In vitro properties of neurons in the rat pretectal nucleus of the optic tract

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Abstract

The nucleus of the optic tract (NOT) has been implicated in the initiation of the optokinetic reflex (OKR) and in the modulation of visual activity during saccades. The present experiments demonstrate that these two functions are served by separate cell populations that can be distinguished by differences in both their cellular physiology and their efferent projections. We compared the response properties of NOT cells in rats using target directed whole-cell patch clamp recording in vitro. To identify the cells at the time of the recording experiments, they were pre-labeled by retrograde axonal transport of WGA-apo-HRP-gold (15 nm), which was injected into their primary projection targets, either the ipsilateral superior colliculus (iSC), or the contralateral NOT (cNOT) or the ipsilateral inferior olive (iIO). Retrograde labeling following injections in single animals of either WGA-apo-HRP-gold with different particle sizes (10 nm and 20 nm) or two different fluorescent dyes distinguished two NOT cell populations. One projects to both the iSC and cNOT. These cells are spontaneously active in vitro and respond to intracellular depolarizations with temporally regular tonic firing. The other population projects to the iIO and consists of cells that show no spontaneous activity, respond phasically to intracellular depolarization, and show irregular firing patterns. We propose that the spontaneously active pathway to iSC and cNOT is involved in modulating the level of visual activity during saccades and that the phasically active pathway to iIO provides a short latency relay from the retina to premotor mechanisms involved in reducing retinal slip.

Keywords: visual system, saccade, spontaneous activity, retrograde tracing, target-directed recording
**Introduction**

The nucleus of the optic tract (NOT) comprises a small and inconspicuous population of neurons intercalated among optic tract fibers passing through the pretectum. Considering its small size and apparent lack of distinct nuclear subdivisions, NOT has been associated with a surprising variety of functions that appear remarkably conserved across mammalian species. The most extensively studied function of NOT is its role in the control of compensatory eye movements during the horizontal optokinetic reflex (OKR; marsupials, Ibbotson et al. 1994; Volchan et al. 1996; rat, Benassi et al. 1991; Cazin et al. 1984; rabbit, Collewijn 1975a; cat, Hoffmann and Fischer 2001; monkey, Kato et al. 1986; Mustari and Fuchs, 1990; Schiff et al. 1988, 1990). In addition, NOT cells can modulate thalamocortical activity (cat, Fischer et al. 1998; Sudkamp and Schmidt, 1995; monkey, Bickford et al 2000; Wilson et al 1995) and provide inhibition to visuosensory neurons in the superior colliculus (Baldauf et al. 2003; Born and Schmidt 2004).

The efferent connections of NOT are correspondingly diverse (reviewed in Gamlin 2005; Ibbotson and Dreher, 2005; Simpson et al. 1988). They include pathways to ipsilateral visuosensory thalamic nuclei, including the dorsal lateral geniculate nucleus, the lateral posterior thalamic nucleus and the pulvinar, to the ipsilateral superior colliculus (iSC), to the ipsilateral inferior olive (iIO) and nucleus prepositus hypoglossi (iNPH), and to the contralateral NOT (cNOT). The pathway from the NOT to the thalamus seems to carry signals related to ongoing saccades, because NOT cells that project to either the dorsal lateral geniculate nucleus or the lateralis posterior/pulvinar complex *in vivo* respond to saccade-induced retinal image shifts 30 to 50ms after saccade onset (cat, Fischer et al. 1998; Schmidt 1996; Sudkamp and Schmidt 1995; monkey, Fuchs et al. 1992). In contrast, the type of information relayed to iSC and to cNOT remains to be determined, partly because cNOT
projecting cells seem unresponsive in anesthetized animals (Schmidt et al. 1995). Finally, the 
preoculomotor structures iIO and iNPH receive NOT signals that are related to the control of 
slow phase eye movements during the OKR (Büttner-Ennever et al. 1996, Magnin et al. 1989; 
Schmidt et al. 1995). In particular, NOT cells that project to iIO and iNPH generate 
directionally selective responses to low velocity global retinal image movements from 
temporal to nasal in the contralateral hemifield (for review, see Gamlin 2005; Ibbotson and 
Dreher 2005; Simpson et al. 1988).

Although a common property of NOT cells is that they preferentially respond to 
moving rather than to stationary visual stimuli, significant differences occur in their preferred 
stimulus velocity range. OKR-related neurons prefer stimulus motion below 100°/s 
(marsupials, Ibbotson et al. 1994; Volchan et al. 1989; rat, Cazin et al. 1980; Schmidt et al. 
1993; rabbit, Collewijn 1975b; ferret, Klauer et al. 1990; cat, Hoffmann and Schopmann 
1981; monkey, Ilg and Hoffmann 1996; Mustari and Fuchs, 1988) while other neurons are 
only responsive to much faster stimulus velocities (marsupials, Ibbotson et al. 1994; Price and 
Ibbotson 2001; cat, Missal et al. 2002; Schweigart and Hoffmann 1992; Sudkamp and 
Schmidt 1995; monkey, Inoue et al. 2000). In addition, in monkey neurons have been 
described whose activity is completely blocked by a saccadic eye movement (Mustari et al. 
1997).

