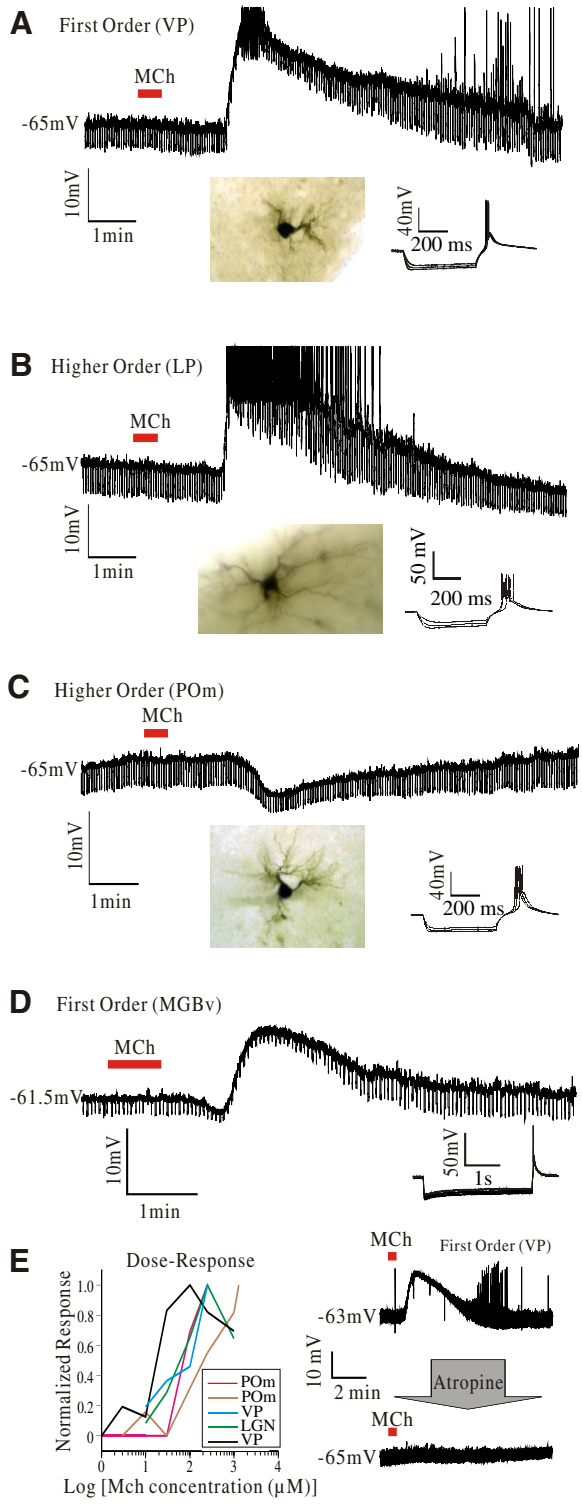
TABLE 2. *Sample size and nucleus of origin for cells used on each experiment and control*

	First Order			Higher Order		
	VP	MGBv	LGN	POm	MGBd	LP
Measure MCh effect	17;1M	8; 1M	24	34,13HP,1M	31, 8HP	20,13HP,2M
Atropine	1	0	0	2, 1 HP	0	0
Dose-response	2	0	1	1, 1 HP	0	0
Input resistance	1	1	6	6, 3 HP	3, 1 HP	6, 6 HP
TTX	1	1	3	3, 2 HP	1	1
Low Ca^{2+} high Mg^{2+}	4	4	4	4, 4 HP	4, 2 HP	4, 3 HP
Histology	2, 1U	1U	1	5,3HP,2NR,4U	3,1HP,1NR,4U	1, 1HP, 1NR,2U

Summary of number of cells used for quantification in each of the indicated experiments (rows; see text for details), origin of the cells in columns. *HP*, cell for which the effect of muscarinic agonist is hyperpolarization; *M*, muscarinic effect is mixed (hyperpolarization followed by depolarization); *NR*, no response to agonist; *U*, agonist not applied. If nothing is indicated the effect was depolarization. Other abbreviations as in Table 1.

response was not significantly different across higher order relays ($P > 0.2$; Kruskal-Wallis).

Finally, Fig. 2*D* displays a representative example for the least frequent of the effects, a hyperpolarization followed by a depolarization (i.e., "mixed" response). This type of response was only found in three first order and five higher order cells (see Table 1 for cell origin). When using MCh at 250 μM, the average hyperpolarization was -4.8 ± 1.5 mV ($n = 3$), and the average depolarization was 8.4 ± 4.5 ($n = 3$).



Every one of the first order relay cells that responded ($n = 72$) was purely depolarized by the MCh application ($n = 69$; 95.8%) or showed a mixed response ($n = 3$; 4.2%). None of the first order relay cells showed a pure hyperpolarizing response or an initial depolarization followed by a hyperpolarization. In contrast to the response pattern of first order relay cells, the 162 higher order relay cells that responded to MCh showed either a pure depolarization ($n = 118$; 72.8%; Fig. 2*B*), a pure hyperpolarization ($n = 39$; 24.1%; Fig. 2*C*), or a mixed response ($n = 5$; 3.1%; Fig. 2*D*). The frequency of the various responses (depolarization, hyperpolarization, no effect, and mixed responses) was significantly different in first versus higher order thalamic relays ($P \ll 0.0001$ on a χ^2 test), and the number of hyperpolarizing cells was significantly larger among the higher order relay cells ($P \ll 0.0001$ on a χ^2 test). Similarly, the number of relay cells that were not responsive to MCh was significantly higher in higher than in first order relays ($P < 0.01$; χ^2 test).

