Sensitization to Morphine Induced by Viral-Mediated Gene Transfer

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Repeated administration of morphine sensitizes animals to the stimulant and rewarding properties of the drug. It also selectively increases expression of GluR1 (an AMPA glutamate receptor subunit) in the ventral tegmental area, a midbrain region implicated in morphine action. By viral-mediated gene transfer, a causal relation is shown between these behavioral and biochemical adaptations: Morphine's stimulant and rewarding properties are intensified after microinjections of a viral vector expressing GluR1 into the ventral tegmental area. These results confirm the importance of AMPA receptors in morphine action and demonstrate specific locomotor and motivational adaptations resulting from altered expression of a single localized gene product.

Repeated exposure to drugs of abuse such as morphine, cocaine, or amphetamine increases their stimulant (*1*) and rewarding (*2*) properties in animal models of addiction. The molecular mechanisms underlying these sensitized locomotor and motivational responses are unknown, but the neural adaptations that result in sensitization in animal models are likely the same that result in some forms of addictive behavior in humans (*3*). The ventral tegmental area (VTA) of the midbrain contains the cell bodies of the mesolimbic dopamine system, which is a major neural substrate for many drugs of abuse (*4*) that has been implicated in behavioral adaptations that occur with repeated drug exposure (*1*). Repeated exposure to morphine increases expression of GluR1 (an AMPA receptor subunit; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) in the VTA without altering expression of other AMPA subunits (*5*). Because glutamatergic inputs regulate the activity of VTA dopamine neurons via AMPA receptors (*6*), it is possible that altered composition of glutamate receptors (*7*) in this region contributes to neural adaptations that underlie drug addiction. Although AMPA antagonists block drug sensitization (*8*), AMPA agonists and antagonists cannot be used to study the role of GluR1 specifically because they affect AMPA receptor function generally and therefore do not mimic morphine's actions. To establish a causal relation between GluR1 expression and sensitization, we assessed the consequences of directly and selectively increasing expression of GluR1 in the VTA by microinjecting a herpes simplex virus (HSV) vector that encodes this protein (HSV-GluR1).

We first evaluated the ability of viral-mediated expression of GluR1 in the VTA to alter morphine's stimulant properties. On day 0, the locomotor activity of rats was quantified in activity chambers (*9*) after subcutaneous (s.c.) administration of 1.0 mg/kg morphine, which causes a small increase in activity. Rats then received unilateral VTA microinjections of HSV-GluR1, HSV-GluR2 (expressing GluR2, an AMPA receptor subunit not regulated by morphine), or HSV-LacZ (expressing β-galactosidase, a control protein), or sham injections (*10*, *11*). After 2 days recovery, rats were retested with 1.0 mg/kg morphine (days 3 and 4). Rats given HSV-GluR1 were significantly more active in response to morphine on days 3 and 4 than rats given the other treatments (Fig. 1A, left); the overall increases in the activity of animals given HSV-GluR1 were approximately double those of the three other groups (Fig. 1A, right). Increased activity in rats given HSV-GluR1 was evident only with morphine treatment; when rats given HSV-GluR1 or HSV-LacZ were treated with saline, there were no differences in activity (Fig. 1B, left and right). Increased sensitivity to morphine was specific to up-regulation of GluR1 rather than due to a general effect on AMPA receptor subunits, because rats given HSV-GluR2 tended to be less active than those given HSV-LacZ or sham surgery (Fig. 1A, left and right).

Protein immunoblot analysis confirmed that GluR1 expression in the VTA (*12*) was significantly increased in rats given HSV-GluR1 (Fig. 2), reaching a level comparable to that observed previously after a regimen of repeated morphine treatment (*5*). Histological examination was used to further confirm viral-mediated gene expression. In rats given HSV-LacZ (Fig. 3A), expression of β-galactosidase (*13*) peaked between days 3 and 4, was restricted to an area in the injected VTA of ~1.5 mm in diameter, and was accompanied by minimal damage (Fig. 3B) indistinguishable from that caused by injection of vehicle (10% sucrose). Expression of the LacZ transgene dissipated by day 7, after a time course similar to that seen in vitro (*14*). In rats given HSV-GluR1, moderate numbers of