Thus, the known response properties in vivo suggest that, even though NOT cells as a 
group prefer moving visual stimuli, some selectively respond to high velocity movements and 
others to slow movements. However, with currently available in vivo methods, it has been 
difficult to determine with certainty whether NOT makes a similar contribution to each of its 
diverse targets or, instead, it consists of several distinct cell populations, each with its own 
function and pattern of connections. In the present experiments, we addressed these questions 
in rats by performing in vitro whole-cell patch-clamp recordings on NOT cells that were pre-
labeled by retrograde axonal transport following injections of neuronal tracers in the
destinations of their efferent projections. This approach allowed direct comparisons of the
intracellular properties of pretectal cell populations with their identified projection targets.
The results demonstrate that NOT includes several cell populations, which can be
distinguished both by their intrinsic membrane properties and by their efferent connections.
Material and Methods

Retrograde labeling

Experiments were performed on 27 Long Evans hooded rats between 16 and 32 days postnatal age. Experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Duke University Institutional Animal Care and Use Committee. For stereotaxic surgery, the animals were deeply anesthetized by an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine hydrochloride (1 mg/kg), and the level of anesthesia was maintained by additional injections of ketamine. Using stereotaxic coordinates, the terminal zones of NOT neurons were injected with 0.5 μl of the retrograde axonal tracer wheat germ agglutinin (WGA)-apo-HRP-gold (15 nm particle size; Sanbio, Beutelsbach, Germany, and E-Y Laboratories, San Mateo, CA). As a cell label, the (WGA)-apo-HRP-gold has the advantage over fluorescent labels of being resistant to fading, nontoxic to the neurons and visible under bright field illumination (Lee et al. 2001). In some experiments, two injections of different size gold particles (10 and 20 nm), each in a different site, were made in single animals to determine if one cell type projects to both sites. After surgery, animals were allowed to recover and survive for a period of 5-7 days, which is sufficient for retrograde axonal transport of the tracer (Lee et al. 2001). Although the particle clusters that appear in labeled cells are of much larger size than the gold particles, control tracer injections with a single particle size revealed that smaller particles lead to considerably smaller clusters than do the larger particles (Fig. 1). This allows a clear distinction between label from different particle sizes in double-label experiments.

The results obtained with WGA-apo-HRP-gold were confirmed using double injections of fluorescent tracers. We used red textmarker dye ("Stabilo Red", Stabilo, Heroldsberg,
Germany,) for cNOT and iIO injections and green textmarker dye (“Stabilo Green”) for injections into the iSC.

**Slice preparation for patch clamp recording**

Following injection of an overdose of ketamine and xylazine hydrochloride, animals were transcardially perfused with ice cold artificial cerebrospinal fluid (ACSF) containing the following components (in mM): NaCl, 124; KCl, 5; NaH$_2$PO$_4$, 1.25; NaHCO$_3$, 26; MgSO$_4$, 2; CaCl$_2$, 2 and glucose, 10. The ACSF was continuously gassed with carbogen (5% CO$_2$/95% O$_2$). After removal of the brain from the skull, 350 μm-thick coronal slices were cut and kept at 35°C in ACSF for at least 1h to allow the tissue to recover from the slicing procedure. For whole-cell patch clamp recording they were transferred to a submersion type recording chamber and superfused at 3 ml/min with room temperature ACSF.

**Whole-cell patch clamp recording**

Whole-cell patch clamp recordings from WGA-apo-HRP-gold labeled neurons in the NOT were performed on a fixed stage microscope (Olympus BX51WI) under visual control with a 40x long working distance objective, using infrared differential interference microscopy (Dodt and Zieggläsnsberger 1998). For recording, borosilicate glass micropipettes (impedance 5-8 MΩ) were filled with internal solution containing (in mM): potassium gluconate, 130; sodium gluconate, 2; HEPES, 20; MgCl$_2$, 4; Na2ATP, 4; NaGTP, 0.4 and EGTA, 0.5. To confirm that the recordings were made from the pre-labeled cell and also for later morphological characterization of the cell, 0.5% biocytin was added to the pipette solution. The biocytin diffused into the cell during the recording and was later reacted with 3’3’-diaminobenzidine (DAB), without heavy metal intensification. Neuronal signals were amplified and filtered by an EPC 9 amplifier (HEKA, Lambrecht, Germany), digitized at 10
kHz and displayed, stored and analyzed using Pulse/Pulsefit software (HEKA). Measured membrane potentials were corrected for the junction potential of -10 mV.