Morphological correlates and resting membrane potential

The photomicrographs included in Fig. 2, *A–C,* correspond to the recorded neurons after filling with biocytin and appropriate tissue processing (see METHODS). All recorded cells recovered for anatomical analysis in this fashion ($n = 33$) displayed the morphology typical of relay cells and not interneurons (Gabbott et al. 1986; Webster and Rowe 1984). This is not surprising because interneurons are essentially absent from the thalamus of rats outside of the lateral geniculate nucleus (Arcelli et al. 1997). We believe that our sample from the lateral geniculate nucleus were also relay cells: not only did all recovered geniculate cells for morphological analysis show relay cell characteristics, but all in addition exhibited relay cell physiological properties. That is, interneurons can be distinguished physiologically from relay cells based on interneurons showing an absence of an obvious rebound burst response after the application of hyperpolarizing current pulses (McCormick and Pape 1988). A protocol including such pulses was used in all the recorded cells. As shown in the *inset traces* next to the photomicrographs of Fig. 2, *A–D,* all recorded cells showed a rebound burst, indicative of relay cells. Thus the combination of the findings of Arcelli et al. (1997), the morphology for many of the cells, and the test for rebound bursting in all lead to the conclusion that all cells recorded in our sample were relay cells.

Because there were no obvious morphological differences among our sample, we limited our morphological measure-

FIG. 2. Effects of acetyl-β-methylcholine (MCh) on first and higher order relay cells. *A*: representative example of a first order relay cell's response to MCh (250 μM, 30 s); the labeled cell and its responses to steps of current injection shown below the response trace. Downward deflections correspond to negative current pulses (-10 pA, 400 ms, 3–4 s between pulses) used to measure input resistance; upward spikes are truncated action potentials. *B*: example representative of depolarizing responses observed in higher order relay cells; conventions as in *A*. *C*: example representative of hyperpolarizing responses observed in higher order relay cells; conventions as in *A*. *D*: example of a first-order cell showing a mixed response (MCh was used at 187.5 μM in this particular case). *E, left*: dose-response curves of 5 cells: 3 first order relay cells (black, green and blue), 1 higher order relay cell with hyperpolarizing (brown), and 1 higher order with depolarizing (red) responses included. *Right*: response to MCh (250 μM, 30 s) blocked by atropine (250 nM). Abbreviations for nuclei as in Fig. 1.

ments to the soma area and number of primary dendrites. We compared these measurements between cells showing depolarization to MCh application and cells showing hyperpolarization. Cells that were depolarized by MCh had an average cross-sectional area of 211.2 ± 76.8 μm^2 (n = 12; see Table 2 for details), whereas cells that were hyperpolarized measured 266.0 ± 49.7 μm^2 (n = 5; see Table 2 for details). These values are not significantly different either in terms of mean (Mann-Whitney test, P = 0.16) or variance (Brown-Forsythe test P = 0.17).

Depolarizing cells had an average number of primary dendrites of 3.6 ± 1.1 (n = 12), and hyperpolarizing cells an average of 3.6 ± 0.9 (n = 5). Again, the differences are not significant (Mann-Whitney, P = 1.0; Brown-Forsythe test, P = 0.84). We thus failed to find any obvious morphological features correlated with the sign of response to MCh.

Similarly, no significant morphological differences were found when comparing first and higher order relay cells regardless of their response to muscarinic activation. Thus first order relay cells had an average soma size of 235.4 ± 60.5 μm^2 (n = 5), which was not significantly different (Mann-Whitney, P = 0.71) from the higher order average of 222.9 ± 66.1 μm^2 (n = 28). The number of primary dendrites was 3.6 ± 0.9 μm^2 (n = 5) for first order and 4.1 ± 1.3 μm^2 (n = 28) for higher order; again, the values were not significantly different (Mann-Whitney, P = 0.42).

Furthermore, resting membrane potential (as measured within the 1st minute after achieving the whole cell configuration) was not significantly different for cells with different muscarinic responses. The average resting potential of 21 first order relay cells was −69.2 ± 7.3mV (all cells depolarized with MCh), whereas that of 36 higher order cells was −66.7 ± 6mV for cells depolarized with MCh, −64.9 ± 5.9 mV for 15 cells showing hyperpolarization, and −66.1 ± 4.8 mV for 14 higher order cells that did not respond to the muscarinic agonist. Differences in membrane potential across populations from all nuclei independent of response signature were not found to be significant (P = 0.14; Kruskal-Wallis test). Only cells in the lateral geniculate nucleus had a membrane potential that was significantly different (P < 0.01; Mann-Whitney) from other first and higher order cells (–72.7 ± 5.7 mV; n = 14).