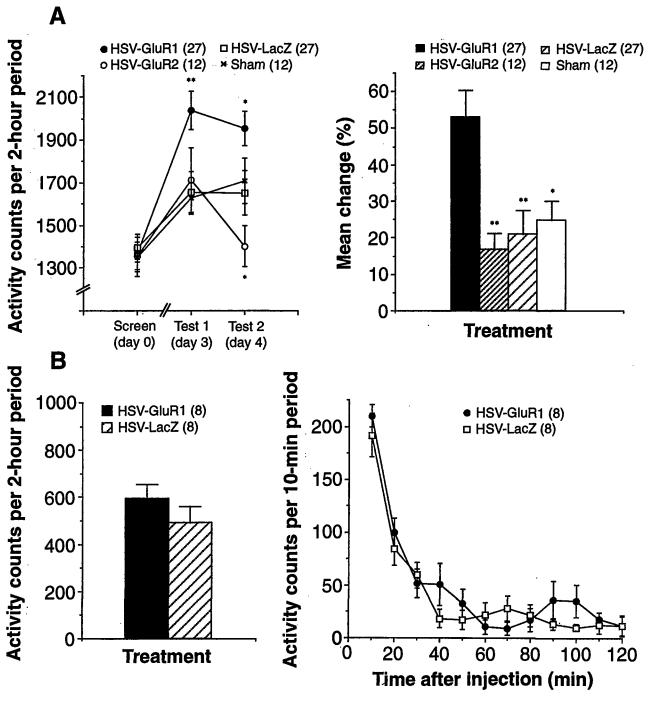

Fig. 1. Sensitivity to morphine after treatment with HSV vectors. (**A**) (Left) Rats given HSV-GluR1 into the VTA had heightened sensitivity to morphine's activating effects (mean ± SEM) (treatment × days interaction: $F_{6,148} = 3.82$, $P < 0.01$) on days 3 and 4. Rats given HSV-GluR2 had attenuated sensitivity on day 4. (Right) Overall, HSV-GluR1 doubled the locomotor-stimulating efficacy of morphine ($F_{3,74} = 6.32$, $P < 0.01$). (**B**) There were no significant differences in mean activity (left; $t_7 = 1.66$, not significant) or in the time course of activity (right; treatment × time interaction: $F_{11,154} = 1.02$, not significant) after saline. Number of rats in each group is in parentheses; $^*P < 0.05$, $^{**}P < 0.01$ (Fisher's t test).

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highly GluR1-immunoreactive cells (*15*) were seen in the VTA on the side of the injection (Fig. 3, C and E), whereas GluR1 was detected at normal low levels in the noninjected VTA. Similar low levels of GluR1 were seen in VTA injected with HSV-GluR2 or HSV-LacZ, or after sham surgery, confirming that increased GluR1 expression is not a nonspecific reaction to surgery or viral infection. Approximately 1000 cells showed high levels of GluR1 labeling after HSV-GluR1 microinjections. The time course of GluR1 expression was similar to that seen with HSV-LacZ: Expression was maximal on day 3 to 4 and had dissipated by day 7. A similar time course was observed in studies of HSV-GluR1 in spinal cord (*16*). Expression of GluR1 was associated with little toxicity: There was no evidence of damage to tyrosine hydroxylase (that is, dopamine)–containing cells in the VTA (*17*) of animals given HSV-GluR1 (Fig. 3D). To confirm that increased GluR1 expression resulted in formation of functional AMPA receptors, we stimulated fresh VTA tissue slices with kainate in a cobalt-containing perfusate (*18*). Cobalt uptake was evident in the injected VTA of rats given HSV-GluR1 only (Fig. 3F). Double-labeling immunohistochemistry (*19*) revealed that the large majority of GluR1-immunoreactive cells (Fig. 3G) were also immunoreactive for tyrosine hydroxylase (Fig. 3H), indicating infection of dopamine-containing cells. Increased GluR2 expression (*20*) was evident in rats given HSV-GluR2, but this subunit had higher basal levels of expression.

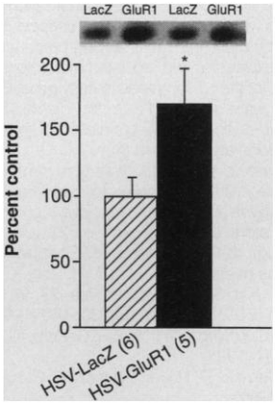

Fig. 2. Protein immunoblot of GluR1 in the VTA after treatment with HSV vectors. HSV-GluR1 significantly increased GluR1 immunoreactivity (t_9 = 2.40, $P < 0.05$). Data are expressed as percentage (mean ± SEM) of GluR1 expression after HSV-LacZ treatment.

