Receptor Endocytosis and Dendrite Reshaping in Spinal Neurons After Somatosensory Stimulation

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In vivo somatosensory stimuli evoked the release of substance P from primary afferent neurons that terminate in the spinal cord and stimulated endocytosis of substance P receptors in rat spinal cord neurons. The distal dendrites that showed substance P receptor internalization underwent morphological reorganization, changing from a tubular structure to one characterized by swollen varicosities connected by thin segments. This internalization and dendritic structural reorganization provided a specific image of neurons activated by substance P. Thus receptor internalization can drive reversible structural changes in central nervous system neurons in vivo. Both of these processes may be involved in neuronal plasticity.

Receptors coupled to heterotrimeric GTP-binding proteins (G protein–coupled receptors) make up a large family of receptors that is widely distributed in the mammalian central nervous system (CNS). Members of this family include receptors for classical neurotransmitters such as norepinephrine, acetylcholine, dopamine, and serotonin, as well as receptors for neuropeptides (*1*). In transfected cells in culture, after the initial agonist-receptor interaction that induces signal transduction, these G protein–coupled receptors typically undergo phosphorylation, endocytosis, and dissociation from ligand in the endosome, and finally the receptors are recycled to the plasma membrane (*2*). We investigated whether CNS activity in vivo produced a similar agonist-induced receptor translocation.

We examined substance P (SP)–induced internalization of the SP receptor (SPR; also known as the tachykinin NK-1 receptor) in the spinal cord. Dorsal root ganglion neurons synthesize and transport SP to the spinal cord (*3*), where it is released upon noxious stimulation of the innervated peripheral tissue (*4*). When SP is released in the spinal cord, it diffuses from the presynaptic terminal and interacts with postsynaptic SPRs, which are expressed by a subset of spinal cord neurons (*5*, *6*). SP also excites spinal cord nociresponsive neurons (*7*) and evokes a short-lived pain behavior (*8*).

In vitro studies in transfected cells show that the SPR is a prototypical G protein–coupled receptor that undergoes agonist-induced phosphorylation and internalization (*9*). In the spinal cord, the SPR is distributed in a lamina-specific manner, being concentrated in superficial lamina I (Fig. 1) where SP-containing primary afferent fibers also terminate (*3*). Optical sectioning with the confocal microscope or electron microscopy demonstrates that in the unstimulated spinal cord, most of the SPR immunoreactivity is present in the plasma membrane of neuronal cell bodies and dendrites, so that more than 70% of the somatic and dendritic plasma membrane of these neurons is imbued with SPR immunoreactivity (*6*). Although some SP terminals directly contact SPR-immunoreactive plasma membranes, no more than 15% of the SPR-containing membrane in spinal neurons is apposed to synaptic terminals (*6*), which suggests that SPRs are present at synaptic and nonsynaptic sites.

To test whether in vivo somatosensory stimulation could induce the internalization of the SPR, anesthetized rats received either a pinch of one hindpaw or injection of 100 μg of capsaicin into one hindpaw (*10*), and then the spinal SPR was localized immunohistochemically (*11*). In the normal unstimulated lumbar spinal cord, or in the spinal cord contralateral to the pinch or capsaicin injection, we detected no lamina I SPR-positive neurons with more than five SPR-positive endosomes per cell body. However, 5 min after injection of capsaicin, the distribution of SPR immunoreactivity in ipsilateral lamina I spinal cord neurons was significantly altered, and 70% of lamina I SPR-positive cell bodies (Figs. 2 and 3) exhibited more than 20 endosomes per cell body (*12*); this effect was dose-dependent (*13*). Similar results were obtained with a pinch; 5 min after the pinch, 67% of the lamina I SPR-positive cell bodies had more than 20 endosomes per cell body. Furthermore, after both pinch and injection of capsaicin, this internalization of the SPR was accompanied by a substantial decrease in the SPR immunoreactivity on the plasma membrane of the same neuron (Fig. 2) and by an increase in the intensity of the total fluorescence (*14*). Thus, the SPRs at synaptic and nonsynaptic sites (*6*) were functional, because SPR immunoreactivity was lost over the entire surface of the lamina I SPR-positive neurons that were exposed to SP.