**Anatomical analyses**

Twelve additional animals were used to examine the efferent connections of NOT using retrograde tracers. After a survival period of 5-7 days for retrograde tracer uptake and axonal transport, animals were injected with an overdose of ketamine hydrochloride and xylazine, and perfused transcardially with ACSF mixed with 0.1% heparin at room temperature. ACSF was followed by 4.0% paraformaldehyde in 0.1 M phosphate buffer and a rinse in 10% sucrose in 0.1 M phosphate buffer. Brains were removed from the skull and then stored in 30% sucrose buffer over night at 4°C for cryoprotection. The brains with injections of WGA-apo-HRP-gold were cut with a freezing microtome into 40 µm thick coronal slices through both the pretectum in its entire anterior-posterior extent and the tracer injection sites. Sections were collected in 0.1 M phosphate buffered saline, put on gelatin-coated slides and stained with 0.03% cresyl violet. After injections of fluorescent dyes, the same procedure was performed, except the sucrose rinses were omitted and 40 µm thick coronal slices were cut using a vibratome. Then, the brain slices were coverslipped in 0.01 M phosphate buffered saline containing 1% glycerol to avoid drying. Injection sites and labeled somata were identified and photographed with an oil immersion objective lens (60x) on a light microscope. Soma sizes of labeled neurons as well as sizes of gold particles were analyzed using a computer-aided reconstruction system (Neurolucida, MicroBrightField, Williston, VT). All chemicals used were obtained from Sigma-Aldrich (Deisenhofen, Germany).
Results

Two populations of NOT cells were distinguished in these experiments. One population projects to the iIO whereas the other projects to both the iSC and the cNOT. Whole-cell patch clamp recordings from cells that were identified by their efferent projections confirmed that these are two distinct cell populations; the cells that project to iSC and cNOT are tonically active in vitro whereas the cells that project to iIO generate phasic responses.

Tonically active cells

All of the clamped cells that projected to the iSC were tonically active (n = 15). For example, Figure 2 illustrates the results from one cell that was pre-labeled by an injection of WGA-apo-HRP-gold into the iSC (0.5 μl, 15 nm gold particles). The arrows in Figure 2A indicate gold particles that were transported to the cell soma from the injection site. The homogeneous background in the soma and primary dendrite (asterisk) was produced by the biocytin that diffused into the cell from the patch pipette during the experiment, and confirmed that the recordings were obtained from the pre-labeled cell. Figure 2B shows, in a current clamp recording, that action potentials evoked by current injections were sustained for the entire duration of the depolarizations (500 ms) and varied in frequency with the injected current or membrane potential. Figure 2C illustrates that action potential frequency increased with stepwise increases in the injected current. Figure 2D plots the relationship between the response frequency and the membrane potential; the action potentials increased in frequency with the amount of depolarizing current from less than 5 spikes/s when the cell was near its resting potential (-61.3 mV) to a peak of approximately 27 spikes/s when the membrane potential approached -25 mV. The mean input resistance of the tonically active cells was 323.7 MΩ (SD = 119.7), the mean time constant τ, which was conventionally calculated from
the onset of long lasting hyperpolarizing voltage steps, was 0.93 ms (SD = 1.09) and the mean resting potential was -55.1 mV (SD = 3.4). The membrane potential threshold for the generation of action potentials, defined as the potential at which the first spike appeared when the cell was continuously depolarized from -90 mV by increasing the amplitude of the current injections, was -59.6 mV (SD = 3.8).

Figure 3 illustrates the results from an experiment on a cell pre-labeled following an injection of WGA-apo-HRP-gold in cNOT (Fig. 3A). Similar to the NOT-iSC cell illustrated in Figure 1, the cell in Figure 2 generated sustained action potentials that varied in frequency with the amplitude of the injected current in a current clamp recording (Fig. 3B). Figure 3C shows trains of action potentials (2 and 18 spikes/s, respectively) that were generated when the cell was clamped at a membrane potential of -60 mV (left) or -40 mV (right). Similar results were obtained for all cells that projected to cNOT (n = 8). The mean input resistance of NOT-cNOT cells, R_{\text{mem}}\text{, was 429.5 M}\Omega (SD = 106.0), the mean time constant, \( \tau \) was 1.81 ms (SD = 2.96), the mean resting potential was -53.5 mV (SD = 3.6), and the mean spike threshold was -56.6 mV (SD = 2.4). These parameters were not significantly different from those of the cells that projected to the iSC (p = 0.84 for membrane resistances, p = 0.35 for time constants, and p = 0.45 for resting potentials, p = 0.15 for spike thresholds, t-test).

