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ORIGINAL RESEARCH ARTICLE

Neurotrophin-3 modulates noradrenergic neuron function and opiate withdrawal

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Somatic symptoms and aversion of opiate withdrawal, regulated by noradrenergic signaling, were attenuated in mice with a CNS-wide conditional ablation of neurotrophin-3. This occurred in conjunction with altered cAMP-mediated excitation and reduced upregulation of tyrosine hydroxylase in A6 (locus coeruleus) without loss of neurons. Transgene-derived NT-3 expressed by noradrenergic neurons of conditional mutants restored opiate withdrawal symptoms. Endogenous NT-3 expression, strikingly absent in noradrenergic neurons of postnatal and adult brain, is present in afferent sources of the dorsal medulla and is upregulated after chronic morphine exposure in noradrenergic projection areas of the ventral forebrain. NT-3 expressed by non-catecholaminergic neurons may modulate opiate withdrawal and noradrenergic signalling. *Molecular Psychiatry* (2001) **6,** 593–604.

Ongoing drug use in an opiate-addicted individual could be reinforced by withdrawal reactions that occur when drug use is halted abruptly.[1] Overactivity and disinhibition of brainstem noradrenergic neurons is thought to be a key mechanism for the hyperautonomic state and for many of the somatic symptoms that occur during acute withdrawal[2] and also for the acquisition of avoidance behaviors after occurrence of withdrawal reactions.[3]

Little is known about endogenous factors that regulate noradrenergic neuron function and opiate withdrawal-related behaviors. Molecules of the nerve growth factor family may influence neuronal health and plasticity both in the developing and in the adult brain, including the reward and addiction circuitry.[4] Brainstem noradrenergic neurons of the adult rodent and primate brain express the neurotrophin-3 (NT-3) high affinity receptor, TrkC,[5–7] as well as the brain derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) receptor TrkB. In selected neuronal populations, NT-3 may promote growth and survival by binding to TrkB or TrkA, the high affinitity receptor for nerve growth factor (NGF).[8–10]

Although levels of NT-3 and TrkC mRNA are altered in several brain areas during opiate withdrawal,[5] *in vivo* evidence of a role for NT-3 in opiate withdrawal and noradrenergic neuron function is still lacking. The perinatal lethality of conventional NT-3 knock-out mice,[11,12] due to major cardiovascular malformations,[13] prevented the use of these mice to study NT-3 in the adult brain. Using a conditional knock-out strategy with Cre/loxP mediated NT-3 gene deletion that results in complete loss of NT-3 in the central nervous system in mice with normal viability, fertility and lifespan,[14] we show here for the first time that NT-3 modulates electrophysiological responses and neurochemistry of brainstem noradrenergic neurons and that NT-3 is essential both for opiate withdrawal-induced avoidance behavior and for the somatic symptoms that occur during acute withdrawal.

NT-3 modulates expression of tyrosine hydroxylase but is not essential for survival of noradrenergic neurons

We used two transgenic lines, a conditional NT3 mutant[14] and a transgenic line carrying an NT3 transgene under the control of the DBH promoter to examine the consequences of NT3 deficiency as well as NT3 overexpression in opiate withdrawal reactions and in noradrenergic neuron function *in vivo*. Expression of the recombinase cre under control of the nestin promoter results in complete deletion of NT-3 during prenatal development of the brain and spinal cord.[14] To ascertain ubiquitous nestin-cre expression, a reporter line that expressed beta-galactosidase in all tissues upon cre-mediated removal of a stop translation sequence[15] was crossed with the nestin-cre transgenic line. The results shown in Figure 1a–c indicate that cre-mediated recombination is complete throughout the brain, consistent with previous Northern blot results.[14]

In adult brain, NT-3 has been implicated in survival of noradrenergic neurons[16] and therefore neuronal numbers and expression of selected neurochemical markers were analyzed in adult mutants and controls. Coronal sections from the brainstem of 3-month-old

