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15. Spontaneous optic nerve impulses were recorded from isolated experimental and control eyes of the same animals. *Aplysia californica* were entrained to cycles of 12 light hours and 12 dark hours before they were used in experiments. Eyes were isolated at CT 12 hours and kept at 15.5°C in constant darkness in buffered filtered seawater (BFSW) (10 ml). The last offset of light experienced by intact animals was at CT 12 hours. Thus, CT 24 or CT 00 hours is the projected time of light onset while the isolated eyes are maintained in constant dark conditions. The frequencies of nerve impulses were obtained with a personal computer that allowed us to count, print out, and plot data over the 5 days of experiments. Chemical treatments were administered while we kept the eyes in darkness by completely exchanging the solution surrounding the eyes. We terminated treatments by removing the experimental solution and rinsing the eyes with ten exchanges of BFSW over a 1-hour interval. Chemical treatments (2-hour), which were used to obtain the PRC, were administered during an interval that began 6 hours after eyes were isolated and extended for about one cycle from this point. Further details of experimental procedures and media have been described [A. Eskin, *J. Neurobiol.* **8**, 273 (1977)]. Five cycles of rhythms were recorded. We measured phase shifts by comparing the peaks of rhythms of matched experimental and control eyes during the fourth cycle of the rhythm. Phase shifts produced by DRB appeared stable; the value obtained for the third, fourth, and fifth cycles were not significantly different from one another. For example, for DRB given at CT 02 to 04 hours, the phase shifts for the third, fourth, and fifth cycles were 3.6 ± 0.2 hours (±SEM), 4.0 ± 0.2 hours, and 3.5 ± 0.4 hours (n = 4), respectively.
16. To measure the effect of various treatments on transcription, we determined the incorporation of [^{3}H]uridine (ICN Radiochemicals, Irvine, CA) into TCA-precipitable material. Groups of three control and three contralateral experimental eyes were used for each experiment. The experimental group was treated with the drug 15 min before being exposed to [^{3}H]uridine (40 μCi/ml) and then exposed to the drug and [^{3}H]uridine for 2 hours. Except as noted in the text, exposure of eyes to [^{3}H]uridine began at CT 06 hours. At the end of experiments, eyes were rinsed with ice-cold BFSW and homogenized on ice in a tris-HCl buffer that contained EDTA, EGTA, protease inhibitors, urea, CHAPS, and β-mercaptoethanol. Details of this buffer and the method of TCA precipitation have been described (*2*). Incorporation of [^{3}H]uridine into total RNA (TCA-precipitable material) was corrected for an effect of DRB on the uptake of uridine as described [D. Granick, *J. Cell Biol.* **65**, 398 (1975); I. Tamm, R. Hand, L. A. Caliguiri, *ibid.* **69**, 229 (1976)]. In this procedure, the ratio of experimental to control TCA-precipitable radioactivity was divided by the ratio of experimental to control total radioactivity of the homogenized samples.
17. We measured the effect of DRB on protein synthesis by determining its effect on the incorporation of [^{3}H]leucine (ICN Radiochemicals) into TCA-precipitable material. Experimental eyes were exposed for 2 hours to [^{3}H]leucine (40 μCi/ml) and 10^{-4} M DRB, and then the TCA-precipitable radioactivity was measured as described (*16*). The [^{3}H]leucine incorporation in DRB-treated and control eyes differed by 8 ± 12%, n = 4.
18. To measure the effect of DRB on the period of the rhythm, we exposed experimental eyes continuously to DRB a few hours after the eyes were isolated. The period difference between experimental and matched control eyes averaged 2.1 ± 0.8 hours (cycle 1 to 2), 1.4 ± 0.2 hours (cycle 2 to 3), 1.1 ± 0.2 hours (cycle 3 to 4), and 1.7 ± 0.1 hours (cycle 4 to 5) (n = 4). The average free-running periods of the control eyes during the same cycles were 21.5, 24.4, 23.8, and 23.2 hours, respectively.
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Concurrent Processing and Complexity of Temporally Encoded Neuronal Messages in Visual Perception

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The intrinsic neuronal code that carries visual information and the perceptual mechanism for decoding that information are not known. However, multivariate statistics and information theory show that neurons in four visual areas simultaneously carry multiple, stimulus-related messages by utilizing multiplexed temporal codes. The complexity of these temporal messages increases progressively across the visual system, yet the temporal codes overlap in time. Thus, visual perception may depend on the concurrent processing of multiplexed temporal messages from all visual areas.