To determine whether the NOT cells with \textit{in vitro} tonic firing patterns comprise a single population that projects to both iSC and cNOT, single animals (n = 3) were injected with 20 nm-sized gold particles in iSC and 10 nm-sized particles in cNOT (Fig. 4A). Although the size of gold particles themselves are below the resolution limit of the light microscope, the two tracers can be distinguished by the size of the aggregates that occur in the labeled cells. Aggregates in iSC, where 20 nm-sized gold particles had been injected, had diameters above 2.5 \( \mu \text{m} \) (mean size 3.0 \( \mu \text{m} \), SD = 0.3), while aggregates of the 10 nm particles in iIO were below 2.5 \( \mu \text{m} \) in diameter (mean 1.8 \( \mu \text{m} \), SD = 0.2), which was significantly smaller
The distribution of retrogradely labeled cells within the NOT in one representative midbrain section is depicted in Figure 4B. Of the cells labeled from iSC (n = 267), 76% were double labeled, of the cells labeled from cNOT (n = 226) 90% were double labeled. Thus, the majority of labeled cells contained both tracers indicating that they projected to both targets. Figure 3C illustrates a cluster of 5 cells in rostral NOT, each of which contained both sizes of gold particles, confirming that single NOT cells project to both targets. The same conclusion was reached in the experiments illustrated in Figure 5. In this case, the retrograde fluorescent tracer Stabilo Red was injected into the cNOT and Stabilo Green was injected into the iSC, and clusters of cells with both labels (arrows) were present in NOT.

We also compared soma sizes of single labeled and double labeled cells. The average soma area of NOT neurons labeled only from iSC was 191.0 μm² (SD = 44.1), that of cells labeled only from cNOT was 212.3 μm² (SD = 46.1). Double labeled cells had a mean soma area of 221.7 μm² (SD = 48.3). These differences were not statistically significant.

**Phasic cells**

Neurons pre-labeled with WGA-apo-HRP-gold following injections in iIO (0.1 μl, 15 nm-sized particles) generated phasic or phasic-tonic, fast adapting or bursting firing patterns in response to intracellular current injection, and never showed spontaneous activity at resting potential. An example is shown in Figure 6. Figure 6A shows a pre-labeled cell containing the gold particles (arrows) and the homogeneously distributed biocytin (asterisk). Figure 6B is a current clamp recording showing that the cell generated a phasic-tonic firing pattern in response to depolarizing current injections leading to irregular maintained firing when higher depolarizing currents were injected. In general, NOT neurons pre-labeled from the iIO showed less uniform responses to intracellular depolarizations than neurons pre-labeled from
either iSC or cNOT. Figure 6C shows the irregular firing pattern of another pre-labeled cell when injected currents induced small depolarizing steps of the membrane potential. In Figure 6D, the response frequency is plotted against the membrane potential; the average firing rate increased with the amount of depolarizing current from less than 5 spikes/s at -40 mV to a peak of approximately 30 spikes/s at -25 mV. More positive membrane potentials resulted in a reduced firing rate and no spikes occurred above -15 mV.

The mean input resistance of the cells that projected to iIO neurons (n = 17) was 200.7 MΩ (SD = 59.4), the mean time constant \( \tau \) was 3.07 ms (SD = 2.28), the mean resting potential was -57.9 mV (SD = 4.9), and the spike threshold was -39.9 mV (SD = 7.9). In comparison to the tonic cells that projected to iSC and cNOT, the phasic iIO-projecting cells were characterized by significantly lower input resistances (\( p = 0.00012; \) t-test), larger time constants (\( p = 0.0122 \)), more negative resting potentials (\( p = 0.015 \)), and, in particular, significantly more positive spike thresholds (\( p < 0.0001 \)).

A striking difference between iSC/cNOT- and iIO-projecting NOT neurons \textit{in vitro} concerned the regularity of their ongoing firing. In order to analyze the temporal firing pattern, interspike intervals (ISI) were calculated from the ongoing firing of spontaneously active cells and from activity of non-spontaneously active cells during maintained intracellular depolarization over a 10 s period. ISIs of NOT neurons that project to iSC and cNOT (Fig. 7 A, D, G) form a narrow unimodal Gaussian distributions with only a little variation (Fig. 7 B, E, H), indicating a high degree of equally sized ISIs. The temporal regularity of the spontaneous firing was easily detectable in autocorrelograms of the activity where multiple peaks appeared at constant time intervals (Fig. 7 C, F, I). This distribution was in contrast to the ISI distributions of iIO projecting NOT neurons (Fig. 7 J, M, P). The interval histograms showed a much less symmetrical distribution (Fig. 7 K, N, Q). Furthermore, the lack of regularity in firing can be derived from the autocorrelograms which showed no distinct peaks (Fig. 7 I, L, R).
To further confirm that the phasic NOT neurons comprise a population that is distinct from
the tonically active cells, 10 nm gold particles were injected in the iIO and 20 nm particles
were injected in iSC (Fig. 8A). In 3 experiments, no cells were found that contained both
labels. This result is shown in Figure 8B where the distribution of retrogradely labeled cells is
shown in a representative midbrain section. Although the labeled cells were intermingled
throughout NOT, none of the cells (n = 90 and n = 60, labeled from iSC and iIO, respectively)
was double labeled. Similar results were obtained when Stabilo green tracer was injected in
iSC and Stabilo red in iIO, that is, no cells contained both tracers (Fig. 9A-D, n = 2).

