been questioned because many of the conserved acidic amino acids in the ligand-binding repeats coordinate Ca^{2+} and are completely or partially buried in a Ca^{2+} cage. Hence they are presumably unavailable to bind to apoB100 or apoE. Instead, a hydrophobic concave face on the opposite side of the Ca^{2+} cage was proposed to interact with the lipoproteins (*6*).

The crystal structure reported by Rudenko *et al.* (*2*) resolves this conundrum by illustrating that some conserved acidic amino acids that coordinate Ca^{2+} also participate in the formation of salt bridges with basic residues of the β propeller. Although the negative charge potential is somewhat attenuated, the three disulfide bonds and the Ca^{2+} coordination lock the negatively charged side chains of R4 and R5 in place for optimal interaction with the basic residues of the β-propeller region (*2*).

Rudenko *et al.* (*2*) point out that the ligand-binding repeats are not in contact with each other and can accommodate different-sized ligands. ApoE (relative molecular mass 33,000) and apoB100 (relative molecular mass 550,000) differ dramatically in size and have no common structural features or amino acid sequence similarity with the exception of a short sequence that serves as the receptor binding site and main proteoglycan binding site in both (*5*, *7*, *8*). Extensive studies on apoE show that basic residues in this region are critical for receptor binding (*4*, *5*, *9*), and a three-dimensional structure of the 22-kD LDLR binding domain of apoE shows that the receptor binding site is a positively charged, amphipathic helix (*10*).

The larger apoB100 is less well understood, but an analogous sequence is the likely site for receptor binding. Mutation of basic amino acids in this site to neutral amino acids abolishes receptor binding (*8*). Because only one small site common to both apoE and apoB100 appears critical for receptor binding, it is likely these proteins have critical interactions with only one or two ligand-binding repeats of the LDLR. This is analogous to the β-propeller, which only interacts with two ligand-binding repeats (R4, R5).

The model of acidic-triggered ligand release by binding to an alternate tethered site will probably be the paradigm for other members of the LDLR family. Will other receptors engaged in receptor-mediated endocytosis outside of the LDLR family have a similar mechanism? Finally, although this study provides insights into the binding of the LDLR with its ligands, a definitive answer will only come from the cocrystallization of a receptor-binding active fragment of apoE with the seven ligand-binding repeats of the LDLR.

References

1. M. S. Brown, J. L. Goldstein, *Science* **232**, 34 (1986).
2. G. Rudenko *et al.*, *Science* **298**, 2353 (2002); published online 28 November 2002 (10.1126/science.1078124).
3. C. G. Davis *et al.*, *Nature* **326**, 760 (1987).
4. R. W. Mahley, *Science* **240**, 622 (1988).
5. K. H. Weisgraber, *Adv. Protein Chem.* **45**, 249 (1994).
6. D. Fass, S. Blacklow, P. S. Kim, J. M. Berger, *Nature* **388**, 691 (1997).
7. J. Borén *et al.*, *J. Clin. Invest.* **101**, 2658 (1998).
8. J. Borén *et al.*, *J. Clin. Invest.* **101**, 1084 (1998).
9. R. W. Mahley, S. C. Rall Jr., *Annu. Rev. Genomics Hum. Genet.* **1**, 507 (2000).
10. C. Wilson, M. R. Wardell, K. H. Weisgraber, R. W. Mahley, D. A. Agard, *Science* **252**, 1817 (1991).