The most striking SP-induced change in the SPR-positive spinal cord neurons occurred in the distal dendrites less than 4 μm in diameter (Figs. 2 and 4). Somatosensory stimulation in the form of either pinch or capsaicin injection induced not only SPR internalization but also a structural reorganization of the distal dendrites. In the unstimulated spinal dorsal horn, the distal dendrites of SPR-positive neurons varied little in diameter along their length (±1 μm over 20 μm), and the SPR immunoreactivity essen-

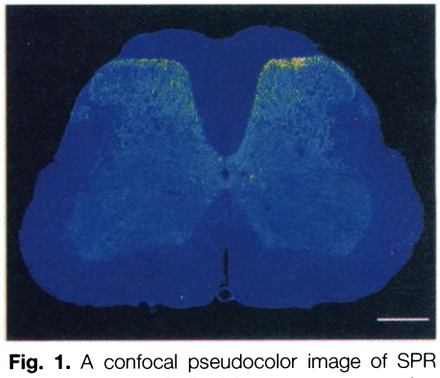

Fig. 1. A confocal pseudocolor image of SPR immunoreactivity (yellow) in a transverse section of the rat lumbar spinal cord (L4 level) 5 min after a unilateral injection of capsaicin into the right footpad. There is an apparent increase in SPR immunoreactivity in the medial superficial lamina on the stimulated (right) side, which corresponds to the area of the lumbar cord that is known to receive nociceptive inputs from the right footpad. The apparent increase in SPR on the stimulated side is probably due to the fact that as the SPR is internalized, it is concentrated in endosomes, which makes it easier to detect (*14*). Scale bar, 0.4 mm.

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tially outlined the plasma membrane (Figs. 2A, 4A, and 5). Five minutes after either pinch or injection of capsaicin, the majority of SPR-positive distal dendrites in lamina I appeared as strings of swollen varicosities (2.0 to 8.0 μm in diameter) connected by thin fibers whose diameters were 10 to 50% of those of the swollen varicosities (Figs. 2D and 4B). When the dendrites were viewed at high magnification (Fig. 2D), the swollen SPR-positive varicosities were found to contain many SPR-positive endosomes (4 to 10 per varicosity), whereas the thin fibers that connected these varicosities were virtually devoid of endosomes (less than two per 10 μm of length).

Although SPR internalization appeared to occur in all dendrites in which the SPR was occupied by the agonist, only the thin, distal dendrites showed a structural reorganization. One possible explanation for this is that in the smaller diameter dendrites, the fraction of the total plasma membrane that is removed with each internalized receptor is much higher than that for larger diameter dendrites. Because the adjacent plasma membrane is internalized together with the receptor (*2*), we hypothesize that when a certain critical percentage of the plasma membrane is internalized, the dendrite undergoes a structural reorganization, because the surface area decreases while the volume stays the same or increases. Also, because changes in the diameter and morphology of a dendrite should alter its passive electrical properties (*15*), these data lead us to suggest that morphological reorganization of the distal dendrites will alter the integrative properties of CNS neurons.

The internalization of SPRs was greatest in the region of maximal SP release, laminae I and II. This finding was particularly evident in the SPR-positive neurons with cell bodies in lamina III, which send dorsally directed dendrites into laminae I and III (Fig. 5). Although internalization of the SPR was evident in the dorsally directed dendrites of the lamina III neurons, no statistically significant increase in SPR-positive endosomes was observed in the cell bodies at 1 min to 24 hours after capsaicin injection. These data also suggest that, unlike the neurotensin receptor, the SPR-positive endosomes in these neurons were not retrogradely