Dose-response curves

We assessed the dose-response relationship of MCh in a subset of five cells (including first and higher order cells and cells that either depolarized or hyperpolarized to MCh application; see Table 2 for further details). These cells were recorded in continuous single electrode voltage clamp (voltage held at −60 mV) and were exposed to different concentrations of MCh, from 1 μM to 1.5 mM, bath applied to the cell in order of increasing or decreasing concentrations. Not all the concentrations were used in all the cells. The maximum current evoked by each application was recorded and the absolute maximum evoked for each cell was used to normalize its responses. These normalized data were plotted against the logarithm of MCh concentrations in Fig. 2*E* (*left*). The curves were used to select a concentration of 250 μM to evaluate the effect of muscarinic activation across relay cells (this concentration should evoke a maximal or close to maximal response). The *right side* of Fig. 2*E* is shown as a confirmation of the specificity of the muscarinic agonist; the response (either hyperpolarizing or depolarizing) evoked by MCh was completely blocked by the general muscarinic antagonist atropine (250 nM, n = 4, 1 first order cell and 3 higher order).

Population effects

Table 1 and Fig. 3 summarize the effects observed in the population of cells by nuclei. Among the first order relays (results summarized in Table 1 and Fig. 3), the ventral posterior nucleus (VP) displayed the most consistent responses to muscarinic activation with 22 cells showing depolarization, 1 mixed response, and no cells unresponsive. The ventral portion of the medial geniculate body (MGBv) showed two types of effects as well as unresponsive cells: 12 cells (80%) showed

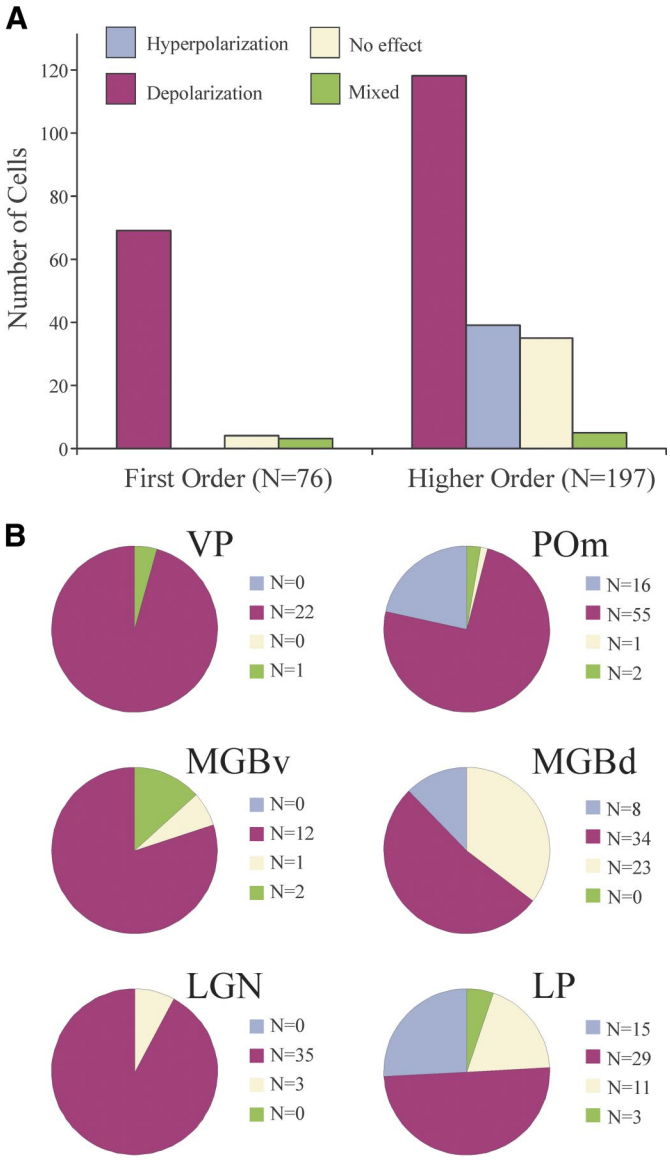


FIG. 3. Summary of responses to MCh. *A*: distribution of the different effects of MCh on first and higher order relay cells. *B*: distribution of the effects in individual nuclei (*left,* first order; *right*, higher order). Abbreviations as in Fig. 1.

depolarization, 2 (13.3%) showed mixed responses, and 1 (6.7%) showed no response. The lateral geniculate nucleus (LGN) sample included 35 cells (92.1%) showing depolarization, three (7.9%) showing no response, and none showing mixed responses. Among the higher order nuclei (Table 1; Fig. 3*B, right*), relay cells in the posterior medial nucleus (POm) exhibited the highest proportion responding to MCh application (73 of 74; 98.7%), with 55 (74.3%) depolarizing, 16 (21.6%) hyperpolarizing, and 2 (2.7%) exhibiting mixed responses. The dorsal division of the medial geniculate body (MGBd) and the lateral posterior (LP) nucleus had more nonresponders. For the former cells, 23 of 65 (35.4%) were unresponsive, 8 (12.3%) showed hyperpolarizing responses, 34 (52.3%) showed depolarizing responses, and none showed mixed responses. Of the latter, 11 of 58 (19%) were unresponsive, 15 (25.8%) showed hyperpolarizing responses, 29 (50%) showed depolarizing responses, and 3 (5.2%) showed mixed responses. Statistical analysis shows that the number of hyperpolarizing cells is not significantly different across higher order relays ($P > 0.14$; χ^2 test). However, the number of nonresponsive cells is significantly higher in the dorsal portion of the medial geniculate body ($P \ll 0.01$ when compared with the posterior medial nucleus, and $P < 0.05$ when compared with the lateral posterior; χ^2 test) and in the lateral posterior nucleus ($P \ll 0.01$ when compared with the posterior medial; χ^2 test).