PERSPECTIVES: CIRCADIAN RHYTHMS

Carbon Monoxide and Clocks

Darren Boehning and Solomon H. Snyder

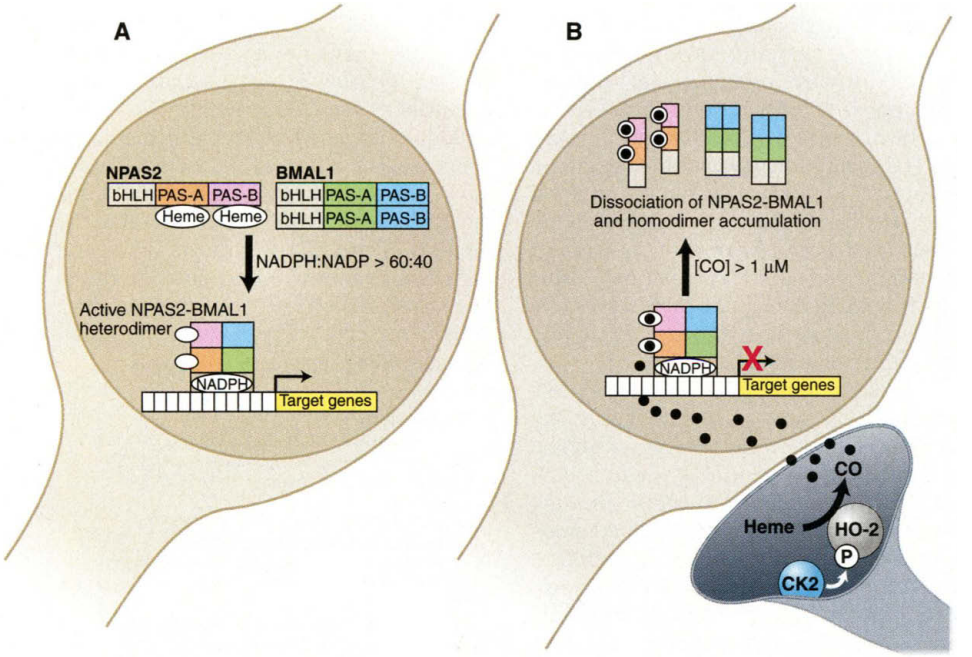
The gases nitric oxide (NO) and carbon monoxide (CO) are increasingly appreciated as major neurotransmitters (*1*). One of the principal means by which NO and CO transmit signals between neurons is through binding to a heme moiety at the active site of soluble guanylyl cyclase. This enzyme then becomes activated, leading to an increase in the intracellular second messenger molecule cGMP (guanosine 3′,5′-monophosphate). NO and CO have been implicated in long-term neural alterations such as learning and memory, and thus it has been presumed that these gases could influence events in the nucleus such as transcription. Independent lines of research have shown that the proteins Clock and NPAS2 are transcription factors that regulate circadian rhythms. On page 2385 of this issue, Dioum *et al.* (*2*) unite the fields of circadian rhythms and neurotransmission by showing that NPAS2 is a hemoprotein whose DNA binding activity is selectively regulated by CO.

NPAS2 (neuronal PAS domain protein 2) was first identified as a member of the basic helix-loop-helix (bHLH) family of transcription factors (*3*). When Takahashi and colleagues (*4*, *5*) identified Clock as a crucial regulator of circadian rhythms, they noted its close sequence similarity to NPAS2. Clock and NPAS2 regulate the activating portion of the circadian transcriptional feedback cycle by forming a heterodimeric complex with another bHLH transcription factor, BMAL1. NPAS2-BMAL1 and Clock-BMAL1 heterodimers direct the transcription of period (Per) and cryptochrome (Cry) proteins, which are the negative regulatory components of the circadian clock. Per and Cry inactivate the Clock-BMAL1 and NPAS-BMAL1 heterodimers, thus completing the transcriptional loop.

One of the salient features of circadian clocks is their entrainment by environmental stimuli such as light, temperature, activity, and food intake. The molecular mechanisms that enable environmental stimuli to abruptly alter circadian rhythms remain obscure. However, modulation of Clock and NPAS2 activity according to the redox state of the cell may provide a clue. The reduced cofactors NADH and NADPH (nicotinamide adenine dinucleotide and its phosphate) greatly enhance binding of Clock-BMAL1 and NPAS2-BMAL1 heterodimers to DNA, whereas the oxidized forms of the same molecules, NAD and NADP, inhibit the DNA binding of these dimers (*6*). Alterations in DNA binding activity occur abruptly with modest changes in the ratio of oxidized to reduced cofactors. This implicates the cofactors as molecular switches that direct NPAS2-BMAL1 and Clock-BMAL1 dimers to bind to DNA in response to changes in cellular redox state.