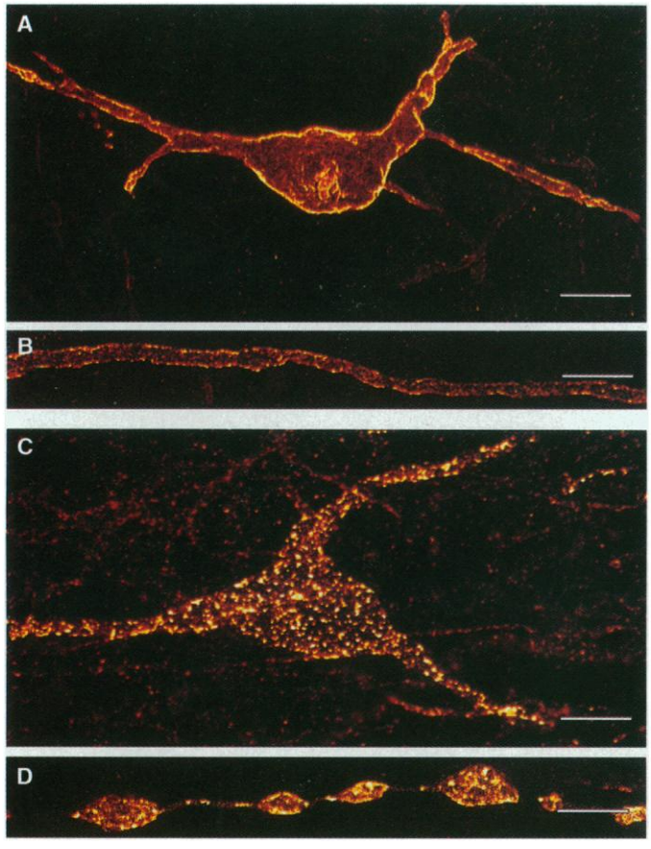

Fig. 2. Fluorescent confocal image of lamina I spinal neurons in the lumbar cord (L4 level). In the contralateral unstimulated dorsal horn of the spinal cord (**A** and **B**), the SPR immunoreactivity (red and yellow) is associated with the somatic (A) and dendritic (B) cell surface of the neurons; few intracellular SPR-positive endosomes are visible. Five minutes after injection of 100 μg of capsaicin into the hindpaw of the rat, the neuronal cell bodies (**C**) and dendrites (**D**) in the ipsilateral dorsal horn show a loss of SPR immunoreactivity from the cell surface and an increase in intracellular SPR-positive endosomes. Additionally, the thin, distal dendrites in laminae I and II show a morphological reorganization by changing from a structure of rather uniform diameter (B) to one characterized by large swollen varicosities (packed with SPR-positive endosomes) connected by thin fibers (D). Images in (A) and (C) are the result of projection of 35 optical sections taken at 0.6-μm intervals, whereas (B) and (D) are the result of projection of 8 optical sections at 0.6-μm intervals. Scale bar in (A) and (C), 20 μm; in (B) and (D), 10 μm.

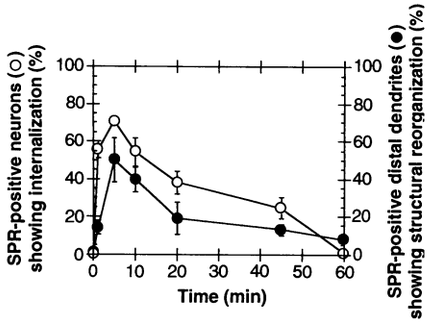

Fig. 3. Time course of capsaicin-induced (100 μg) SPR internalization in cell bodies and structural reorganization of distal dendrites in ipsilateral lamina I neurons. Neuronal cell bodies (open circles) that contained >20 SPR-positive endosomes per cell body were considered to have internalized the SPR. SPR-positive distal dendrites (solid circles) that had at least three varicosities >3 μm in diameter, connected by fibers less than 1.5 μm in diameter over a distance of 50 μm, were considered to have undergone a structural reorganization.