Further analysis of responses of relay cells to muscarinic activation

A number of experiments were done to characterize further the response to muscarinic activation. The number of cells from each nucleus used for each of the following tests is recorded in Table 2.

CHANGES IN INPUT RESISTANCE. During the current-clamp recordings, a short (400 ms), negative, current pulse of −10 pA was applied every 3 or 4 s to assess input resistance. In some cells where input resistance was high enough, these pulses were sufficiently long and hyperpolarizing to evoke rebound activation of I_T channels, sometimes resulting in low-threshold Ca^{2+} bursts; examples of this are shown during the last minutes of recording in Fig. 2*A*. To estimate input resistance changes evoked by muscarinic receptor activation and minimize any voltage-dependent effects, the membrane voltage was brought back to initial baseline level with appropriate DC current injection while the test pulses were applied (e.g., Fig. 4, *A* and *B*).

We observed an increase in input resistance in cells that were depolarized by muscarinic activation (as measured when the effect reached its peak) both in first and higher order relay cells. The increase in input resistance was quite variable with an average of 27.3% ($n = 8$) in first order (Fig. 4*C*, red filled circles) and 31.9% ($n = 15$) in higher order cells (Fig. 4*C* plot,

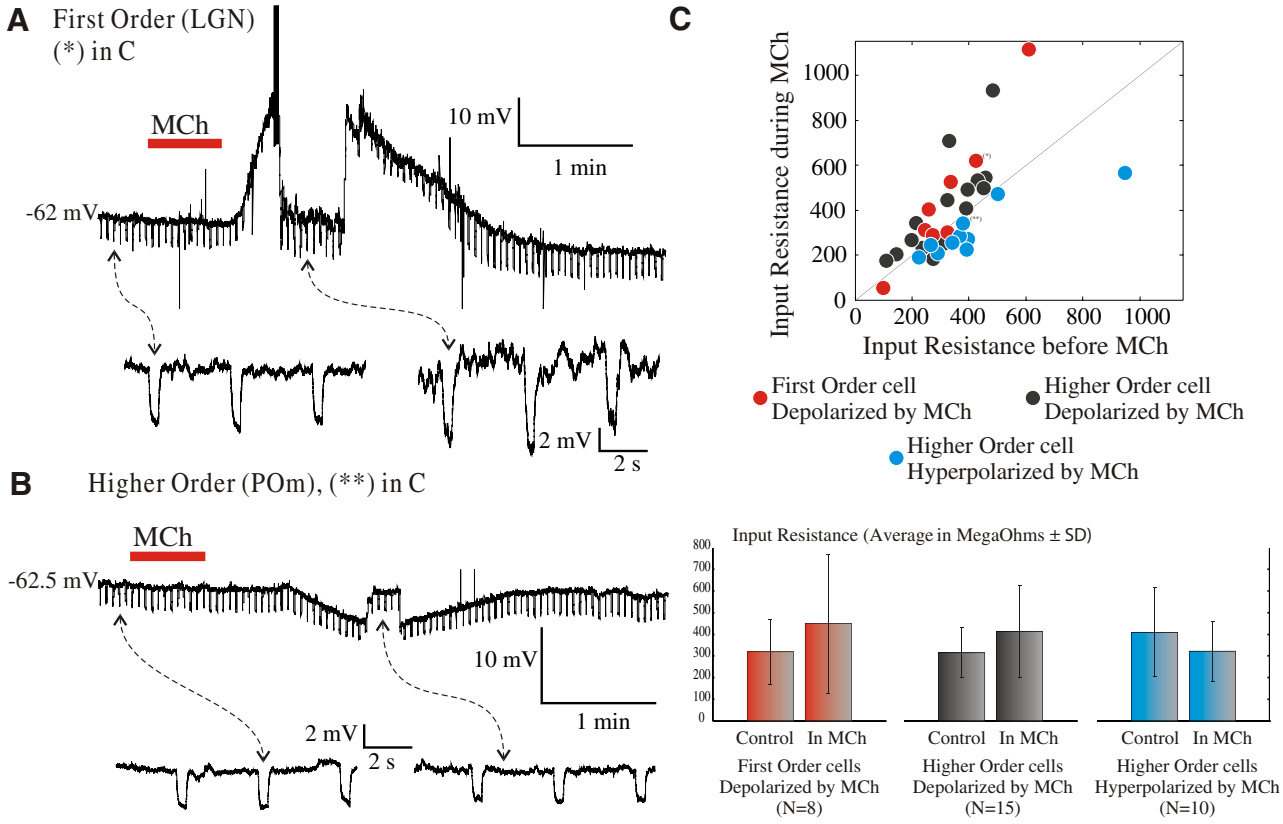


FIG. 4. Effects of MCh on input resistance. *A* and *B*: expanded traces below each main trace show the negative current pulses (−10 pA, 400 ms; 3–4 s between pulses) used to measure input resistance. DC current injection during the peak of the effect was manually adjusted to bring the potential back to the resting membrane potential to compare input resistance at the same voltage level. *C*: input resistance before and at the peak of the MCh effect for all the cells measured: red filled circles correspond to first order relay cells depolarized by MCh; black filled circles, to higher order relay cells depolarized by MCh; and blue filled circles, to higher order relay cells hyperpolarized by MCh. *Below*: histograms show average input resistance (±SD) before and during the effect of MCh for first order depolarizing (*left*), higher order depolarizing (*middle*), and higher order hyperpolarizing (*right*) cells.

black filled circles; see Table 2 for distribution of cells by nucleus) and the difference with the control input resistance was not significant for either group ($P > 0.19$ for higher order and $P > 0.38$ for first order, Mann-Whitney) However, during the hyperpolarizing response to the activation of muscarinic receptors in higher order relay cells, the input resistance was decreased an average of 20.1% ($n = 10$; Fig. 4*C* plot, blue filled circles), although the difference with control was not significant ($P > 0.12$, Mann-Whitney). These changes are in agreement with previously reported results showing that the depolarization is, at least partly, the effect of closing K^+ channels, thereby increasing input resistance (rat: Mooney et al. 2004; Zhu and Uhlrich 1998; guinea-pig and cat: McCormick and Prince 1987). Similarly, the decrease in input resistance during the hyperpolarization suggests that the mechanism could be the same as previously reported for interneurons, cells of the ventral portion of the medial geniculate body, and thalamic reticular nucleus cells: namely, the result of opening K^+ channels (McCormick and Pape 1988; McCormick and Prince 1986; Mooney et al. 2004).