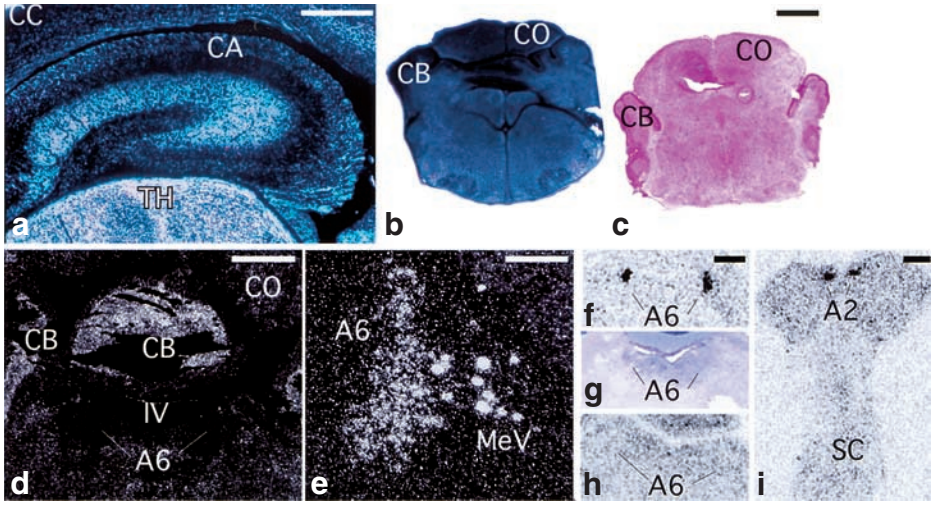


Figure 1 Regional pattern of Nestin-Cre transgene expression and of DBH-NT-3 transgene expression. (a) Representative part of forebrain and (b) rostral hindbrain of newborn animal showing ubiquitous activation of the Rosa-26 Cre/lox lacZ reporter by the Nestin-Cre transgene. (c) Control section (caudal pons/rostral hindbrain) of Rosa-26 Cre/lox lacZ +; Nestin-Cre – animal. Emulsion-dipped coronal sections of dorsal pons/rostral cerebellum from 10-week-old wild-type mouse (d) and from DBH-NT-3/wt transgenic mouse (e), hybridized with ^{35}S-labeled NT-3 cRNA. Note strong signal over cerebellar cortex but not over A6 in wild-type (d) and strong signal over A6 and MeV in DBH-NT-3 transgenic mouse (e). Film autoradiograms of sections from dorsal pons (f) and medulla (i) from 14-month-old DBH NT-3/wt transgenic mouse and dorsal pons (h) of 4-month-old wild-type mouse, hybridized with ^{33}P-labeled NT-3 cRNA, showing strong labeling bilateral over A6 and A2 in the DBH-NT-3 transgenic mouse (f, i) but not the control (h). (g) Same section as shown in (f) after counterstain with cresyl violet. Abbrevations: IV = IVth ventricle; CA = CA fields of Ammon; CB = cerebellar cortex; CC = cerebral cortex; CO = colliculi; TH = thalamus; and SC = spinal cord. Magnification bars in mm (a) 0.5; (b, c) 1; (f, g, h, i) 1; (d) 0.8; (e) 0.1.

NT-3 conditional mutant mice (Figures 2b, d, f and 4b, d) and littermate controls (Figure 4a, c) were stained for tyrosine hydroxylase (TH) immunohistochemistry and total numbers of A6 neurons in the dorsal pons (Figure 2b) and of A2 neurons in the medial solitary tract nucleus (Figure 2d, f) were counted, as outlined by Kalia *et al*.[17] TH is the rate-limiting enzyme in the biosynthesis of norepinephrine. The difference in neuronal numbers between the conditional mutants and the controls was less than 15% and not significant (Figure 3a).

Previous studies have shown that expression of TH is upregulated during chronic morphine exposure, presumably as a compensatory mechanism for increased neuronal inhibition.[18] This transcriptional regulation is mediated, in part, by cAMP-dependent transcription factors and repressors.[19] Western blot analysis of microdissected A6 from wild-type mice that received morphine (total of 500 mg over 60 h) showed increased TH immunoreactivity in comparison to drug-free animals. In contrast, the conditional NT-3 mutant mice revealed no up-regulated TH immunoreactivity within this brain region upon chronic morphine administration (Figure 3b). This difference to morphine-treated controls was consistent for 5/5 morphine-treated mutants tested (mean ± SE: controls 193.3 ± 18.3; mutants 84.8 ± 22.2).