To determine whether behavioral adaptations followed a time course similar to that of transgene expression, we tested some rats given HSV-GluR1 or HSV-LacZ with morphine only on days 7 and 8 after microinjection (when GluR1 expression had dissipated). Under these conditions, there were no differences between treatment groups (Fig. 4, left). However, when some rats given HSV-GluR1 or HSV-LacZ were tested with morphine on days 7 and 8 in addition to days 3 and 4, significant increases in sensitivity to morphine's locomotor-stimulating actions persisted in the group given HSV-GluR1 (Fig. 4, right), despite the fact that GluR1-labeling in the VTA had dissipated. Thus, when morphine is given while GluR1 expression in the VTA is elevated, increased sensitivity to morphine can outlast vector-induced increases in GluR1 expression, confirming a role for the VTA in the initiation of long-term changes in drug sensitivity.

We next examined the ability of increased expression of GluR1 in the VTA to alter morphine's rewarding properties using conditioned place preference, a classical conditioning paradigm (*21*) in which animals learn to associate the rewarding consequences of a drug with a particular environment. Rats given HSV-GluR1 into the rostral VTA spent more time in an environment associated with morphine administration than did control rats (Fig. 5), indicating a potentiation of morphine's rewarding properties. Because previous treatment with morphine intensifies its rewarding actions in this paradigm (*2*), these data suggest that the behavioral consequences of morphine pre-exposure are mimicked by

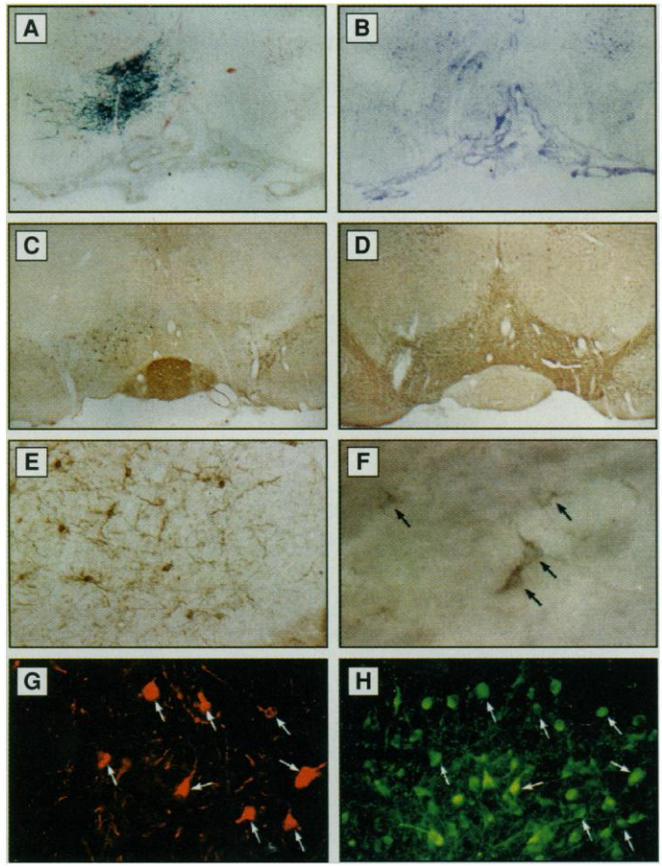

Fig. 3. Histological examination of the VTA after treatment with HSV vectors. (**A**) Expression of β-galactosidase 3 days after injection of HSV-LacZ into the left VTA. Brain slices were reacted in sodium phosphate buffer solution (pH 7.4) containing X-Gal (0.2 mg/ml; Boehringer-Mannheim) overnight. (**B**) An adjacent, Nissl-stained slice from the same brain. (**C**) Expression of GluR1 4 days after injection of HSV-GluR1 into the left VTA (magnification, ×40). Brains were incubated with anti-GluR1 (Chemicon; 1:100), biotinylated goat antibody to rabbit (anti-rabbit) immunoglobulin G (IgG) (Vector Laboratories; 1:500), and avidin-biotin (ABC Elite; Vector). (**D**) Tyrosine hydroxylase immunoreactivity (Eugene Tech International; 1:2000) in an adjacent slice from the same brain. (**E**) Higher (200×) magnification of slice in (C). (**F**) Neuronal uptake of cobalt after stimulation with kainate, indicating formation of functional AMPA receptors in rats given HSV-GluR1 (400×). (**G**) VTA cells labeled with anti-GluR1, visualized with Texas Red–conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:50). (**H**) In the same slice, cells labeled with a monoclonal tyrosine hydroxylase antibody, visualized with fluorescein-conjugated horse anti-mouse IgG (Jackson ImmunoResearch; 1:100). Arrows indicate colabeled cells.