In their current study, Dioum *et al.* (*2*) have discovered a new way in which NPAS2 responds to environmental stimuli. PAS domains—modules of 130 amino acids previously characterized in organisms ranging from bacteria to mammals—respond to variations in stimuli including oxygen, voltage, light, and redox potential. The PAS domains of some bacterial proteins operate as oxygen sensors via a heme prosthetic group. During purification of NPAS2, Dioum and colleagues discovered that both PAS domains of the NPAS2 monomer contain a heme molecule. Heme was not required for the NPAS2-BMAL1 dimer to bind to DNA, nor did it affect the regulation by redox cofactors. The authors noted that the absorption spectrum of heme-bound NPAS2 resembles that of gas-sensing proteins from bacteria, including the CO-sensor protein CooA from *Rhodospirullum rubrum*. Therefore, they examined the effects of CO, NO, and O_2 on the DNA binding activity of NPAS2. CO bound to heme-containing NPAS2 with a dissociation constant of ~1 μM but failed to bind to NPAS2 lacking heme. In addition, CO inhibited the DNA binding capacity of NPAS2-BMAL1 heterodimers with a similar molar potency. By contrast, NO did not bind to NPAS2 at physi-

The authors are in the Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA. E-mail: ssnyder@jhmi.edu



Gas-regulated gene transcription. (**A**) NPAS2 is a member of the bHLH-PAS domain family of transcription factors. The bHLH domain is important for forming dimers and for binding to DNA, whereas both PAS domains contain a heme prosthetic group (*2*). Upon changes in cellular redox state, NPAS2 forms a heterodimer with BMAL1, another bHLH-PAS transcription factor, and binds to DNA, activating the transcription of various target genes, including those critical for circadian rhythms. Inactive BMAL1 normally exists as a homodimer. (**B**) CO is generated in neurons exclusively by HO-2, which is activated by CK2 (*10*). Dioum *et al.* (*2*) demonstrate in vitro that the DNA binding activity of NPAS2-BMAL1 is greatly reduced by binding of CO to the heme molecule of NPAS2. Because CO can passively diffuse through cell membranes, CO generated by one neuron may inactivate NPAS2-BMAL1 heterodimers in a target neuron.

ologic concentrations, and O_2 bound irreversibly. These findings contrast with those for soluble guanylyl cyclase, which is activated by nanomolar concentrations of NO but requires high micromolar concentrations of CO for activation. The Dioum *et al.* study of CO regulation of NPAS2 is all in vitro; thus, it would be prudent to ascertain whether CO regulates NPAS2 in intact cells and organisms. If CO regulates NPAS2 in vivo, it would be the first example of a mammalian protein selectively regulated by CO.

Might CO, acting as a neurotransmitter, be a physiologic regulator of NPAS2? CO is formed in neurons exclusively by heme oxygenase–2 (HO-2), which cleaves the heme ring, releasing biliverdin, expelling iron from the heme ring, and releasing a one-carbon fragment as CO. HO-2 occurs in neuronal populations in numerous parts of the brain and partially overlaps with the distribution of NPAS2 (*3, 7–9*). HO-2 activity is dynamically regulated by neuronal impulses through a kinase cascade in which protein kinase C activates casein kinase 2 (CK2), which in turn phosphorylates and activates HO-2 (*10*). HO-2 activity generates low micromolar concentrations of CO in the intact brain, which is sufficient to regulate the DNA binding activity of NPAS2 (*11*).

A gaseous neurotransmitter such as CO is a particularly attractive candidate for regulating a transcription factor that is found in both the cytoplasm and nucleus. Most neurotransmitters are highly charged molecules that bind to receptor proteins on the external surface of the cell membrane. By contrast, CO is freely diffusible, and its known target, soluble guanylyl cyclase, resides in the cytoplasm. Presumably, CO could also diffuse into the nucleus. Dioum and colleagues point out that the production of CO ebbs and flows according to the circadian rhythm of heme metabolism (heme breakdown requires HO-2). Although Dioum and co-workers only examined interactions of CO with NPAS2, Clock displays significant sequence homology to NPAS2 in both PAS domains, and therefore might also bind to heme and respond to CO.

