well as the cytosolic protein GFP, to the AIS. However, additional motifs probably play a role in establishing the differential distribution of certain types of sodium channel in vivo. We also obtained evidence for the association of ankyrin G with the motif we have identified. From a mechanistic point of view, ankyrin G could be primarily involved in anchoring sodium channels at the plasma membrane. However, we cannot exclude the possibility that the two partners could be preassembled early in biogenesis and cotransported during sorting, as observed in the case of presynaptic proteins (*29, 30*).

Note added in proof: During revision of this report, Lemaillet *et al*. (*31*) identified a conserved ankyrin-binding motif located within the AIS motif.

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Supporting Online Material

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Figs. S1 to S3

1 April 2003; accepted 12 May 2003

Facilitation of Spinal NMDA Receptor Currents by Spillover of Synaptically Released Glycine

Seifollah Ahmadi,[1*] Uta Muth-Selbach,[2*] Andreas Lauterbach,[1*] Peter Lipfert,[2] Winfried L. Neuhuber,[3] Hanns Ulrich Zeilhofer[1]†

In the mammalian CNS, *N*-methyl-D-aspartate (NMDA) receptors serve prominent roles in many physiological and pathophysiological processes including pain transmission. For full activation, NMDA receptors require the binding of glycine. It is not known whether the brain uses changes in extracellular glycine to modulate synaptic NMDA responses. Here, we show that synaptically released glycine facilitates NMDA receptor currents in the superficial dorsal horn, an area critically involved in pain processing. During high presynaptic activity, glycine released from inhibitory interneurons escapes the synaptic cleft and reaches nearby NMDA receptors by so-called spillover. In vivo, this process may contribute to the development of inflammatory hyperalgesia.

Glycine serves a dual role in central neurotransmission. It is not only the primary inhibitory neurotransmitter in the spinal cord and brain stem, but also an obligatory coagonist at excitatory glutamate receptors of the NMDA type (*1–3*). Although glycine concentrations in the cerebrospinal fluid are in the micromolar range (*4–6*), which would fully activate this site (*7*), glycine transporters located on glial cells can lower extracellular glycine in the vicinity of synaptic NMDA receptors to subsaturating concentrations (*8–10*). A physiological role of glycine in the modulation of NMDA receptor–mediated synaptic transmission therefore appears possible. We have previously shown that in the spinal cord dorsal horn the neuropeptide nocistatin (NST) (*11*) specifically inhibits the synaptic release of glycine [and γ-aminobutyric acid (GABA)] without affecting the release of L-glutamate (*12*). We have now used this peptide to show that spinal NMDA receptors can be facilitated by spillover of synaptically released glycine both in spinal cord slices and in vivo.

Glutamatergic and glycinergic input was activated simultaneously by electrical field stimulation. Excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were isolated pharmacologically and recorded from superficial dorsal horn neurons in transverse rat spinal cord slices (*13*). At a saturating concentration of 10 μM (*12*), NST reversibly reduced the amplitude of glycinergic IPSCs (gly-IPCSs) by 36.5 ± 7.2% ($n = 15$, $P \leq 0.01$, paired t test). By contrast, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor–mediated EPSCs (AMPA-EPSCs) remained completely unchanged [96.7 ± 4.3% of control amplitudes, $n = 11$, $P = 0.33$, see also (*12*)], whereas NMDA-EPSCs were reversibly reduced by 29.2 ± 3.2% ($P \leq 0.001$) in 15 out of 17 neurons (Fig. 1A).

Inhibition of NMDA-EPSCs was completely prevented when the slices were continuously superfused with glycine (100 μM) to permanently saturate NMDA receptors (Δ NMDA-EPSC: 0.16 ± 5.9%, $n = 6$, $P = 0.67$, ANOVA followed by Scheffé's post hoc test). A significant contribution of GABA acting on ionotropic $GABA_A$ or G protein–coupled $GABA_B$ receptors could be excluded, because all experiments were performed in the continuous presence of the $GABA_A$ receptor blocker bicuculline (10 μM) and additional application of the $GABA_B$ receptor antagonist CGP55845 (100 μM) did not prevent inhibition (Δ NMDA-EPSC: 37.2 ± 10.2%, $n = 8$, $P \leq 0.01$) (Fig. 1B).