Evidence that MCh effects are direct

As a control for the possibility that the effects noted in the preceding text from bath applied drugs could be presynaptic, either due to activation of presynaptic axons or terminals, we performed experiments in the presence of the sodium channel blocker, TTX (to block firing in presynaptic axons) or low-Ca^{2+} and high-Mg^{2+} (to eliminate any presynaptic contributions, see following text). TTX (1 μM) was added to the bath to block spike generation within the slice and therefore eliminate any contributions from active presynaptic axons. The results are shown in Fig. 5, and the subset of cells used in this control ($n = 12$) can be found in Table 2. Figure 5*A* shows a representative posterior medial relay cell, and as shown, TTX has only a small effect on the amplitude of the response to MCh application. Note that the sodium spikes are blocked in the presence of TTX as shown by the *insets* below the traces in Fig. 5*A*. The fact that the response remained with the same polarity in the presence of TTX indicates that it is not dependent on the activation of presynaptic axons, although such activation without TTX could contribute to the overall amplitude of the response. Results were similar for higher order relay cells hyperpolarized by MCh, as shown in Fig. 5*B*, which displays an example from the posterior medial nucleus. The results for all the cells tested in the presence of TTX are included in the plot of Fig. 5*C*. The black, filled circles represent depolarizing responses to MCh application and include five first order and five higher order cells, and the blue, filled circles represent two higher order cells that hyperpolarized to MCh application. On average, the maximum depolarizing response to MCh application was reduced by 11.5% in the presence of TTX for first order relay cells and by 7.5% for higher order cells. The higher order relay cells showing a hyperpolarization presented a response reduced by 2.2% when TTX was in the solution. Overall, the change in membrane potential evoked by MCh was not significantly different in control than in the presence of TTX ($P > 0.66$, Mann-Whitney).

TTX was also used in two of the cells presenting a mixed response (a cell from the ventral portion of the medial geniculate body and another from the lateral posterior; data not shown), and it almost completely blocked the hyperpolarization in the cell from the ventral medial geniculate body (without notably affecting the depolarization), whereas it did not affect either part of the response in the lateral posterior cell. This suggests that in at least some cells with a mixed response, the hyperpolarization could be caused by the MCh activating cells of the thalamic reticular nucleus or local interneurons that inhibit the recorded cell.