We determined total numbers of TH+ neurons in A2 and A6 of mice which expressed an NT-3 transgene under control of the 5.8-kb fragment of the human dopamine-beta hydroxylase gene (DBH) promoter[20] (see also Methods). In these mice, NT-3 was expressed at high levels in A6 and A2 noradrenergic cell groups (Figure 1e, f, i), in the proprioceptive mesencephalic trigeminal tract nucleus, peripheral sympathetic ganglia, and the adrenal medulla by *in situ* hybridization and ELISA (data not shown). Activity of the DBH promoter in sensory neurons and other tissues was previously described for a transgenic lacZ reporter line.[20] In sections obtained from wild-type mice, no specific labeling was observed over noradrenergic cell groups including A6 (Figure 1d).

In the DBH-NT-3 transgenic mice, total numbers of TH+ cells were increased in A2 by 30% (unpaired two-tailed *t*-test (T); $P < 0.05$) but in A6 did not differ from controls (Figure 3a). It is not clear if the observed increase in TH+ cells in A2 reflects a true increase in total number of neurons or if, alternatively, more neurons express detectable levels of TH. In the DBH-NT-3 transgenic mice, TH immunoreactivity both in somata and in the neuropil of the A2 region appeared more robust (Figure 2c), in comparison to NT-3 conditional mutants (Figure 2d).

In the conditional NT-3 mutant mice, strong and ubiquitous nuclear staining for phosphorylated CREB,[21] a transcription factor known to be involved in opiate withdrawal[22,23] was preserved in A6, A2 and all other noradrenergic cell groups such as A1 in the ventral medulla (Figure 4c, d) and no significant differences were found in levels of CREB immunoreactivity in TH-positive neurons of mutants and controls (data not shown). The CREB transcription factor regulates expression of the proto-oncogene c-fos.[21] Opiate withdrawal stimulates strong c-fos expression in norad-

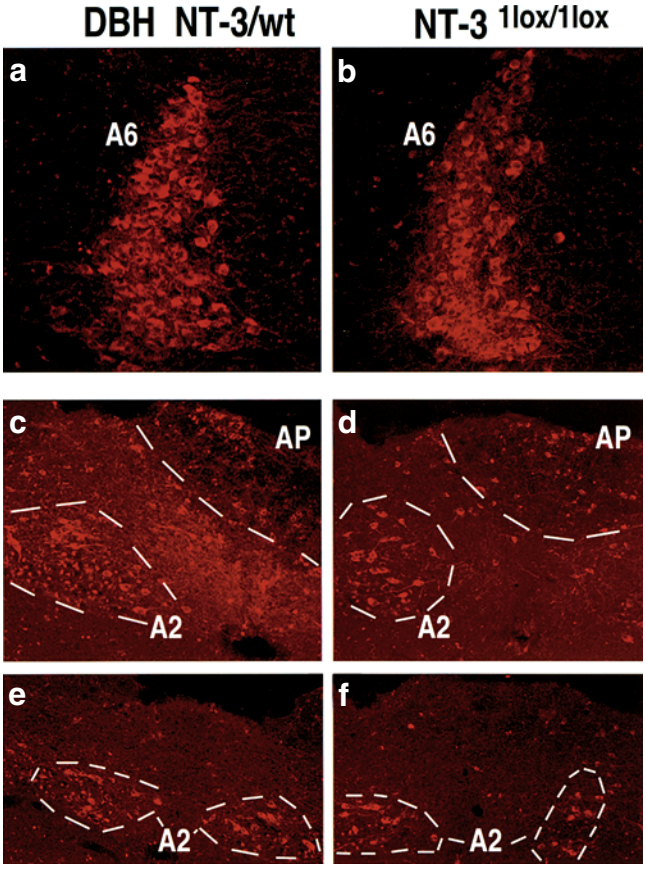


Figure 2 Immunohistochemistry of noradrenergic neurons in NT-3 mutant mice. (a, b) Representative example of Texas Red-labeled TH immunoreactive neurons of DBH-NT-3/wt transgenic mouse (a, c, e) and of NT-3 conditional mutant mouse (b, d, f). The A6 cell group is shown in (a, b), the A2 cell group at the level of area postrema (AP) shown in (c, d) and caudal parts of A2 at the level of the obex are shown in (e, f). Area of the A2, as outlined by Kalia *et al*[17] marked by dotted line. Notice subtle increase in TH immunoreactivity in (c, e) in comparison to (d, f). (a–f) Images taken at 10 × 10 magnification.