A reduction in glycine release could interfere with NMDA receptor–mediated synaptic transmission by at least two mechanisms. First, glycine might act through presynaptic strychnine-sensitive glycine receptors to facilitate the release of glutamate, an action that has been demonstrated in the brainstem (*14*). A significant contribution of this process to the inhibition of NMDA-EPSCs described here appears unlikely, because NMDA-EPSCs were recorded in the presence of strychnine (300 nM). Second, glycine might

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Erlangen-Nürnberg, Fahrstrasse 17, D-91054 Erlangen, Germany. [2]Klinik für Anästhesiologie, Moorenstrasse 5, Universitätsklinikum Düsseldorf, D-40225 Düsseldorf, Germany. [3]Institut für Anatomie, Krankenhausstrasse 9, Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: zeilhofer@pharmakologie.uni-erlangen.de

act postsynaptically by activating the glycine binding site of NMDA receptors. To prove this hypothesis, we used D-serine, another agonist at the glycine binding site of NMDA receptors (*15*, *16*), which is ineffective at strychnine-sensitive glycine receptors. Superfusion of the slices with D-serine (100 μM) completely prevented inhibition of NMDA-EPSCs (Δ NMDA-EPSC: –1.9 ± 4.0%, $n = 9$, $P = 0.47$), whereas L-serine was ineffective (Δ NMDA-EPSC: –60.0 ± 6.6%, $n = 8$, $P \leq 0.01$) (Fig. 1C).

Results obtained with D-serine also argue against the possibility that glycine directly activated particular subtypes of NMDA receptors containing NR3A or NR3B subunits, which are activated by glycine in the absence of glutamate, but blocked by D-serine (*17*). D-Serine did not inhibit NMDA-EPSCs in our experiments (Δ NMDA-EPSC: –3.3 ± 5.8%, $n = 4$; $P = 0.39$, four presynaptic stimuli). A direct glycine-independent effect of NST on NMDA or glycine receptors could also be excluded, because NST did not inhibit membrane currents evoked by exogenous NMDA or glycine [fig. S1 and (*12*)].

Glycinergic interneurons could either form direct synaptic contacts with postsynaptic NMDA receptors or, alternatively, synaptically released glycine could diffuse out of the synaptic cleft of glycinergic synapses and could reach