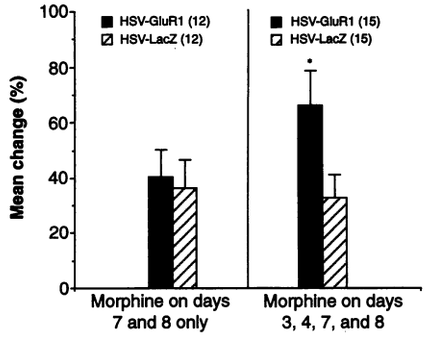

Fig. 4. Time course of heightened locomotor sensitivity to morphine after treatment with HSV vectors. (Left) There were no differences between rats given HSV-GluR1 or HSV-LacZ when tested with morphine on days 7 and 8 only (mean ± SEM) (t_{11} = 0.27, not significant). (Right) Differences persisted on days 7 and 8 (right; t_{14} = 2.11, $P < 0.05$) when rats were tested with morphine on days 3, 4, 7, and 8.

HSV-mediated expression of GluR1 in the VTA.

As noted above, there is little evidence of toxicity associated with injection of the HSV vector preparations into the VTA, which is consistent with our previous findings in vitro (*16*). There are no detectable differences in Nissl staining (Fig. 3B) or tyrosine hydroxylase immunohistochemistry (Fig. 3D) among brain sections of rats given HSV-GluR1, HSV-LacZ, or vehicle. The transient nature of the behavioral changes, which parallel transgene expression, provides further evidence against a toxic effect of HSV-GluR1, because the behavioral effects of even partial VTA lesions appear permanent (*22*). There are previous reports involving HSV vectors (*23*) in which viral toxicity may have contributed to behavioral changes (*24*). However, the vector preparations used in our studies have diminished toxicity (*25*) because they contain no detectable wild-type HSV, are purified of cellular debris, and have increased ratios of amplicon (virus particles that carry the transgene) to helper virus (virus particles necessary to package amplicon).

Our data support a causal relation between increased GluR1 expression in the VTA and increased sensitivity to morphine's actions. The VTA is a trigger-zone for acute behavioral actions of morphine: VTA microinjections of morphine increase locomotor activity (*1*) and are rewarding (*26*). Moreover, the VTA is a critical region for induction of sensitized behavioral responses to morphine (*1*). As with morphine, repeated administration of cocaine, alcohol, or stress also increases GluR1 expression in the VTA (*5*). These treatments cross-sensitize (*1*), consistent with the notion that they produce their effects via common mechanisms. The fact that increased expression of GluR1 and not of GluR2 in the VTA enhances sensitivity to morphine suggests that changes in subunit composition of AMPA receptors contribute to drug-induced behavioral adaptations. The subunit composition of AMPA receptors is directly related to their permeability to Ca^{2+}: Heteromeric receptors composed of GluR1 and GluR2 are Ca^{2+}-impermeable, whereas GluR1 homomeric receptors are Ca^{2+}-permeable (*7*). Cultured motor neurons infected with HSV-GluR1 have inward-rectifying (rather than linear) current-voltage relations, which is consistent with increased Ca^{2+} permeability (*16*).

Selective increases in GluR1 expression in the VTA, and the associated increases in Ca^{2+} entry, could trigger other changes known to occur in the VTA with chronic morphine exposure, including up-regulation of tyrosine hydroxylase, decreased levels of neurofilaments, and visible changes in neuronal morphology (*27*). Altered functioning of VTA dopamine neurons associated with these biochemical changes may underlie altered dopamine release (*28*), and subsequent changes in receptor sensitivity (*29*), in terminal regions such as the nucleus accumbens. Nonetheless, these data demonstrate that specific changes in the motivational state of an animal can result from altered expression of a single, localized gene product.