renergic neurons.[24] The expression of c-fos was up-regulated in the wild-type mice of the present study after morphine withdrawal (Figure 4a), which was induced with the μ-(opioid) receptor antagonist naloxone. This up-regulation was found to be unperturbed in the conditional null mutant (Figure 4b). Ligand binding autoradiography with nisoxetine, a ligand for noradrenergic re-uptake transporters selectively expressed by noradrenergic neurons, showed a labelling pattern in the fore- and hindbrain that was indistinguishable between conditional mutant and control (data not shown). Taken together these findings suggest that NT-3 modulates TH expression in noradrenergic neurons during chronic opiate exposure but this neurotrophin is not essential for neuronal survival and noradrenergic innervation.

NT-3 deficiency causes abnormal cAMP-mediated signal transduction in A6 neurons

In order to clarify if loss of NT-3 alters the functional state of brainstem noradrenergic neurons we conducted electrophysiological recordings in A6. The focus of these experiments was on standard measures for the cAMP signal transduction pathway that activates Ca^{2+} and Na^{+} channels, causing depolarization and excitation. In wild-type animals, the activity of this intracellular signal transduction pathway is upregulated under conditions of increased neuronal inhibition, including chronic opiate exposure.[25] This mechanism is thought to compensate for drug-induced neuronal hypoactivity. When drug use is halted abruptly, these and other neurochemical adaptations[26] result in overexcitation of noradrenergic neurons by intrinsic mechanisms and by increased excitatory afferent input,[4] causing the withdrawal syndrome.

Coronal slices containing the A6 were obtained from 10–16-week-old conditional NT-3 mutant and control mice treated with saline or morphine. Using extracellular recordings from single A6 neurons, the effects on firing rate of substances applied in the perfusate were measured. Neurons recorded from 10-week-old mice did not differ from those recorded in 16-week-old mice. Examples of recordings are shown in Figure 5.

The cAMP pathway mediates neuronal excitation in noradrenergic neurons and was examined in conditional mutants and controls treated with saline or chronic morphine. Forskolin stimulates adenylyl cyclases and when it was added to the slice perfusate, neuronal firing in saline-treated wild-type A6 neurons was increased (Figure 5a, e). This forskolin-mediated increase in neuronal firing was significantly reduced in saline-treated conditional mutants (Figure 5b, e).

After 60 h of a progressive treatment schedule for morphine (total dose = 500 mg kg^{-1}), forskolin caused a robust increase in neuronal firing in wild-type mice which was, on average, stronger than the forskolin-induced neuronal firing in saline-treated control mice (Figure 5c, e). This apparent upregulation of the cAMP pathway after chronic opiate exposure is consistent with previous results.[23] Interestingly, morphine-treated conditional mutants showed at baseline a significant elevation in neuronal firing, in comparison to morphine-treated controls (Figure 5d, e). However, addition of forskolin to the perfusate caused a further increase in neuronal firing in the conditional mutants (Figure 5d) and was similar to the firing rate of forskolin-treated controls (Figure 5e).