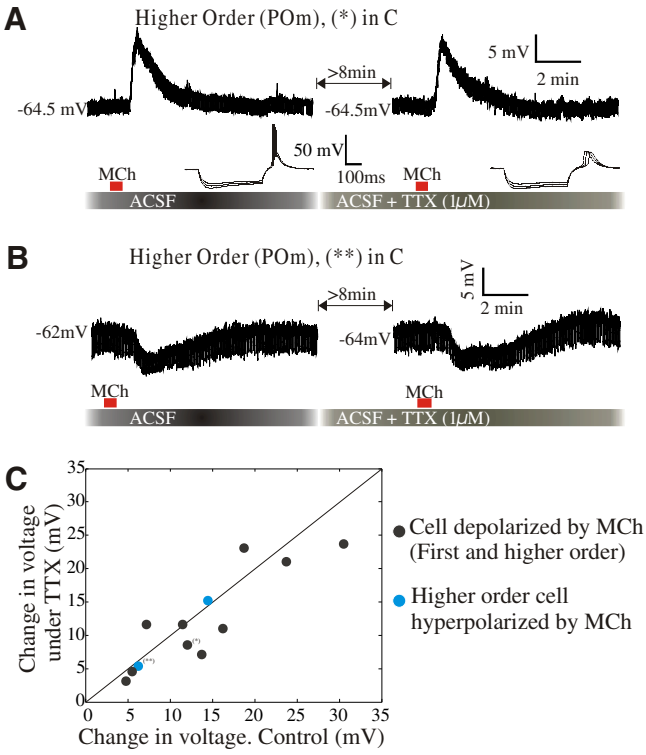


FIG. 5. Effects of TTX (1 μM) on MCh effect. *A*: TTX effect on the response of a higher order relay cell depolarized by MCh application. *B*: TTX effect on the response of a higher order relay cell hyperpolarized by MCh application. *C*: population results for the TTX experiments.

Because the use of TTX does not rule out the contribution of presynaptic receptors to the observed responses, we repeated the MCh application in the presence of a bathing solution (ACSF) containing low Ca^{2+} (0.5 mM) and high Mg^{2+} (8 mM) concentrations. Normal neurotransmitter release from nerve terminals requires extracellular Ca^{2+} (for a recent review, see Oheim et al. 2006), and it is antagonized by extracellular Mg^{2+} (Douglas 1968). It is also well known that by neutralizing the membrane negative surface charge, divalent cations can influence gating and permeation in most voltage-dependent channels (Piccolino and Pignatelli 1996). In particular, changing the extracellular Ca^{2+} concentration has effects on voltage-dependent channels and the threshold for electrical excitation of nerves (Frankenhaeuser and Hodgkin 1957). All divalent ions, including Mg^{2+}, have these effects (Piccolino and Pignatelli 1996). To block synaptic transmission without modifying excitability, we decreased the Ca^{2+} concentration of the ACSF and increased the Mg^{2+}. This allows us primarily to ensure that observed responses are all postsynaptic. Figure 6*A* illustrates that MCh application caused a depolarization for a first order relay cell in the ventral posterior nucleus that survived the blockade of synaptic transmission. A similar