THE ABILITY OF MONKEYS TO RECOGnize pictures depends on a large number of cortical and subcortical brain regions (*1*). To understand how neurons within these regions contribute to visual perception, we must learn what codes they use to transmit information. Despite many years of studying visual neurons, researchers have failed to define this intrinsic code. In the classical hypothesis, neurons encode information about images only in the number of action potentials (spikes) in their responses by using a mean firing rate or strength code (*2*). However, neurons in four brain regions spanning the visual system in the monkey also carry information about images in the distribution of spikes within the response (*3, 4, 5*). This temporal code is two to three times more efficient than the strength code in representing visual information. Furthermore, the proportion of the information carried by the temporal modulation increases in regions more distant from the retina. These findings show that temporal modulation that is dependent on the stimulus is a common neuronal mechanism, and we hypothesize that it may be important for visual perception.

We recorded electrophysiologically from the following: ganglion cell fibers (RET), which carry visual information out of the retina; neurons in the parvocellular division of the lateral geniculate nucleus (LGN), which receive input from the retina and which project to the primary visual cortex (V1); complex cells in layers two and three of V1, which receive input from layer four of V1 and project to later visual areas; and neurons in the inferior temporal cortex (IT), which receive input from earlier visual areas and project beyond the visual system to the limbic system (*6*). Awake monkeys were trained to fixate on a small spot while stimuli were presented on a video monitor. The stimuli used in these experiments (Fig. 1) consisted of a complete set of two-dimensional black-and-white pictures based on Walsh functions (*7*). Neuronal responses were quantified by the Karhunen-Loève transform (KLT), which is similar in principle to a Fourier transform but does not use sine waves as its basis. Instead, the basis of the KLT is a set of waves of excitation and inhibition, called principal components (Fig. 2, ϕ_0 through ϕ_3), that must be computed separately for each neuron. Because no principal component can be represented by a sum of the others, each can be interpreted as a separate element in a temporal code.

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Neuronal responses can be synthesized by weighting the principal components and adding them to the mean response of the neuron (*3, 5, 7*). The average responses and first principal components of neurons within RET and LGN were sustained during the stimulus interval. The average responses and first principal components of neurons within V1 and IT could be either sustained or transient (Fig. 2, Avg and ϕ_0). Although the neurons had shorter response latencies in earlier visual areas, their responses continued well after the responses in later visual areas had started. Thus, the code elements formed by the principal components of all visual areas overlapped in time. This observation supports the idea that visual processing occurs concurrently in all areas (*8*).

One can use information theory to calculate how much stimulus-related information is transmitted by neurons if their code is known (*9*). Because the intrinsic neuronal code is not known, we compared the efficiency of two different response codes in carrying information about the Walsh pictures. The first, a univariate code, was based on the number of spikes in the response. The other, a multivariate code, was based on the coefficients of the first four principal components. Transmitted information serves as a measure of the amount by which knowledge of the response reduces the uncertainty about the identity of the stimulus (*4*). The average transmitted information about the Walsh stimuli declined at higher levels of the visual system (Fig. 3A). The decline was greatest for the code based on the spike count, T_s, and least for the code based on the coefficients of the first four principal components, T_{0123}. The ratio of the information transmitted by the multivariate temporal code to that transmitted by the univariate spike count code was greater than 1.0 for all the neurons we sampled in each of the four brain regions studied, and the ratio increased in the later areas (Fig. 3B). This increase was accompanied by an increase in the number of higher principal components carrying stimulus-related information, which means that the codes in the later areas were more complex.