Clock was discovered by Takahashi and colleagues (*5*) in a mass mutational analysis of mice. These authors sought to discover new genes regulating circadian rhythms. Clock is highly concentrated in the suprachiasmatic nucleus, and mice deficient in Clock have profound circadian rhythm defects (*5*). However, Clock is also found in many other brain regions. Surprisingly, NPAS2 is not present in the suprachiasmatic nucleus but is concentrated in the somatosensory cerebral cortex, especially in whisker barrel fields (which mediate tactile sensation) and in areas mediating emotional behavior (*3, 8*). Behavioral studies of NPAS2-deficient mice indicate selective abnormalities in emotional behavior and memory triggered by tactile stimuli (*8*). Thus, Clock and NPAS2 may regulate neuronal activity independently of the circadian clock. This is supported by the observation that more than 100 genes are regulated by NPAS2, only a portion of which are circadian-linked (*12*).

Might NPAS2 and Clock mediate behavioral influences of the CO neurotransmitter system? Studies with mice deficient in HO-2 have established functional roles for CO in the peripheral autonomic nervous system. These knockout mice manifest a major loss of nonadrenergic/noncholinergic (NANC) neurotransmission in the intestines and urogenital system, where CO is a transmitter (*13–15*). NPAS2 and Clock are expressed in tissues outside of the brain, but their detailed localization in the autonomic nervous system has yet to be explored. In behavioral studies, HO-2–deficient mice are normal in olfactory ability, motor coordination, motor strength, and visual activity (*14*). Their only behavioral abnormality is observed in an open-field model that assesses "anxiety" in terms of the reluctance of animals to move about in an arena. In this model, HO-2 knockout mice display increased movement. Similarly, the mutant animals display less "fear of falling" when suspended from a wire. Conceivably, such alterations in emotional behavior relate in some way to the behavioral abnormalities of NPAS2-deficient animals. A direct link between these two animal models awaits studies of NPAS2 activities, including binding to DNA, in HO-2 knockout animals.

The discovery that a neurotransmitter may directly regulate a transcription factor reveals a new mechanism for transducing signals that is particularly appropriate for a gaseous messenger. Explorations of such functions for NO and hydrogen sulfide, another recently discovered gaseous modulator, may reveal that multiple gases regulate transcriptional activity in the brain.

References

1. D. E. Baranano *et al.*, *Trends Neurosci.* **24**, 99 (2001).
2. E. M. Dioum *et al.*, *Science* **298**, 2385 (2002); published online 21 November 2002 (10.1126/science.1078456).
3. Y. D. Zhou *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 713 (1997).
4. D. P. King *et al.*, *Cell* **89**, 641 (1997).
5. M. H. Vitaterna *et al.*, *Science* **264**, 719 (1994).
6. J. Rutter *et al.*, *Science* **293**, 510 (2001).
7. A. Verma *et al.*, *Science* **259**, 381 (1993).
8. J. A. Garcia *et al.*, *Science* **288**, 2226 (2000).
9. S. R. Vincent *et al.*, *Neuroscience* **63**, 223 (1994).
10. D. Boehning *et al.*, paper presented at the 32nd annual meeting of the Society for Neuroscience, Orlando, Florida, 7 October 2002.
11. T. Ingi *et al.*, *J. Neurosci.* **16**, 5621 (1996).
12. M. Reick *et al.*, *Science* **293**, 506 (2001).
13. R. Zakhary *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14848 (1997).
14. A. L. Burnett *et al.*, *Nature Med.* **4**, 84 (1998).
15. L. Xue *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1851 (2000).

CREDIT: KATHARINE SUTLIFF/*SCIENCE*