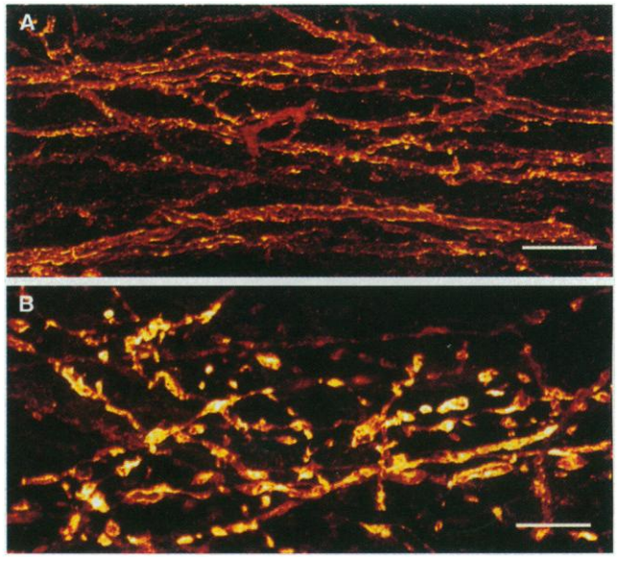

Fig. 4. Structural reorganization of lamina I SPR-positive distal dendrites after somatosensory stimulation. In the unstimulated contralateral dorsal horn (**A**), SPR-positive dendrites varied little in diameter along their length; the SPR-positive immunoreactivity essentially outlines the outer cell membrane. Five minutes after injection of 100 μg of capsaicin (**B**), most of the SPR-positive thin, distal dendrites have undergone a structural reorganization and now are characterized by swollen varicosities connected by thin fibers. Both images were developed by projection of 40 optical sections taken at 0.6-μm intervals. Scale bar, 20 μm.

transported to the cell body after internalization (*16*).

Agonist-induced SPR internalization and dendritic structural reorganization were reversible (Fig. 3). Sixty minutes after capsaicin injection, the number of SPR-positive neuronal cell bodies that showed high concentrations of internalized SPR-positive endosomes had returned to the normal unstimulated levels seen on the contralateral side. The structural changes observed in the SPR-positive distal dendrites followed a similar time course; the distal dendrites returned to a tubular morphology within 1 hour after the injection (Fig. 3).

Because internalization is activity dependent, it is possible that translocation of G protein–coupled receptors could be used as a pharmacologically specific index of neuronal activity. Thus, agonist-dependent endocytosis of a signal-transducing receptor not only provides a method for identifying the anatomical components of highly specific neuronal pathways, but because the extent of receptor endocytosis is dose-dependent, this technique also offers the possibility of quantifying neurotransmitter release locally at the physiologically relevant synapses or at the postsynaptic signaling sites.

The finding that activation of a G protein–coupled receptor produced a structural reorganization of the dendrite within minutes of receptor activation agrees with observations that suggest that neurons within the spinal cord display substantial plasticity after somatosensory stimulation (*17*). Because repeated receptor activation has been reported to produce long-term cytoskeletal changes (*18*), our results also may be important in linking short-term neurochemical events to long-term changes in neuronal morphology.

Our results also suggest that many of the findings concerning G protein–coupled receptor internalization and recycling in in vitro systems (*2*) are directly applicable to the CNS in vivo. Determination of how each G protein–coupled receptor interacts with cytoskeletal elements as the receptor is internalized and definition of the cytoskeletal elements involved in the stimulus-evoked structural reorganization of the dendrites should shed light on the cellular mechanisms underlying neuronal desensitization and plasticity.