We conclude that cAMP mediated signal transduction in A6 neurons is altered in NT-3 deficient mice both under drug-free conditions and after chronic morphine exposure. In saline-treated mutant mice, the cAMP pathway is hypoactive because the forskolin-induced increase in neuronal firing was reduced. In morphine-treated mutants, cAMP-mediated signaling is dysregulated because the baseline firing rate was abnormally elevated, which made further elevation of

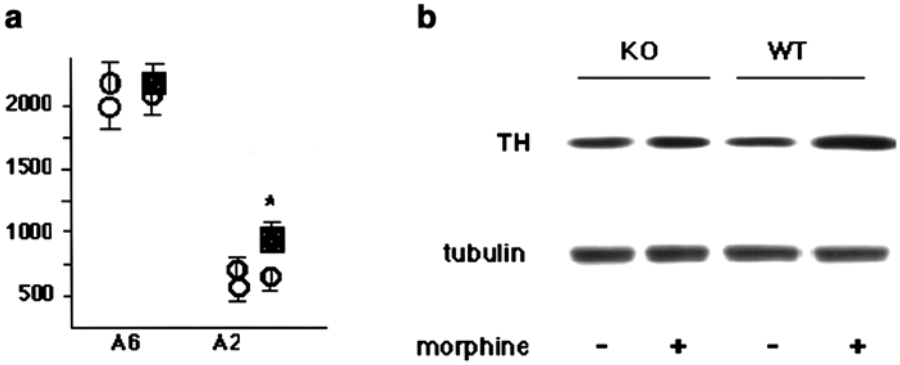


Figure 3 Regulation of TH expression in noradrenergic cell groups of NT-3 mutants. (a) total numbers of TH+ neurons (*y*-axis) in bilateral A6 and A2 noradrenergic cell groups. (■) = DBH-NT-3/wt transgenic mice; (○) = NT-3 conditional mutant mice; (⦶) = controls. NT-3 deficient mice have no loss of neurons. Data collected in pairwise fashion (mutant and wild-type control) and therefore two sets of controls are used. Data expressed as mean ± SE. $^*P < 0.05$. (b) Representative example of Western blots for TH of A6 region in drug-free (– morphine) and drug-treated (+ morphine) conditional mutant (KO) and wild-type (wt) mice, with loading control (tubulin). Notice that KO mice maintain at baseline TH levels similar to control but fail to upregulate TH after drug-treatment.

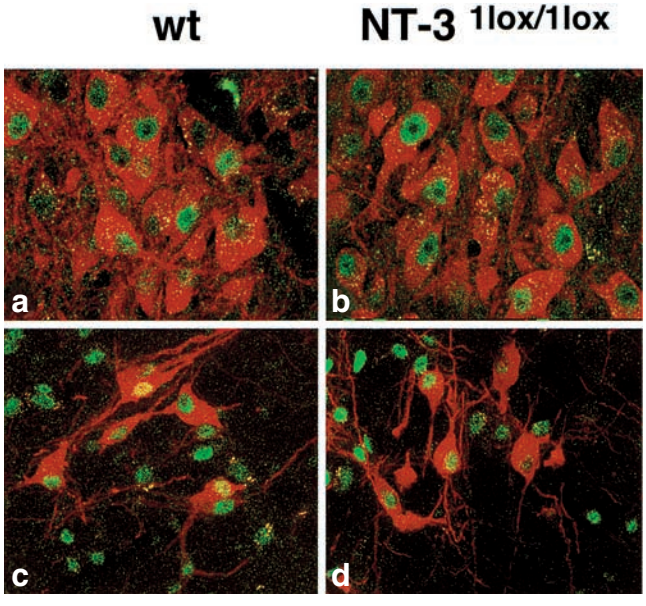


Figure 4 Regulation of c-fos and CREB expression in NT-3 conditional mutants. Sections double stained for Texas Red-labeled TH immunoreactivty and FITC-labeled c-fos immunoreactivity of A6 (a) control and (b) NT-3 conditional mutant mouse 110 min (a, b) after naloxone-induced morphine-withdrawal, showing robust c-fos expression TH neuron nuclei. (c, d) Sections from ventral medulla double stained for Texas-Red labeled TH immunoreactivity and FITC-labeled CREB immunoreactivity in A1 neurons of (c) control and (d) NT-3 conditional mutant mouse after chronic opiate exposure. Note robust expression of c-fos and CREB in nuclei of mutant and control. (a–d) Images taken at 40 × 10 magnification.