Comparisons of the soma sizes of labeled cells revealed that the average soma area of NOT
neurons labeled from iSC was 227.4 μm² (SD = 49.9), while that of cells labeled from iIO
was 154.3 μm² (SD = 40.2). Thus, iIO-projecting NOT cells had significantly smaller soma
areas than iSC-projecting cells (p < 0.001). When comparing soma shapes (Figs. 8B and 9) it
appeared that iSC-projecting cells had spherical cell bodies while somata of iIO-projecting
cells were more elongated, particularly along the medio-lateral axis.
Discussion

We examined the in vitro activity patterns of neurons in the NOT of the rat pretectal nuclear complex that were distinguished by differences in the destinations of their axons. Specifically, we examined NOT neurons that project to one or more of three structures, the iIO, the iSC, and the cNOT. These NOT cells could be distinguished by their responses to intracellular depolarization: one group responded with a transient fast adapting pattern of activity or burst firing while a second group responded tonically with only minor adaptation. The latter neurons also were spontaneously active when clamped at their resting potential, which was a unique feature of this population. These two response patterns were clearly associated with the connections of the cells; the neurons that projected to iIO responded transiently while those that projected to iSC and cNOT were tonically active. This distinction between NOT cells that project to iSC and cNOT and those that project to iIO was confirmed by anatomical experiments. That is, injections of different retrograde axonal tracers into cNOT and iSC in single animals produced NOT cells with both labels, indicating that at least some NOT cells project to both structures. In contrast, following similar injections into either iIO and iSC or iIO and cNOT, individual NOT cells contained only one of the two labels, indicating that the cells that project to iIO are distinct from those that project to iSC and cNOT. These results demonstrate that NOT comprises at least two cell populations and that these populations differ in both their firing properties and their patterns of connections.

NOT cell populations

Data in the literature on NOT anatomy and physiology obtained from a variety of mammalian species demonstrate that cell properties are very similar across different mammals (Gamlin...
2005; Ibbotson and Dreher 2005; Simpson et al. 1988). Therefore, it seems reasonable to
generalize results between mammalian species.

Previous *in vitro* experiments in the rat demonstrated differences in the responses of NOT
cells to intracellular depolarization (Prochnow and Schmidt 2004). One population of cells
was characterized by tonic firing to intracellular depolarizations, an almost linear relationship
between membrane potential and firing rate, and the generation of spontaneous activity. The
activity pattern is characterized by a high degree of regularity leading to periodic peaks in
activity autocorrelograms. The association of these properties was confirmed in the present
study. Although we did not specifically test for an autonomous generation of spontaneous
activity, all cells that generated spontaneous activity at their resting membrane potential also
responded with tonic firing to intracellular depolarizations. Furthermore, the spike frequency
of the cells in this population was a linear function of the membrane potential and the
spontaneous firing showed strong periodicity. Because these properties always appeared
together in individual neurons, we conclude that they define a distinct population of NOT
cells. The experiments with two retrograde tracers demonstrated that at least some of the cells
in this population project to both iSC and cNOT. In contrast, NOT cells that project to iIO
must be regarded as a separate NOT cell population, as can be derived from fundamental
differences in their temporal activity pattern, which lacks any periodicity. This conclusion is
consistent with earlier results from cat and rat showing NOT cells that project to iIO send
collaterals to the iNPH but not to the cNOT (Schmidt et al. 1995). Furthermore, NOT cells
that project to iSC have been characterized as being GABAergic (rat, Baldauf et al. 2003;
Born and Schmidt 2004; rabbit, Nunes Cardozo et al. 1994). In contrast, NOT cells that
project to iIO are not GABAergic (cat, Horn and Hoffmann 1987) and show
immunoreactivity for glutamate (rat, Lewald et al. 1994).
A comparison of electrophysiological properties reveals that NOT cells which project to iSC and cNOT have higher input resistances, more positive resting potentials, and more negative spike thresholds than do cells that project to iIO. This constellation of properties is similar to the properties of spontaneously active NOT cells in an earlier study (Prochnow and Schmidt 2004), which also showed higher input resistance, more positive resting potentials, and lower spike thresholds than did NOT cells with phasic firing characteristics. Because the differences in firing were still present when the cells were pharmacologically isolated from synaptic input, by a substitution of calcium in the extracellular solution with cobalt (Prochnow and Schmidt 2004), they must result from differences in intrinsic physiological properties of the two cell populations.