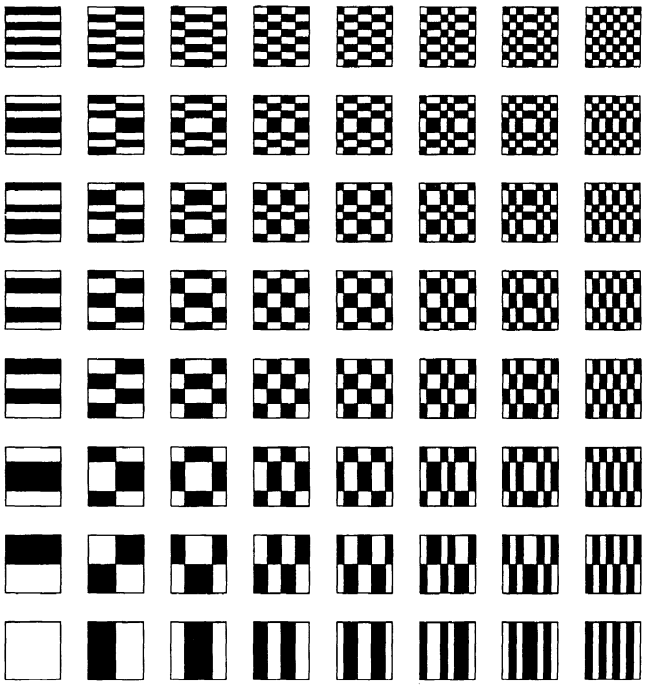

Fig. 1. Walsh pictures. The stimulus set also contained the pictures with the contrast reversed. The stimuli subtended 2° for RET and LGN, 2.5° for V1, and 40° for IT.

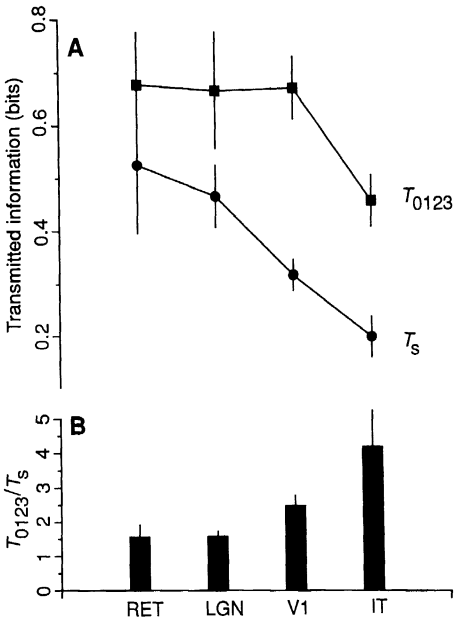

Fig. 3. Information analysis. (**A**) The average information carried by neurons calculated with different codes. These values were compiled from 5 RET, 13 LGN, 21 V1, and 21 IT neurons. (**B**) The average ratio of T_{0123} to T_s in each of the four brain regions studied. The vertical bars through each symbol represent SEM.

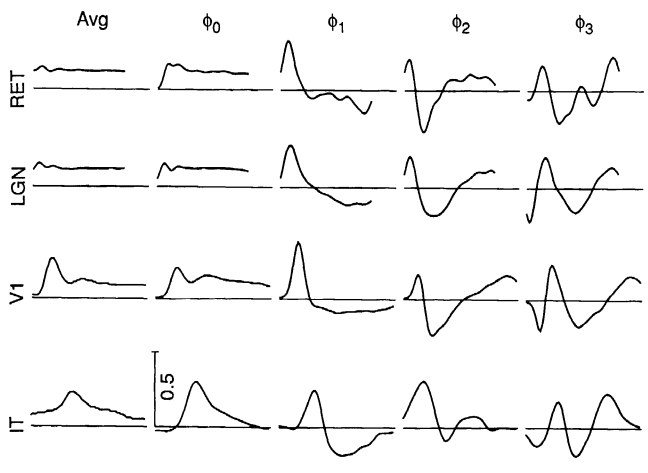

Fig. 2. Waveform analysis. The average response waveform (Avg) and the first four principal component waveforms (ϕ_0 through ϕ_3) for a neuron in each of RET, LGN, V1, and IT. All of the waveforms were normalized to unit length. The horizontal line in each plot represents the zero level. The vertical calibration bar indicates a probability of 0.5 that the neuronal discharge will deviate from the average. Each plot spans 320 ms. Stimuli were presented for 256 ms for experiments in RET and LGN, 320 ms in V1, and 384 ms in IT.

The importance of temporal encoding does not lie solely in its increased efficiency over mean-rate encoding. Temporal codes are also important because their multiple components can keep separate information that a single component code would confound. Consider the problem of determining whether one or two stimuli are in the receptive field of the neuron. Two dim stimuli could elicit a response of the same magnitude as one bright stimulus. In this case, a discrimination could not be made on the basis of the response strength only. However, the temporal modulation of a response is not correlated with its strength (*3, 5*). Therefore, the waveform of the response would allow differentiation between the two cases. The confounding of stimuli within receptive fields by a single component code such as we have described would become worse at later stages of visual processing because the sizes of neuronal receptive fields increase progressively throughout the visual system as a consequence of converging inputs (*10*). The increasing complexity of the temporal codes in neurons

with larger receptive fields could prevent this confounding of stimulus properties.