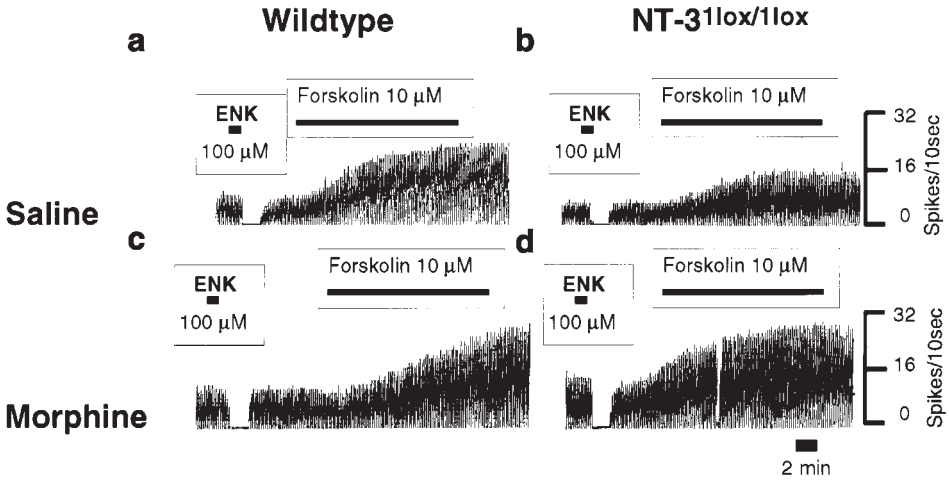
neuronal firing by activation of the cAMP pathway less effective.

G-protein coupled inhibitory receptors are intact in mutant noradrenergic neurons

Hyperpolarization and functional inhibition of noradrenergic neurons is, in part, mediated through G-protein coupled opioid- and α2-adrenoreceptors. Brief exposure to enkephalin (100 μM), an endogenous opioid receptor agonist, caused total neuronal inhibition lasting approximately 2 min in all neurons recorded from A6 of controls (Figure 5a, c) and conditional mutants (Figure 5 b, d). In addition, the suppression of neuronal firing by successively increased concentrations of the synthetic μ-(opioid) receptor-agonist DAMGO was evaluated in 10-week and 16-week-old mice, and no differences were found between mutants and controls (Figure 5f). The IC_{50} was very similar in both groups of mice (mutants: 46.9 nM; controls: 47.9 nM). Also, all mutants and controls showed robust α2-adrenoreceptor mediated inhibition (data not shown). Therefore, the mechanisms of cellular inhibition appear to be fully preserved in A6 neurons of NT-3 conditional mutants.

NT-3 deficiency attenuates opiate withdrawal and withdrawal-induced aversion

We next tested whether the observed abnormalities in opiate-induced neurochemical and physiological adaptations of noradrenergic neurons in the conditional mutants resulted in altered opiate withdrawal behavior. A withdrawal reaction was induced in 12-week-old mice after 60 h of a progressive treatment of morphine (total dose 500 mg kg^{-1}) with an s.c. injection of naloxone (1 mg kg^{-1}). Wild-type mice showed symptoms characteristic of opiate withdrawal, including signs of autonomic hyperactivity, wet dog shakes, tremors and digging. As shown in Figure 6a, b, the NT-3 conditional mutant mice scored, in comparison to wild-type mice, 35% lower on ratings for autonomic hyperactivity (ANOVA $F = 15.4$, $P < 0.01$, Fisher's PLSD –1.1, $P < 0.01$) and also had a significant attenuation in various behavioral symptoms, including wet dog shakes (ANOVA $F = 3.36$, $P < 0.05$; Fisher's PLSD –3.28; $P = 0.018$). The total withdrawal score for this



e

A6 neuron firing rate (spikes/10sec)
mean ± SE

recording condition		wildtype	NT-3 1lox/1lox	% difference
saline	baseline	7.8 ± 0.6	7.5 ± 0.5	- 4 % (N.S.)
	forskolin	20.7 ± 1.3	15.7 ± 1.6	**- 25 % (p <0.05)**
morphine	baseline	9.6 ± 1.1	14.8 ± 1.3	**+ 50% (p < 0.05)**
	forskolin	26.9 ± 2.7	27.0 ± 2.2	0