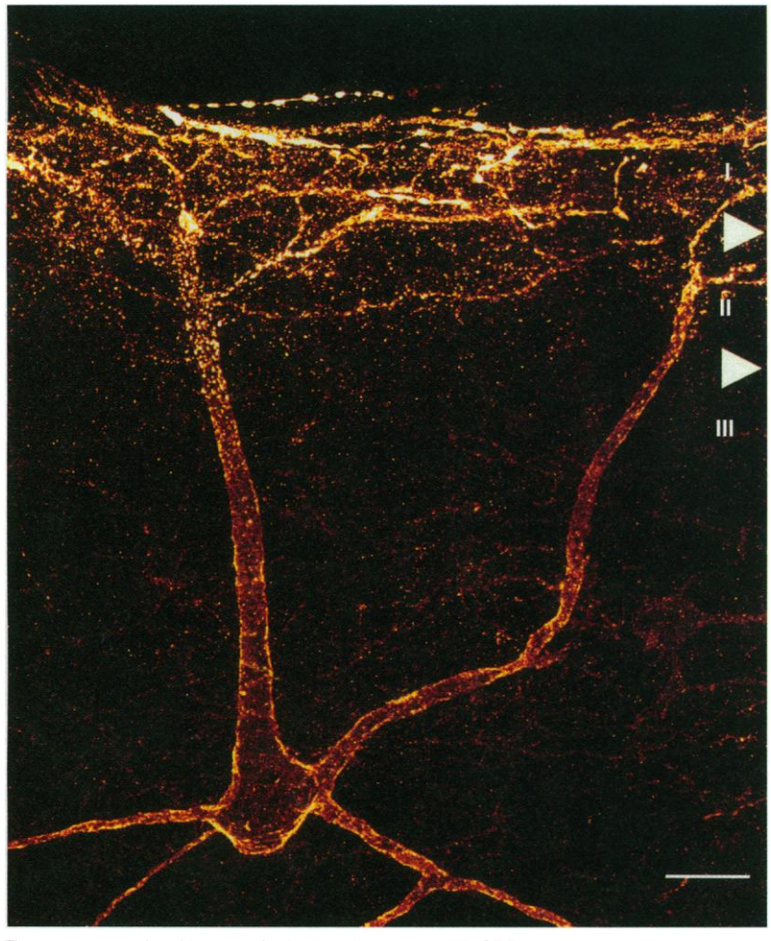

Fig. 5. Fluorescent confocal image of a lamina III neuron, with SPR-positive dendrites projecting dorsally into laminae I and II 5 min after capsaicin injection. Whereas nearly all the SPR-positive dendrites that extend into laminae I and II, where SP is released by primary afferent neurons, show evidence of SPR internalization, only the thin (<4.0 μm in diameter) distal dendrites show evidence of structural reorganization. This image was developed by projection of 35 optical sections taken at 0.6-μm intervals. Scale bar, 22 μm.

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10. The studies were done on male Sprague-Dawley rats (240 to 260 g) that were deeply anesthetized with sodium pentobarbital (60 mg per kilogram of body weight). Capsaicin was prepared in a vehicle containing 5% polyoxyethylenesorbitan monooleate (Tween 80) and saline as previously described [D. A. Simone, J. Y. Ngeow, G. J. Putterman, R. H. LaMotte, *Brain Res.* **418**, 201 (1987); D. A. Simone, T. K. Baumann, R. H. LaMotte, *Pain* **38**, 99 (1989)]. Injections of the vehicle and capsaicin were administered by means of a 0.5-ml insulin syringe with a 28-g needle in a volume of 20 μl. All injections were given intradermally into the center of the plantar surface of one hindpaw that was selected randomly. Noxious mechanical stimulation was applied to the plantar surface of one hindpaw by means of a hemostat with serrated tips. The hemostat was used to pinch a small area of skin (approximately 5 mm in length). With the hemostat locked in its tightest adjustable position, the skin was pinched for a duration of 30 s, after which the hemostat was removed.
11. After the appropriate survival time, the rats were perfused through the ascending aorta with 500 ml of 0.1 M phosphate-buffered saline (PBS) (pH 7.4, 22°C), followed by 1000 ml of PBS containing 4% formaldehyde and 12.5% picric acid (pH 7.4, 4°C). After perfusion, the lumbar spinal cord (L4) was removed, blocked in the transverse plane, postfixed in PBS containing 4% formaldehyde and 12.5% picric acid (pH 7.4, 4°C, 2 hours), and placed in PBS containing 30% sucrose (pH 7.4, 4°C, 24 hours). Next, the spinal cords were serially sectioned at a thickness of 60 μm on a sliding microtome and collected in PBS. The sections were washed three times in PBS containing 1% normal goat serum and 0.3% Triton X-100 (pH 7.4, 22°C), then incubated for 12 hours in the above solution containing the SPR antibody (no. 11884-5) at a concentration of 1:5000 (pH 7.4, 22°C). The antibody used in our study was raised against a 15–amino acid peptide sequence ($SPR_{393-407}$) at the COOH-terminus of the rat SPR. The immunogen con-