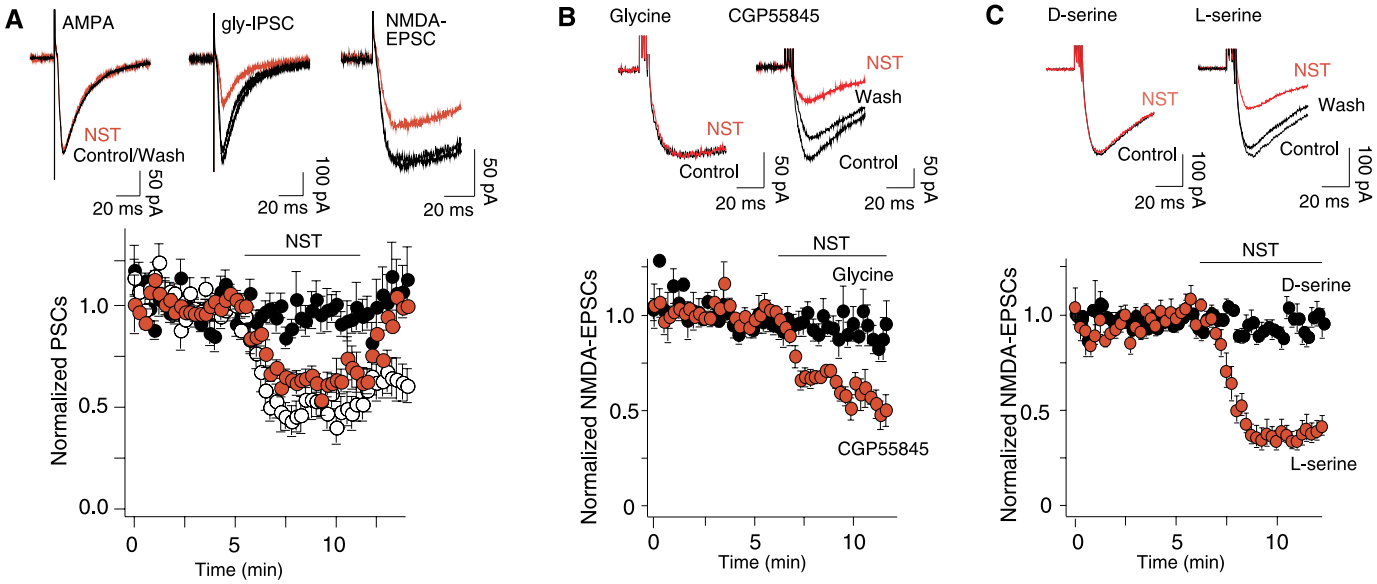


Fig. 1. NST-mediated inhibition of gly-IPSCs and NMDA-EPSCs. (**A**) (Top) Averages of ten consecutive current traces recorded under control conditions, in the presence of NST (10 μM) and after its removal. (Bottom) Time course of inhibition (means ± SEM). AMPA-EPSCs (●, $n = 11$) and NMDA-EPSCs (red circles, $n = 15$) and gly-IPSCs (○, $n = 15$). (**B**) NST-mediated inhibition of NMDA-EPSCs was prevented by continuous superfusion of slices with glycine (100 μM, $n = 6$), but not with the $GABA_B$ receptor antagonist CGP55845 (100 μM, $n = 8$). (**C**) D-Serine (100 μM) mimicked the effect of glycine ($n = 9$). L-Serine (100 μM) was without effect ($n = 8$). Recordings shown in (A) were made at room temperature (20°C) and after a single synaptic stimulation, (B and C) were done at 35°C and after four synaptic stimulations at 500Hz.

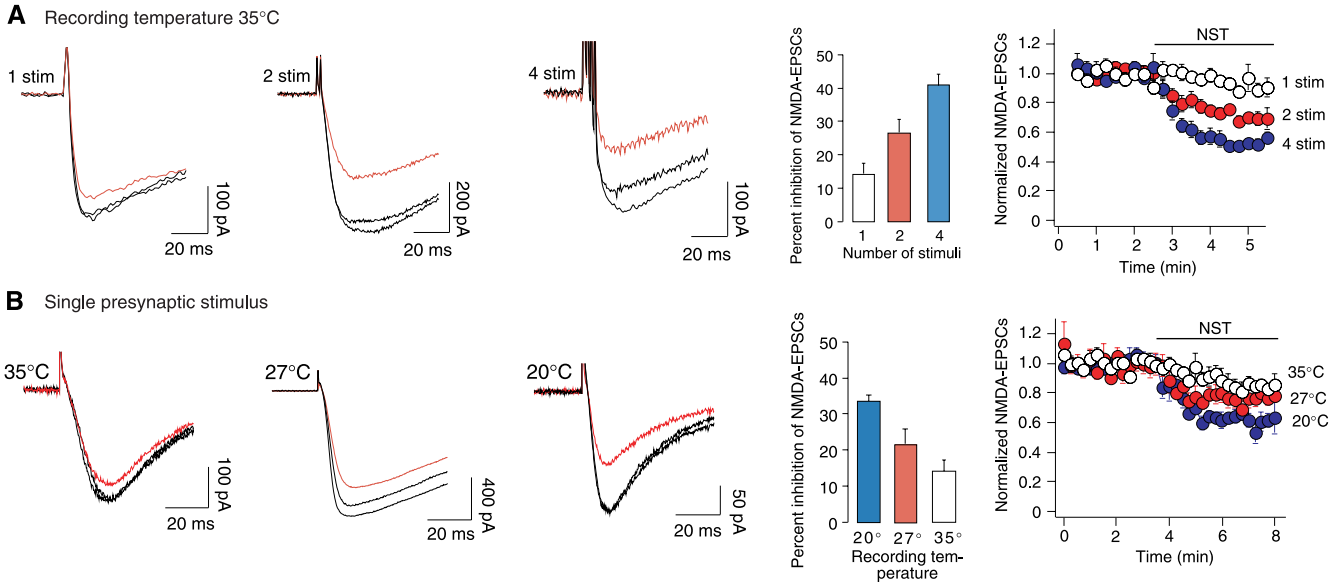


Fig. 2. Inhibition of NMDA-EPSCs by NST is due to reduced glycine spillover. NMDA-EPSC inhibition depended on the number of presynaptic stimuli (**A**) and on the recording temperature (**B**). Left, averaged current responses; middle, statistical analysis; and right, time course (for six, five, and eight cells for one, two, and four stimuli; for five, five, and six cells, for recording temperatures of 20°C, 27°C, and 35°C, respectively).