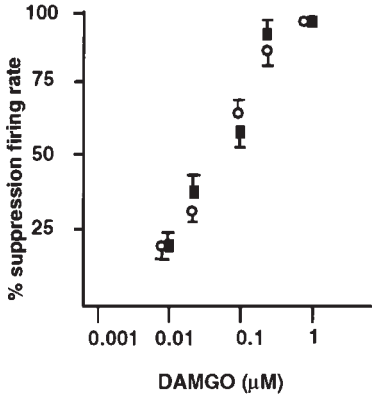


Figure 5 Activity of A6 neurons in NT-3 conditional mutant and control mice at baseline, after stimulation of the cAMP pathway and G protein-coupled receptors. (a–d) Representative traces of single unit recordings of A6 neurons in 15-week-old wild-type animal (a, c) and 15-week-old NT-3 conditional mutant mouse (b, d) treated with saline (a, b) or chronic morphine (c, d). *y*-axis shows firing rate (spikes per 10 s), horizontal axis represents running time, bold bars represent running time period of perfusate application (calibration bar = 2 min). Notice reduced neuronal firing in drug-free conditional mutant after application of forskolin (b) in comparison to wild-type (a) and increased neuronal firing at baseline prior to application of forskolin in morphine-treated conditional mutant (d) in comparison to wild-type (c). Table (e) summarizes recording data and % differences for the four cohorts of saline- and morphine-treated conditional mutants. (f) Dose-response curve for μ-receptor agonist DAMGO, measured as % suppression of baseline firing rate (*y*-axis) after successive increase in DAMGO concentration in perfusate (*x*-axis) in 10-week-old mutant (○) and control (■) mice. Note similar dose-response curves in mutant and control. Data shown as mean ± SE. Significance of differences between mutants and controls was calculated by an unpaired two-tailed *t*-test.