sisted of synthetic peptide conjugated to bovine thyroglobulin with the use of glutaraldehyde. The antiserum recognized a protein band of 80 to 90 kD on protein immunoblots of membranes prepared from cells transfected with the rat SPR [S. R. Vigna *et al.*, *J. Neurosci.* **14**, 834 (1994)]. The cells could be immunostained with the antiserum, and the staining could be blocked by preabsorbing the antiserum with $SPR_{393-407}$. After the incubation with the primary antibody, the tissue sections were washed for 30 min at 22°C in PBS (pH 7.4) and then incubated in the secondary antibody solution (pH 7.4) for 2 hours at 22°C. This secondary antibody solution was identical to the primary antibody solution, except that cyanine (Cy3)–conjugated donkey anti–rabbit immunoglobulin G (no. 711-165-152; Jackson ImmunoResearch Labs, West Grove, PA) was present (at a concentration of 1:600) instead of the SPR antibody. Finally, the tissue sections were washed for 20 min in PBS (pH 7.4, 22°C), mounted on gelatin-coated slides, and coverslipped with PBS-glycerine containing 1.0% *p*-phenylenediamine to reduce photobleaching.

12. To examine the sites of internalization within the cell, sections were examined with an MRC-600 Confocal Imaging System (Bio-Rad, Boston, MA) and with an Olympus BH-2 microscope equipped for epifluorescence (Olympus America, Lake Success, NY). Both of the microscopes were set up as previously described [T. C. Brelje, D. W. Scharp, R. L. Sorenson, *Diabetes* **38**, 808 (1989); P. W. Mantyh *et al.*, *J. Neurosci.* **15**, 152 (1995)]. To determine the percentage of neurons showing intense internalization, sections were examined with a Leitz Orthoplan II microscope (Ernst Leitz Wetzler, Germany) equipped for fluorescence. To determine the total number of cell bodies showing SPR-positive internalization, immunostained sections were viewed through a 1.0-cm^2 eyepiece grid that was divided into 100 1-mm by 1-mm units, and the total number of SPR-positive cell bodies and the number of SPR-positive cell bodies showing significant SPR-positive internalization were counted. SP-induced internalization has been shown to be blocked by nonpeptide SPR antagonists in transfected cells [A. M. Garland, E. F. Grady, D. G. Payan, S. R. Vigna, N. W. Bunnett, *Biochem. J.* **303**, 177 (1994)] and in cultured spinal neurons (*19*).
13. To determine whether the capsaicin-induced SPR internalization in lamina I neurons was dose dependent, 0.1, 1.0, 100, and 300 μg of capsaicin was injected into one hindpaw of rats. The percentage of neurons showing significant internalization (>20 endosomes per cell body) was examined 1 min later. The percentage of L4 lamina I neurons showing significant internalization was 26, 54, 55, and 70% for 0.1, 1.0, 100, and 300 μg of capsaicin, respectively.
14. We have consistently observed an increase in total apparent SPR immunoreactivity per cell or per field after either exogenous injection of SP in the spinal cord (*19*) or after increased release of endogenous SP as observed here. Although this observation may reflect a change in the accessibility of the antiserum to the SPR or a change in SPR immunoreactivity related to possible changes in receptor conformation after agonist binding, both of these possibilities seem unlikely. The most plausible explanation is that the increase in total SPR-positive immunoreactivity observed after SP binding simply reflects the concentration of SPRs into small membrane-bound endosomes, which are present in confocal slices in greater amounts than is plasma membrane, in which the SPR is located before peptide binding. This interpretation is supported by the observation that an early step in agonist-induced endocytosis is the migration of receptors in the plane of the plasma membrane to pits (*2*), thereby effectively concentrating the receptors in the membranes destined to become endosomal.
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20. We thank A. Georgopoulos for helpful comments, R. Elde and the Biomedical Imaging Processing Laboratory for use of the confocal microscope, and C. Garret for a gift of RP-67580. Supported by NIH grants NS23970, NS14627, NS21445, DA08973, NS31223, and GM15904 and by a Veterans Administration Merit Review.