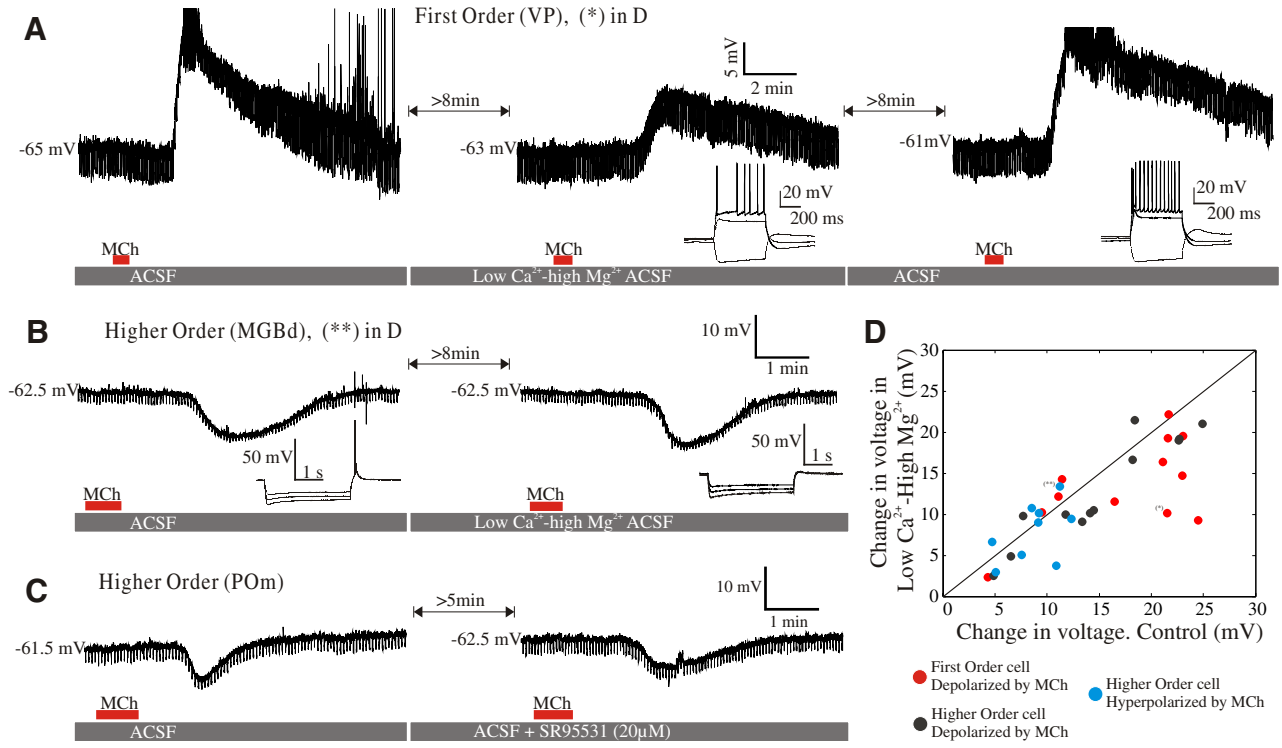


FIG. 6. Effects of synaptic release and $GABA_A$ blockade on MCh response. *A*: effect of low-Ca^{2+} (0.5 mM) high-Mg^{2+} (8 mM) artificial cerebrospinal fluid (ACSF) for a higher order relay cell depolarized by MCh. *B*: effect of low-Ca^{2+} (0.5 mM) high-Mg^{2+} (8 mM) ACSF for a higher order relay cell hyperpolarized by MCh. *C*: response to MCh in the presence of $GABA_A$ antagonist SR95531 (20 μM). *D*: population results for the low-Ca^{2+} high-Mg^{2+} experiments; red, first order cell; black, higher order cell depolarized by MCh; blue, higher order cell hyperpolarized by MCh.

control is illustrated in Fig. 6*B* for a higher order relay cell from the dorsal medial geniculate body for which the hyperpolarizing response to MCh also persisted after blockade of synaptic transmission. The *insets* below the hyperpolarizing traces in Fig. 6, *A* and *B,* show a control for the effect of the low-Ca^{2+} and high-Mg^{2+} solution (notice that the low-threshold calcium spikes are gone during the presence of Ca^{2+} and high Mg^{2+} as the bathing solution). Overall, this control was carried out with similar results for a total of 33 relay cells: 12 first order, 12 higher order relay cells with depolarizing responses to MCh, and 9 higher order with hyperpolarizing responses (see Table 2 for specific nuclei of origin), cells with mixed response were rare (3.9% of first order and 2.5% of higher order) and the control was not performed on this group. The difference between the response amplitude in control and in the presence of low-Ca^{2+} and high-Mg^{2+} solution was not significant for any of the groups ($P > 0.12$ for the first order group, $P > 0.47$ for the higher order depolarizing cells and $P > 0.68$ for the higher order hyperpolarizing cells; Mann-Whitney test). We conclude that most or all of the responses seen in our population of relay cells are caused by the postsynaptic activation of muscarinic receptors on the recorded relay cells although we cannot rule out presynaptic contributions.

Figure 6*D* plots the results of the low-Ca^{2+}-high Mg^{2+} controls for all cells tested. The effect (measured as the maximum change in membrane potential) of MCh is plotted with and without low Ca^{2+} high Mg^{2+} (red filled circles correspond to first order cells, black to higher order cells depolarized by MCh, and blue to higher order cells hyperpolarized by MCh). The average decrease in the low-Ca^{2+} and high-Mg^{2+} control was 17.9% for the first order cells ($n = 12$) and 14.6% for the higher order cells depolarized by MCh ($n = 12$). The decrease was 6.3% for nine higher order cells that hyperpolarized to MCh application. Although we cannot unambiguously explain the reduction in the response under the presence of the low-Ca^{2+} and high-Mg^{2+} solution, an obvious conclusion is that some of the effects are presynaptic (e.g., affecting presynaptic terminals or activating afferent axons). Nonetheless the point remains that the surviving effects of cholinergic activation demonstrate a postsynaptic action of this application.

Finally, as a complement to the low-Ca^{2+} and high-Mg^{2+} control, we tested the effect of the $GABA_A$ antagonist SR95531 (20 μM) in two higher order relay cells hyperpolarized by MCh; their responses remained virtually unchanged in the presence of the blocker, suggesting that there are no GABAergic presynaptic contributions to these hyperpolarizing responses (Fig. 6*C*).

DISCUSSION

Our results indicate that first and higher order thalamic nuclei are affected differently by cholinergic input. All of the relay cells in first order nuclei are depolarized by activation of muscarinic receptors, and although depolarization is the most prevalent response, a significant subset of higher order relay cells are hyperpolarized by muscarinic receptor activation: 19.8% of all recorded higher order neurons were hyperpolarized. Our data extend conclusions from previous results showing that although neurons of the ventral portion of the medial geniculate body (a first order relay) are depolarized by muscarinic activation, most neurons of the dorsal portion of the

medial geniculate body (a higher order relay) are hyperpolarized by muscarinic activation (Mooney et al. 2004).

Other differences between first and higher order thalamic relays

The different effects of muscarinic activation in first and higher order relay cells should be viewed in the context of a growing body of evidence for other differences. The distinction between first and higher order thalamic nuclei was first proposed (Guillery 1995) based on an anatomical criterion, namely, that a first order relay received its basic information (or driver input) from a subcortical source (e.g., retinal input to the lateral geniculate nucleus), whereas a higher order relay received such input from layer 5 of a cortical area (e.g., layer 5 input from primary visual cortex to the pulvinar). Other differences have since been documented. Inhibitory inputs from the zona incerta and the anterior pretectal nucleus selectively target higher order relays (Barthó et al. 2002; Bokor et al. 2005). Likewise, dopaminergic inputs appear to target higher order relays with relative selectivity (Sánchez-González et al. 2005). The relative proportion of modulatory synapses is significantly greater in higher order relays (Van Horn and Sherman 2007; Wang et al. 2002). In addition, the hyperpolarization from muscarinic activation may bias higher order relays to fire in burst mode, whereas the depolarizing effects on first order relays may bias these neurons to fire in tonic mode, and there is evidence from behaving monkeys that indicates that higher order relay cells fire in burst mode much more frequently than do first order relay cells (Ramcharan et al. 2005).