nearby NMDA receptors through diffusion, a process called spillover (*1*, *18*, *19*). Inhibition of NMDA-EPSCs by NST exhibited two characteristic features of spillover (*19*, *20*). Inhibition increased with decreasing recording temperatures and with an increased amount of glycine released (Fig. 2). At a near-physiological temperature of 35°C and when four presynaptic stimulations were applied, inhibition was 43.4 ± 3.3% ($n = 8$, $P \leq 0.01$), which is similar to what was obtained after a single stimulus at 20°C (41.5 ± 2.0%, $n = 5$). However, when a single stimulus was applied at 35°C, inhibition was reduced to 10.6 ± 3.4% ($n = 5$). These results indicate that synaptically released glycine reached NMDA receptors after it had diffused over a certain distance where it was partially taken up by temperature-sensitive glycine transporters (*21*), which at physiological temperatures limit the diffusion of glycine. However, under conditions of repeated presynaptic stimulation, synaptically released glycine can apparently overwhelm these transporters to facilitate synaptic NMDA receptors. The modulation of NMDA-EPSCs by NST also demonstrates that glycine binding to NMDA receptors is not saturated under resting conditions [see also (*22*)]. Indeed, when a pure glutamatergic input was activated by dorsal root stimulation, D-serine (100 μM) potentiated NMDA-EPSCs by 55.4 ± 11.0% ($n = 6$, $P = 0.024$, fig. S2), which suggests that resting glycine was near the median effective concentration (EC_{50}).

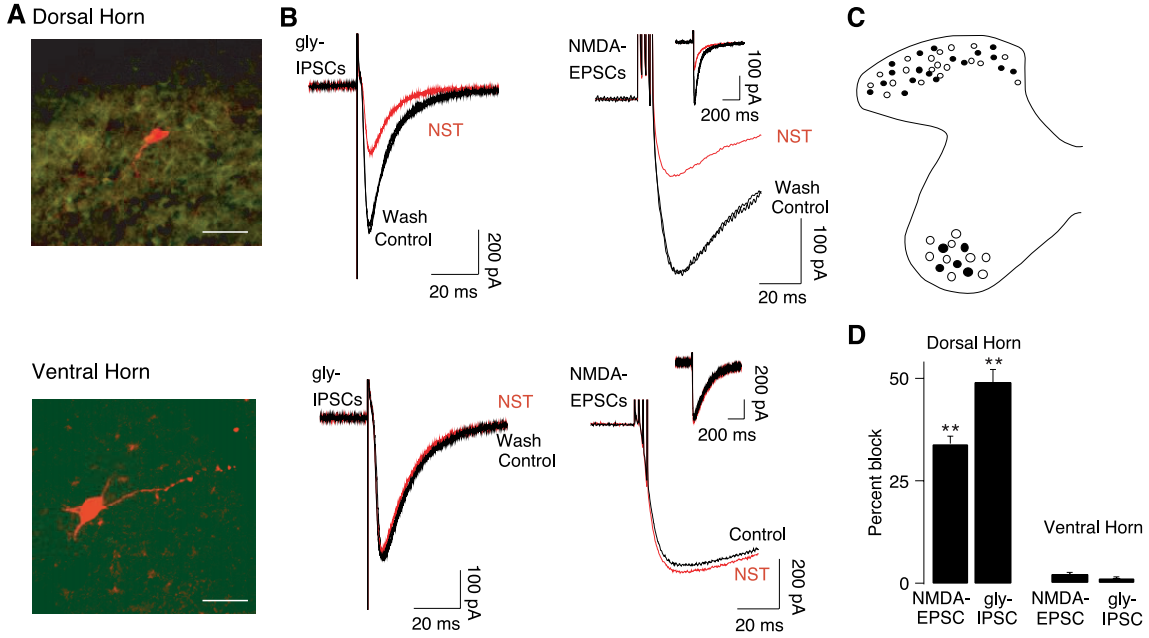
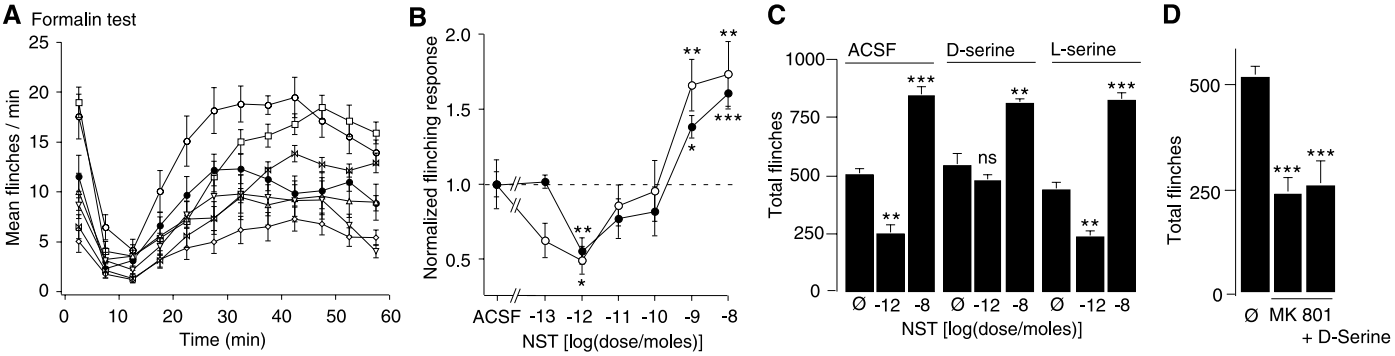