30 September 1994; accepted 2 March 1995

Integration of Visual and Linguistic Information in Spoken Language Comprehension

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Psycholinguists have commonly assumed that as a spoken linguistic message unfolds over time, it is initially structured by a syntactic processing module that is encapsulated from information provided by other perceptual and cognitive systems. To test the effects of relevant visual context on the rapid mental processes that accompany spoken language comprehension, eye movements were recorded with a head-mounted eye-tracking system while subjects followed instructions to manipulate real objects. Visual context influenced spoken word recognition and mediated syntactic processing, even during the earliest moments of language processing.

The two essential properties of language are that it refers to things in the world and that its grammatical structure can be characterized independently of meaning or reference (*1*). The autonomy of grammatical structure has led to a long tradition in psycholinguistics according to which it is assumed that the brain mechanisms responsible for the rapid syntactic structuring of continuous linguistic input are "encapsulated" from other cognitive and perceptual systems (*2*), much as early visual processing often is claimed to be structured by autonomous processing modules (*3*). This contrasts with a second tradition by which language processing is inextricably tied to reference and relevant behavioral context (*4*). The primary empirical evidence that syntactic processing is modular is that brief syntactic ambiguities, which arise because language unfolds over time, appear to be initially resolved independently of prior context. Unfortunately, it has been impossible to perform the crucial test to determine whether strongly constraining nonlinguistic information can influence the earliest moments of syntactic processing, because experimental techniques that provide fine-grained temporal information about spoken language comprehension could not be used in natural contexts. However, by recording eye movements (*5*) as participants followed instructions to move objects (for example, "Put the apple that's on the towel in the box"), we were able to monitor the ongoing comprehension process on a millisecond time scale. This enabled us to observe the rapid mental processes that accompany spoken language comprehension in natural behavioral contexts in which the language had clear real-world referents.

Our initial experiments demonstrated that individuals processed the instructions incrementally, making saccadic eye movements to objects immediately after hearing relevant words in the instruction. Thus the eye movements provided insight into the mental processes that accompany language comprehension. For example, when asked to touch one of four blocks that differed in marking, color, or shape, with instructions such as "Touch the starred yellow square," a person made an eye movement to the target block an average of 250 ms after the end of the word that uniquely specified the target with respect to the visual alternatives (for example, after "starred" if only one of the blocks was starred, and after "square" if there were two starred yellow blocks). With more complex instructions, individuals made informative sequences of eye movements that were closely time-locked to words in the instruction that were relevant to establishing reference. In one experiment, subjects were given a complex instruction such as "Put the five of hearts that is below the eight of clubs above the three of diamonds," with a display composed of seven miniature playing cards, including two fives of hearts. As the person heard "the five of hearts," she looked at each of the two potential referents successively. After hearing "below the," she immediately

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