Direct effect of cholinergic activation

The known inputs to thalamic relay cells include glutamatergic input from cortex, local GABAergic input (from thalamic reticular cells and interneurons), and various inputs from brain stem (reviewed in Sherman and Guillery 2006). We must thus consider the possibility that the responses we observed could be completely explained by cholinergic effects on afferents to relay cells. From a logical point of view, possible presynaptic effects on many of the cell groups, including cortical and brain stem, can be ignored because these cells are not included in our slice; similarly, there are essentially no interneurons to innervate relay cells of our sample outside of the lateral geniculate nucleus (Arcelli et al. 1997). Nonetheless the possibility does exist that ACh can affect presynaptic terminals, and this might apply to any synapses onto recorded relay cells even if the cell bodies giving rise to these synapses are eliminated in the slice. In any case, the results of the experiments with TTX and low Ca^{2+} with high-Mg^{2+} ACSF indicate that the effects of cholinergic activation are mostly evoked by activation of receptors on the relay cells. The participation of some presynaptic receptors is suggested by the fact that the responses to muscarinic activation (both de- and hyperpolarization) are decreased but not completely eliminated in the presence of low-Ca^{2+} and high-Mg^{2+} ACSF. Another possibility is that Ca^{2+} contributes to the current activated by muscarinic receptors (e.g., through I_h channels) (Zhu and Heggelund 2001; Zhu and Uhlrich 1998), explaining why the responses are smaller when the extracellular Ca^{2+} concentration is decreased. Further experiments would be needed to clarify the role of Ca^{2+} and presynaptic activation of cholinergic receptors.

Functional considerations

CLASSIFICATION OF HIGHER ORDER RELAY CELLS. The results raise questions about the higher order cells that hyperpolarize in response to muscarinic activation. Do these cells represent a unique class of relay cell? That is, do these cells differ from others in morphology, afferents, projections targets, and/or intrinsic properties? So far, we have not found any morphological or electrophysiological properties correlated to the hyperpolarizing responses in these cells, but a broader analysis might be required to address this question.

Another interesting possibility requires further study. That is, thalamic relay cells are thought to be depolarized in response to cholinergic activation via two complementary mechanisms: direct depolarization and an indirect process via cholinergic inhibition of inhibitory inputs from the thalamic reticular nucleus and, where present, interneurons (McCormick and Pape 1988; McCormick and Prince 1986). If such a complementary, cooperative pattern is a general property of relay cells, it would also apply to higher order hyperpolarizing cells, suggesting the possibility that cholinergic afferents depolarize the subset of reticular cells or interneurons that innervate higher order relay cells that, in turn, are directly hyperpolarized by such cholinergic input, thereby contributing to further inhibition of these relay cells.

RELATIONSHIP TO FIRING MODE. Thalamic relay cells can respond to incoming input in one of two modes depending on the activation state of an inward voltage-dependent Ca^{2+} current (I_T). If these cells are relatively depolarized beyond roughly -60 mV, I_T is inactivated and the cell responds in *tonic mode*: the response is a train of unitary action potentials, the number of which increases fairly linearly with increasing input amplitude. If these cells are hyperpolarized beyond roughly -65 mV, I_T is deinactivated, and the cell will respond in *burst mode*: the response is a characteristic burst of action potentials, providing a highly nonlinear input/output relationship (Sherman 2001; Smith et al. 2000). However, the pattern of firing in burst mode provides an improved signal-to-noise ratio thought to underlie better detectability (Guido et al. 1995; Reinagel et al. 1999; Sherman 2001), and it also is especially effective at activating postsynaptic cortical cells (Swadlow and Gusev 2001). Together these properties have led to the hypothesis that tonic firing is better suited to a more accurate processing of information during attentive states, whereas burst firing may be largely used as a sort of "wake-up call" during less alert states, signaling a significant change in the incoming signal to be relayed.

The voltage dependency of I_T means that the de- or hyperpolarizing effects of modulatory inputs, such as cholinergic inputs, can strongly influence response mode. Activity in cholinergic brain stem nuclei that innervate thalamus increases monotonically with more awake and alert behavioral states (Kayama et al. 1992; Steriade and McCarley 1990). Our data predict that the waking state, although leading to depolarization and thus promoting tonic firing for most thalamic relay cells, will lead to hyperpolarization and thus burst firing in a

significant subset of higher order relay cells. This in fact, has been observed in monkeys: a higher proportion of bursting has been found in recordings of spontaneous activity of cells in several higher order thalamic nuclei in the awake monkey (Ramcharan et al., 2005). This may be especially interesting given that higher order thalamic relays have been proposed to be a key link in cortico-thalamo-cortical processing of information.

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