Fig. 3. Effects of NST on gly-IPSCs and NMDA-EPSCs in the dorsal versus ventral horn. (**A**) Biocytin-labeled neurons in the superficial layers of the dorsal horn (top) and in the ventral horn, where recordings were made from large diameter presumed motor neurons (bottom). Scale bars, 20 μm (dorsal horn) and 40 μm (ventral horn). (**B**) Averaged current responses in the dorsal (top) and ventral horn (bottom). (**C**) Locations of the recorded neurons, ○, gly-IPSCs, ●, NMDA-EPSCs. (**D**) Statistical analysis. $^{**}P \leq 0.01$, paired *t* test, dorsal horn, $n = 18$ and 19, NMDA-EPSCs and gly-IPSCs, respectively. Ventral horn, $n = 5$ and 9, NMDA-EPSCs and gly-IPSCs, respectively. Recordings were made at 35°C, after a single synaptic stimulus (gly-IPSCs) or after four stimuli (NMDA-EPSCs).



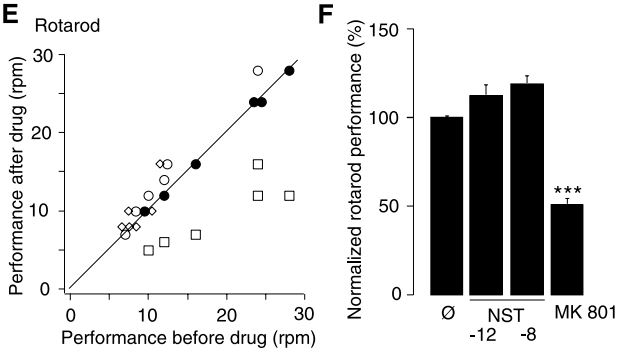


Fig. 4. Dose-dependent effects of NST on pain-related behavior in the rat formalin test. (**A**) Number of flinches per minute (means ± SEM) of rats treated with different doses of NST injected intrathecally versus time: 10 nmol (○),1 nmol (□), 100 pmol (△), 10 pmol (▽), 1 pmol (◇), 100 fmol (▷◁), vehicle (●). (**B**) Dose-response relationship for phase I (1 to 10 min, ○) and phase II (21 to 60 min, ●). Values (means ± SEM) normalized to vehicle-treated animals. (**C**) Total number of flinches (means ± SEM) in rats injected with either 10 nmol or 1 pmol of NST or vehicle (∅) and pretreated with ACSF, D-serine, or L-serine (each 100 μg). (**D**) Same as (C), but treated with vehicle or MK-801 (10 μg) and pretreated with D-serine (100 μg). (**E**) Motor performance of rats (rounds per minute) before (*x* axis) and after (*y* axis) treatment with 10 nmol (○) or 1 pmol (◇) NST, MK-801 (10 μg) (□), or vehicle (●). Each symbol represents an individual animal. (**F**) Statistical analysis (means ± SEM). $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns, not significant (ANOVA followed by Scheffé's post hoc test). $n \geq 6$ rats/group.

We tested whether NST inhibited glycinergic IPSCs and/or NMDA-EPSCs differently in different regions of the spinal cord. Inhibition of NMDA-EPSCs by NST was absent in the ventral horn, the site of spinal motor control (Fig. 3). Because NST was inactive also at ventral horn gly-IPSCs, this finding most likely reflects the lack of expression of NST receptors in the ventral horn and does not contradict the existence of glycine spillover in this region (*23*).

Spillover of a fast neurotransmitter to extrasynaptic receptors or receptors located in neighboring synapses has been reported previously (*24*). However, its significance for in vivo synaptic processing has remained elusive.