While both response properties and efferent connections distinguish between these two populations, other common criteria for differentiating between cell groups, such as differences in spatial distribution and morphology, do not appear to apply to NOT. That is, the cells labeled by transport from iIO, iSC and cNOT were intermingled at similar topographical locations within the NOT. Furthermore, the morphology of the cells revealed by the biocytin label after recording did not reliably distinguish the two populations upon a single-cell analysis. This result agrees with our earlier observation that spontaneously active NOT cells with tonic response patterns show dendritic morphologies similar to cells with phasic response characteristics (Prochnow and Schmidt 2004).

While morphological comparisons of the biocytin-filled cells revealed no obvious differences, our results from the tracing experiments indicate that iIO-projecting cells on average have smaller somata than cells that project to iSC and cNOT. While this might seem contradictory to results from the biocytin-filled cells, the soma sizes of the recorded cells may be biased towards large cells because they are easier to detect and to patch-clamp than are small cells. Thus, because there is overlap of the soma sizes between NOT cells that project to cNOT and
iSC and cells that project to iIO, we probably have primarily recorded from large iIO-projecting cells. On average, NOT cells labeled from iSC and cNOT had soma sizes which are comparable to those of spontaneously active cells recorded from in a previous study (Prochnow and Schmidt 2004).

**NOT cells involved in the optokinetic reflex**

A priori, one might predict that the spontaneously active cells with tonic firing patterns are involved in the generation of slow compensatory eye movements during the horizontal optokinetic reflex. Indeed, *in vivo* the NOT cells that serve this function generate tonic responses to large moving visual stimuli as do functionally related cells in the adjacent dorsal terminal nucleus (DTN) of the AOS (Cazin et al. 1980; for review, see Gamlin 2005; Ibbotson and Dreher 2005; Simpson et al. 1988). Because neurons in the left NOT-DTN generate eye movements only to the left and neurons in the right NOT-DTN generate movements only to the right (Cazin et al. 1980; Collewijn 1975b; Hoffmann and Schoppmann 1981; Mustari and Fuchs 1990; for review see Gamlin 2005; Ibbotson and Dreher 2005), spontaneous activity may stabilize the eyes by maintaining an activity balance between the right and left NOT-DTN in the absence of appropriate visual stimuli. However, the NOT-DTN neurons involved in horizontal OKR generation project to the iIO (Gamlin 2005; Ibbotson and Dreher 2005; Simpson et al. 1988) and in our *in vitro* experiments none of the cells that projected to iIO was spontaneously active. Instead, all NOT neurons that projected to iIO showed phasic responses to intracellular depolarization and we assume similar responses are characteristic of DTN cells.

How might this *in vitro* response property be related to the known function of OKR- NOT neurons? In general, phasic responses allow rapid transmission of afferent spikes with high temporal precision. For the NOT cells that project to iIO, the main driving input arises from...
retinal ganglion cells (rat, Kato et al. 1992; rabbit, Pu and Amthor 1990; ferret, Klauer et al. 1990; cat, Hoffmann and Schoppmann, 1981; Koontz et al. 1985; monkey, Perry and Cowey 1984). In cat, retinal afferents have been classified as directionally selective ganglion cells that respond tonically to visual stimuli that move slowly across the visual field (Hoffmann and Stone 1985). Because the directionally selective ganglion cells are tuned to stimulus velocity (rabbit: Oyster 1968; Oyster et al. 1972) changing the firing rate at the retinal ganglion cell-to-NOT cell relay might confound the information necessary to achieve the required eye velocity. That is, a temporally precise transfer of changes in spike frequency might be better achieved by NOT cells that have phasic response properties than by spontaneously active cells which add self-generated spikes to the firing patterns generated by the retinal input.

**NOT cells that project to cNOT and iSC**

To our surprise, NOT cells that project to the cNOT and the iSC were tonically active *in vitro*. We expected these neurons would respond phasically because the only NOT neurons that are characterized by tonic responses *in vivo* are the OKR-related directionally selective NOT cells that project to the iIO and iNPH (Gamlin 2005; Ibbotson and Dreher 2005). In anesthetized animals, many NOT neurons respond to saccadic eye movements or shifts of the retinal image at saccadic velocities with short, high-frequency bursts (for review see Gamlin 2005; Ibbotson and Dreher 2005). Previous studies demonstrated that neurons with these phasic responses project to either the ipsilateral dorsal lateral geniculate nucleus or to the ipsilateral extrageniculate visual thalamic nuclei (Schmidt 1996; Sudkamp and Schmidt 1995). The response properties of the NOT neurons that project to iSC and/or cNOT have not been characterized *in vivo*. Based on results in marsupials, it has been argued that the NOT cells which project to cNOT contribute to the binocularity of the directionally selective NOT cells.
that project to IO (Ibbotson et al. 2002; Pereira et al. 1995). Because the responses of these cells to monocular stimulation of the ipsilateral eye is tonic, the expectation would be that the commissural NOT cells also are tonically active. In higher mammals, the binocularity of directionally selective NOT cells seems to depend on cortical input (ferret, Sengpiel et al. 1990; cat, Distler and Hoffmann, 1993; monkey, Hoffmann et al. 1992), suggesting that the commissural NOT cells have a function different than conveying input from the ipsilateral eye. Whether cortical input is also responsible for NOT cell binocularity in lower mammals (rat, Schmidt et al. 1993; guinea pig, Lui et al. 1994) remains to be confirmed.