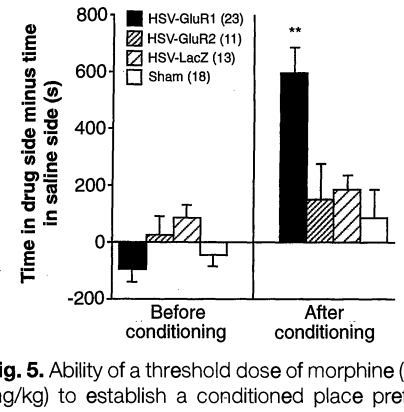

Fig. 5. Ability of a threshold dose of morphine (0.5 mg/kg) to establish a conditioned place preference. Rats given HSV-GluR1 into the VTA showed larger changes in preference than control rats (mean ± SEM) (treatment × session interaction: $F_{3,61} = 4.67$, $P < 0.01$). $^{**}P < 0.01$ (Fisher's *t* test) versus control groups.

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9. Locomotor activity was measured for 2 hours in circular chambers [M. J. D. Miserendino, X. Guitart, R. Z. Terwilliger, S. Chi, E. J. Nestler, *Mol. Cell. Neurosci.* **4**, 440 (1993)]. Male Sprague-Dawley rats (325 to 350 g) were screened with morphine because of within-strain differences in drug sensitivity [V. Deroche, P. V. Piazza, M. Le Moal, H. Simon, *Brain Res.* **623**, 341 (1993)]. This low dose of morphine is not sufficient to cause statistically significant sensitization or to increase expression of GluR1 in the VTA (W. A. Carlezon Jr. *et al.*, unpublished data), although small increases in activity can be observed with repeated treatment (for example, Fig. 1A, left).
10. Rats were anesthetized (65 mg/kg sodium pentobarbital, intraperitoneal) and given atropine (0.25 mg/kg, s.c.) to minimize bronchial secretions. Unilateral microinjections (2.0 μl) of recombinants were delivered over 10 min into the VTA [relative to bregma: AP = −5.3, lat. = ±2.0, DV = 7.6 mm below dura [G. Paxinos and C. Watson, *The Rat Brain in Strereotaxic Coordinates* (Academic, Sydney, Australia, 1986)]]. The injection syringe was angled at 10° from the midline. For sham surgery, the injection syringe was lowered 1 mm into the brain. Rats were retained if the microinjection site was within the VTA.
11. cDNAs for GluR1i (flip), GluR2i, and LacZ were inserted into the HSV amplicon HSVPrpUC and were packaged into virus with the helper 5*dl*1.2 (*16*). Virus was purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose. Average titer of the recombinant virus stocks was 2.0×10^7 infectious units/ml; titers did not differ by more than 10% among preparations. Transgene expression was regulated by the constitutive promoter for the HSV immediate-early gene IE 4/5.
12. VTAs were dissected from the injected hemisphere 4 days after vector microinjection; immunoblotting was done as described (*5*).
13. Rats were overdosed with sodium pentobarbital and perfused with 0.9% saline followed by 4% paraformaldehyde; brains were kept overnight in 20% glycerol before slicing (40 μm, or 20 μm for double-labeling). β-Galactosidase expression was examined as described [N. Min, T. H. Joh, K. S. Kim, C. Peng, J. H. Son, *Mol. Brain Res.* **27**, 281 (1994)].
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19. Immunohistochemical analyses for double-labeling of GluR1 and tyrosine hydroxylase were similar to those described [M. T. Berhow, N. Hiroi, L. A. Kobierski, S. E. Hyman, E. J. Nestler, *J. Neurosci.* **16**, 8019 (1996)].
20. Immunohistochemical analysis for GluR2 (Chemicon GluR2/3; 1: 500) was similar to that described for GluR1 (*16*).
21. Rewarding drugs establish conditioned place preferences [R. A. Wise, in *The Neuropharmacological Basis of Reward*, J. M. Liebman and S. J. Cooper, Eds. (Oxford Univ. Press, Oxford, 1989)]. Place preference conditioning occurred in a three-compartment apparatus. On day 0, rats were allowed to freely explore the entire apparatus for 30 min; rats then received unilateral injections of an HSV vector or sham surgery. After 2 days' recovery, conditioning trials (two per day) were given on two consecutive days (days 3 and 4). Rats first received saline (1 ml/kg, s.c.) and were confined to a side compartment for 1 hour; 3 hours later, rats received morphine (0.5 mg/kg, s.c.) and were confined to the other side compartment for 1 hour. Compartments differed in floor texture, wall patterns, and lighting; rats do not show reliable baseline preferences for any compartment. On day 5, rats were allowed to freely explore the entire apparatus for 30 min. Rats with maximal transgene expression in the rostral VTA (from 4.7 to 5.3 mm posterior to bregma) were included.
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