When NST is injected intrathecally, it modulates spinal pain processing (*11*, *12*). We tested NST in the rat formalin test, an animal model of tonic pain (*13*). Low (picomolar) doses of NST were antinociceptive, whereas higher (nanomolar) doses were pronociceptive (Fig. 4, A and B). At 10 nmol, NST increased the average number of flinches per minute to 173.9% and 159.8% of control in phase I (1 to 10 min) and II (21 to 60 min) of the formalin test, respectively ($P \leq 0.001$ and $P \leq 0.01$, $n = 6$ each). The lower dose of 1 pmol NST significantly reduced the number of flinches per minute to 55.1% and 49.3% ($P \leq 0.05$ and $P \leq 0.01$) in phase I and II, respectively. The reduction by NST of inhibitory neurotransmission probably underlies its pronociceptive effect (*12*). To test whether a reduced availability of glycine at NMDA receptors underlies the antinociceptive effect, we pretreated rats intrathecally with D-serine and, as a negative control, with L-serine (each 100 μg). Antinociception evoked by 1 pmol NST was selectively blocked by D-serine, whereas the pronociceptive effect of the higher dose (10 nmol) remained virtually unchanged in rats treated with either D- or L-serine (Fig. 4C). By contrast, analgesia induced with MK-801 (10 μg), an open channel blocker of NMDA receptors, was unaffected by D-serine (Fig. 4D). D-serine (and L-serine) per se had no significant effect in the formalin test (Fig. 4C), which suggests that glycine binding sites were saturated during intense nociceptive stimulation.

The use of NMDA receptor blockers as therapeutic agents has been hampered by their inhibitory effects in CNS areas other than the spinal cord dorsal horn, where they give rise to severe side effects (*25*, *26*). Because the synaptic effects of NST were limited to the sensory (dorsal) part of the spinal cord, we tested whether inhibition of NMDA receptor–mediated neurotransmission by NST would spare motor function (*13*). Neither the high dose (10 nmol) nor the low dose (1 pmol) of NST impaired motor performance in the rotarod test (Fig. 4, E and F). Unlike NST, MK-801 (10 μg) significantly reduced the rotarod performance (Fig. 4F).

Our finding that glycine released from inhibitory interneurons not only mediates synaptic inhibition, but also contributes to the facilitation of NMDA receptors adds to the emerging role of glycine as an excitatory neurotransmitter (*14*, *17*). During acute pain states, when glycinergic interneurons in the spinal cord dorsal horn are excessively activated, glycine can escape the synaptic cleft and reach nearby NMDA receptors. This spillover causes NMDA receptor facilitation, which apparently contributes to the excessive activation of NMDA receptors, which is thought to underlie the development of chronic pain (*27*). Furthermore, the finding that a suppression of this process induces analgesia reveals that spillover of a fast neurotransmitter in the CNS can indeed occur in vivo. A selective inhibition of this process in the dorsal horn may provide a new and promising strategy for the prevention of chronic pain.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/300/5628/2094/DC1
Materials and Methods
Figs. S1 and S2
References and Notes

27 February 2003; accepted 9 May 2003

Comprehensive Identification of Human bZIP Interactions with Coiled-Coil Arrays

John R. S. Newman* and Amy E. Keating†

In eukaryotes, the combinatorial association of sequence-specific DNA binding proteins is essential for transcription. We have used protein arrays to test 49^2 pairings of a nearly complete set of coiled-coil strands from human basic-region leucine zipper (bZIP) transcription factors. We find considerable partnering selectivity despite the bZIPs' homologous sequences. The interaction data are of high quality, as assessed by their reproducibility, reciprocity, and agreement with previous observations. Biophysical studies in solution support the relative binding strengths observed with the arrays. New associations provide insights into the circadian clock and the unfolded protein response.

In DNA binding transcription factors, autonomously folding domains are often responsible for the formation of homo- and heterodimeric protein complexes. Transcription factor dimerization can increase the selectivity of protein-DNA interactions and generate a large amount of DNA binding diversity from a relatively small number of proteins (*1*, *2*). Dimerization also leads to the establishment of complex regulatory networks (*3*), and dimers can interact with other proteins required for transcription (*4*). The bZIP transcription factors constitute an important class of eukaryotic DNA binding proteins in which dimerization has been shown to occur by way of coiled-coil regions. Although previous studies have described bZIP pairings and the biological effects of these associations (*3*, *5*–*9*), many substantive questions remain