We cannot yet prove that either spike count or temporal modulation are actually used in visual processing. However, there are many advantages in regarding temporal modulation as the intrinsic neuronal code underlying visual perception. For example, the simultaneous encoding of several stimulus features by temporal modulation, a general mechanism found throughout the visual system, could be used to avoid confounding information. Furthermore, the overlap of the temporal codes in all areas would allow concurrent, rather than sequential, visual processing.

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Technical Comments

Counting and Discounting the Universe of Exons

R. L. Dorit *et al.* (*1*), beginning with the premise that all proteins are constructed of modules encoded by discrete exons, undertake to count the "underlying universe" of exon types. They calculate that a reasonable estimate is between 1000 and 7000. I question whether there is a suitable method for counting the members of such a universe, and whether the sequence-alignment methods used by Dorit *et al.* actually identified homologous pairs in most cases. Finally, I challenge whether there is an underlying universe of exons to count.

It is impossible to prove that two sequences have not evolved from a common ancestor; they may just have changed so much by amino acid replacement that the relationship is obscured. All we can do is make statistical judgments about the likelihood that similarities are not due to chance (*2*). Let us assume that the number of exon types has been constant from the time of the first encoded proteins and that all proteins are indeed constructed from that prototypic set. Can the method used by Dorit *et al.* establish the primordial number? Their strategy depends on determining two fundamental numbers: (i) the number of nonhomologous protein types in their database collection of exons, and (ii) the number of pairs of the exons themselves that are homologous but embedded in nonhomologous proteins. Their judgments about which sequences belong in which class depend exclusively on sequence compairson.

Suppose that when Dorit *et al.* compared their exon types they did not find *any* homologous pairs. Should they conclude, as their formula would demand, that the number of exotypes is infinite? The exact opposite could also be correct: there could have been a single exotype that started the entire expansion, but amino acid replacements over the past 3 to 4 billion years have eroded all possible recognition at the pairwise level.

This is not to say that one cannot use amino acid sequence comparison to show that portions of proteins, whether or not they are encoded by exons, have been shuffled about during evolution. There are many cases where parts of proteins are more similar to portions of other proteins than are the parental proteins in which they are embeded. It is also possible that, under carefully specified conditions, sampling may allow an upper bound to be placed on the number of entities being shuffled. No lower bound short of unity can be established, however.

Sequence comparison was the only basis on which Dorit *et al.* determined the two samples sets needed for the combinatorial estimation: in both instances the determinations are vulnerable to errors of judgment. It might seem that compiling the starting list of exons from nonhomologous proteins, the numerator n in the sampling statistic, is straightforward. Although species redundancies are readily removed, homologous entries that result from past gene duplications present a much greater challenge. Nonetheless, let us assume that the culled list generated by Dorit *et al.* represents 1255 exons from nonhomologous proteins.

The objective is to identify any homologous exons among them. The criterion is that a pair of exons be significantly more similar than are the proteins from which they are drawn. To this end, Dorit *et al.* compared exon sequences using a program that did not allow gaps and scored only identities. In line with their premise that exon size was a principal determinant, comparisons were restricted to exons of similar lengths. The cutoff for deciding whether a match was significant was determined by using a global measure based on the overall amino acid composition of the database.

How effective was this search? I subjected each internal measure of the 14 pairs they found to a conventional alignment and scrambling test (*3*) to see how the results fared statistically by an internal measure (Table 1). Of the 14 pairs, four had reasonably significant scores (>4 standard deviations above the random mean). All of these sequences had been reported previously by others as examples of "exon shuffling" and are likely valid. They included the epidermal growth factor (EGF) domain being moved into the blood clotting factors IX and XII (*4*), collagen-like segments being translocated into complement component Clq (*5*) and a mannose-binding protein (*6*), and a shuffle of a segment between thyroglobulin and the Ii-antigen (7). Other than the four, only one other pair reached the generally applied minimum threshold of 3 standard deviations above the random mean, this being an unlikely match between a hydrophobic signal peptide from a chloroplast gene product and a membrane-spanning sequence from the mouse red cell band 3 protein.

None of the remaining cases bears up to scrutiny. Indeed, there is good evidence to reject several of the matches a priori. In the case of the proposed relationship of a collagen exon and an elastin exon, for example, the repeat structures of the two proteins are fundamentally different, even though both are rich in glycine and proline (Fig. 1). The elastin repeat leads to a spiral of β turns (*8*), whereas collagen is a three-stranded cable.

There are other inconsistencies regarding the alleged homologous pairs. In the matchup of a mouse collagen exon 5 (MAC5) with a similar sized complement Clq exon, the complement exon contains the signal peptide region over its first half and must be