Without more information concerning the in vivo response properties of the NOT cells that project to cNOT and iSC, we can only speculate about their functions. The evidence that NOT is not retinotopically organized (Hoffmann and Schoppmann 1981) suggests that its influence is modulatory. Tonic activity of NOT neurons in vivo has not only been reported for OKR-related cells, but also for a population of NOT neurons in monkey which are effectively suppressed by saccadic eye movements in a non-directionally selective fashion (Mustari et al. 1997). These neurons, termed pretectal omnidirectional pause neurons, could represent the spontaneously active cells reported here. We also know that SC-projecting NOT neurons, as many others, are GABAergic and that they project almost exclusively to non-GABAergic projection neurons in the superficial, visuosensory, layers of the superior colliculus (Nunes Cardozo et al. 1994; Baldauf et al. 2003; Born and Schmidt 2004). If iSC-projecting NOT cells are the pretectal pause neurons, they could provide a tonic inhibitory input to collicular cells that might help to suppress the execution of unwanted saccades. Alternatively, iSC-projecting neurons could have properties in vivo different from the pause neurons and might be activated by fast image movements, as many NOT cells are. Then, these GABAergic NOT cells could provide inhibition to collicular cells that increases during saccades and reduces the likelihood that the saccade induced shifts in the visual field trigger subsequent unwanted eye
movements. In contrast, the GABAergic projection from NOT to dorsal lateral geniculate nucleus terminates exclusively on GABAergic interneurons and disinhibits relay cells in the dorsal lateral geniculate nucleus during saccades (Cucchiaro et al. 1991, 1993; Fischer et al. 1998; Wang et al. 2002). Thus, NOT may contribute both to preventing unwanted saccades by attenuating visuomotor activity in the superior colliculus during saccades (Richmond and Wurtz 1980) and to maintaining the relay of visuosensory signals from the thalamus to the cortex during and immediately after a saccade ( Büttner and Fuchs 1973; Judge et al. 1980).

Conclusions

The organization of NOT has been a puzzle. This small and, by usual morphological criteria, homogeneous nucleus has been associated with several apparently diverse functions ranging from modulation of the activity of visual thalamic relay cells during saccades to inhibition of the visual layers of the SC and, through its pathway to iIO and iNPH, to mediation of the OKR. The question addressed by the present experiments is whether NOT is one nucleus that makes a single contribution to all of these diverse structures and functions or whether, in contrast, it comprises several distinct nuclei that overlap spatially but differ in terms of their connections, physiology and functions. The results demonstrate that NOT consists of several cell types that are anatomically and physiologically distinct. Tonic spontaneous activity is propagated by NOT neurons in a branched pathway to the iSC and the cNOT, while the NOT-DTN neuronal population that projects to the iIO is characterized by phasic response properties in vitro.
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Figure legends

Figure 1:
Distribution of gold particle cluster sizes after retrograde transport of WGA-apo-HRP, labeled with 10 nm-sized gold particles (grey bars, arrowheads in A) and 20 nm-sized gold particles (black bars, arrows in B). Although clusters are much larger than the original gold particles retrograde label from either particle size can be easily distinguished. Scalebar in A), 20 μm.

Figure 2:
Results from a representative NOT neuron retrogradely labeled from iSC. A) Clustered gold particles are present in the soma and primary dendrite of the neuron (arrows). The homogeneous background label (asterisk) produced by biocytin diffusion from the patch pipette during recording confirms that the recording was from a prelabeled cell. The inset shows the location of the neuron within the slice (m, medial; l, lateral; d, dorsal; v, ventral). B) Depolarizing current injection under current clamp conditions (duration 500 ms) evokes action potentials in a regular firing pattern. The neuron’s firing rate varies with the membrane potential. C) Depolarizing current injections (adjusted to achieve +5 mV steps of the membrane potential) increase the firing rate of the neuron, which was spontaneously active at resting potential (-62 mV). Interspike intervals remain regular at all membrane potentials. D) Firing rate plotted as a function of membrane potential. The maximum firing rate (27 spikes/s) is reached at -30 mV.
Figure 3:
Representative NOT neuron labeled after an injection of WGA-apo-HRP-gold into the cNOT. 
A) Arrows point to the clustered gold particles in the soma and primary dendrite that prelabeled the cell. The homogenous background of biocytin confirms that the recording was from the prelabeled cell. B) Depolarizing current injections during current clamp (5 and 10 pA, duration 500 ms) increase the firing rate as a function of the amplitude of the injected current. C) A sustained non-adapting tonic firing of action potentials (maximum firing rate: 18 spikes/s) is generated during current clamp recordings at membrane potentials of -60 mV and at -40 mV.

Figure 4:
Double labeling with two retrograde tracers. A) WGA-apo-HRP-gold with 20 nm-sized gold particles were injected into the superficial layer of the iSC, and WGA-apo-HRP-gold with 10 nm-sized gold particles into the cNOT. B) Distribution of retrogradely labeled neurons in a representative midbrain section containing caudal NOT. Double labeled cells are shown in blue, cells labeled only from iSC or cNOT are shown in red and green, respectively. C) In 40μm-thick coronal sections counterstained with cresyl violet, NOT neurons retrogradely labeled with WGA-apo-HRP-gold contain clusters with both particle sizes (inset). Clustered 20 nm-sized particles appear as prominent dots, in contrast to the smaller clusters of 10 nm-sized particles. The coappearance of 10 nm and 20 nm-sized particles within individual NOT neurons (arrows) indicates that the neuron sends axon collaterals to both the iSC and the cNOT.
Figure 5:
Results from double retrograde tracing with fluorescent dyes in 40μm-thick coronal vibratome sections. A), B), NOT neurons labeled following an injection of “Stabilo Green” into the SGS of the iSC and an injection of “Stabilo Red” into the cNOT. The superimposed images for green and red fluorescence display the coincidence of green and red fluorescing tracer in neurons of the iNOT. The presence of both labels in single cells indicates that these cells project to both the iSC and the cNOT.

Figure 6:
NOT neuron prelabeled with WGA-apo-HRP-gold (particle size 15 nm) after an injection into the iIO. A) Gold particles are clustered in the soma and the primary dendrite (arrows). To confirm that the recording was from the prelabeled cell it was filled with biocytin from the patch pipette during whole-cell patch clamp recording(asterisk). B) In this cell, depolarization (5 mV above resting potential, middle trace) generated a phasic-tonic response with irregular action potentials. Increasing the membrane potential (30 mV above resting potential, upper trace) leads to an increase of the number of the evoked action potentials, the firing pattern, however, remains irregular. C) Action potentials generated by current injections that induce small depolarization steps at various holding potentials in another cell. The firing pattern is always irregular. D) Firing rate as a function of membrane potential. Firing rate increases rapidly above spike threshold until maximum firing level. Further depolarization leads to reduced firing.
Figure 7:
Temporal analysis of the firing pattern of NOT neurons. A, D, G) NOT neurons retrogradely labeled from iSC / cNOT are characterized by maintained regular firing. B, E, H) Interspike interval histograms of these neurons show narrow Gaussian distributions with only limited variations of interspike intervals. C, F, I) The regularity of firing is confirmed in the autocorrelograms by the appearance of equally spaced peaks due to the little variation in the interspike intervals. J, M, P) NOT neurons that project to the IO exhibit irregular firing in vitro. K, N, Q) Interspike interval histograms show the spread distribution of events. L, O, R) The irregular firing of this population is further illustrated by the irregular event distributions in the autocorrelograms. Scalebars in A, D, G) and J, M, P), 30 μm.

Figure 8:
Retrograde labeling with two tracers demonstrates that phasic cells are anatomically distinct from tonically active cells A) WGA-apo-HRP-gold with 20 nm-sized particles was injected into the superficial layer of the iSC (left) and WGA-apo-HRP-gold with 10 nm-sized particles was injected into the iIO (right). B) Distribution of retrogradely labeled cells shown in a representative midbrain section through the caudal NOT. Despite the spatial overlap of the retrogradely labeled cells, neurons are either labeled from iSC (shown in red) or from iIO (shown in green). C) Light micrograph of the left iNOT in a 40μm-thick coronal section counterstained with cresyl violet. While large clusters of the 20 nm-sized particles are localized in triangular to ovoid shaped somata, clusters of the smaller 10 nm-sized particles are present only in flattened or spindle shaped somata. No neurons contained both labels, confirming that the in vitro phasically active NOT neurons which project to the iIO are distinct from the tonically active cells that project to SC and cNOT.
Figure 9:

Results with two retrograde fluorescence tracers A) and B), NOT neurons retrogradely labeled after an injection of “Stabilo Green” into the superficial layer of the iSC, and “Stabilo Red” into the iIO. A) and C) show examples for neurons in the left iNOT retrogradely labeled from the iSC with green fluorescent tracer. At the same location, images in B) and D) show NOT neurons labeled from the iIO with the red fluorescent tracer. A comparison of A) and B), and C) and D) indicates no colocalization of the green and the red fluorescent tracers in single NOT neurons, confirming that the *in vitro* tonically active NOT cells are anatomically distinct from the phasically active cells.
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