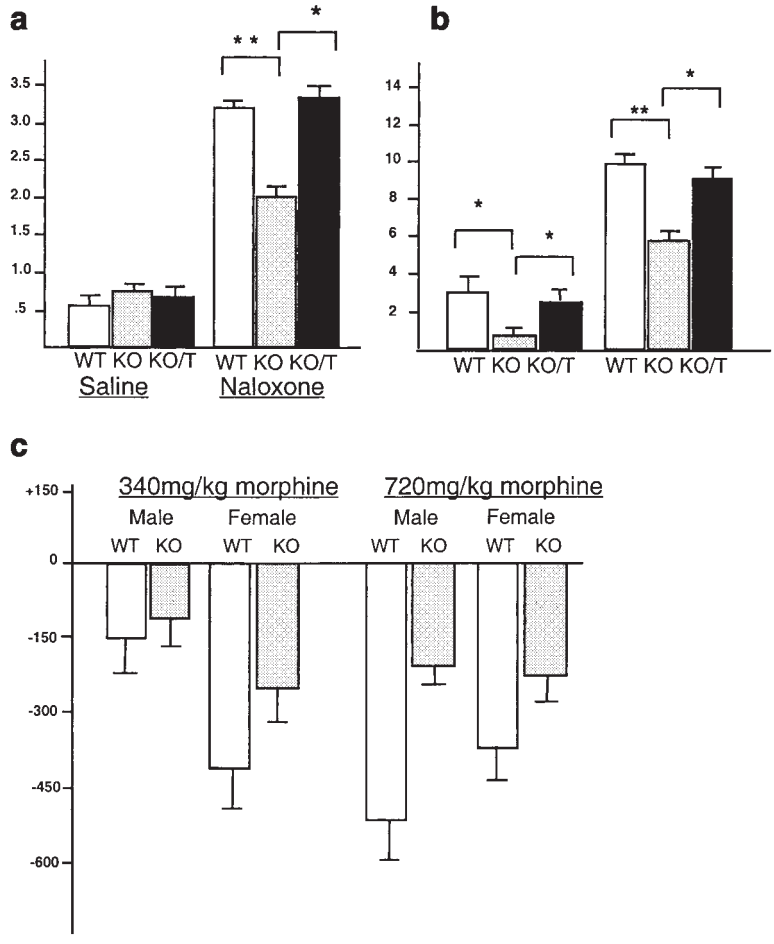


Figure 6 Opiate withdrawal reactions and withdrawal-induced aversion in NT-3 mutant mice. (a, b) Summarize observer-based ratings of naloxone-precipitated withdrawal in the NT-3 conditional mutant mice (gray bars), in comparison to conditional mutants that carried the DBH-NT-3 transgene (black bars) and wild-type controls (white bars). (a) Total number of checked signs (diarrhea, rhinorhea, lacrimation, abnormal posture, irritability, ptosis) in saline-treated animals (left-sided bars) and after chronic morphine administration (500 mg kg^{-1} total dose per 60 h) (right-sided bars). White bar (WT) = wild-type mice, gray bar (KO) = NT-3 conditional mutant mice, black bar (KO/T) = conditional mutant mice carrying the DBH-NT-3 transgene. (b) Total number of wet dog shakes (left-sided bars) and total overall withdrawal score (right-sided bars) in morphine-treated animals. Bar codes as in (a). $*P < 0.05$; $**P < 0.01$. (c) Avoidance (*y*-axis, in seconds) of aversive chamber 24 h after morphine withdrawal in male and female conditional NT-3 mutants and controls. Data are expressed as mean ± SE.

group of mutant mice also was 30% lower in comparison to age-matched controls (total score mutant 6.1 ± 0.47 (mean ± SE); controls 8.9 ± 0.97; (ANOVA) F = 9.69, $P < 0.01$).

To confirm if this behavioral difference was a primary effect of NT-3 on noradrenergic neurons, we tested the opiate withdrawal response of NT-3 conditional mutants that also carried one copy of the DBH-NT-3 transgene. We found that the response was indistinguishable from wild-type animals (Figure 6a, b). This result provides strong genetic evidence for a role of NT-3 in normal opiate withdrawal and suggests that the attenuated withdrawal reaction in NT-3 deficient mice is, at least in part, due to dysfunction of noradrenergic neurons.

Withdrawal acts as a negatively reinforcing stimulus, which is thought to be a component for the development or maintenance of the addicted state.[1] Aversive conditioning to opiate withdrawal was measured by the conditioned place aversion paradigm. In a pre-conditioning test, all animals with a strong side preference at baseline were removed from further testing. Then, mice were placed on a progressive treatment schedule for morphine. One group of mutants and controls received 340 mg kg^{-1} per 60 h, another group received 720 mg kg^{-1} per 72 h. Twenty-four hours after the animals underwent a naloxone-induced withdrawal reaction in one particular chamber, wild-type mice spent less time in that aversive chamber, in comparison to NT-3 conditional mutants (Figure 6c). This difference was significant (mixed factor ANOVA: genotype–F = 4.20, $P < 0.05$; sex–F = 1.22, $P = 0.28$; dose–F = 1.34, $P = 0.26$). After collapsing the data for sex and dose, pairwise comparisons using the Newmans–Keuls test revealed a significant difference between null and wild-type mice ($P < 0.05$).

A simple fear conditioning paradigm was applied, in order to rule out a generalized learning deficit in the

conditional mutant. After repeated exposure to electrical footshocks in a conditioning chamber, both controls and conditional mutant mice developed, within minutes, an approximately five-fold increase in freezing behavior, as quantified by computerized video-monitoring. When returned to the aversive chamber 24 h after receiving shocks, this increase in freezing time was retained in the first 5 min both in mutants (ratio freezing/non-freezing time 0.57 ± 0.08; baseline 0.11 ± 0.02) and controls (0.48 ± 0.07; baseline 0.08 ± 0.01).

Regional distribution pattern of NT-3 expression suggests target-derived modulation of noradrenergic neuron function

It is not known whether NT-3 expression is dynamically regulated in adult mouse brain. Differential expression of NT-3 after opiate exposure may imply a role for NT-3 in drug-induced neuronal and behavioral adaptations. In order to learn more about the regional distribution and transcriptional regulation of NT-3 in opiate-exposed mice, we studied transgenic mice with a lacZ reporter coding sequence integrated into the endogenous NT-3 locus.[27] In the forebrain and midbrain, the regional distribution pattern of lacZ expressing cells in morphine-treated animals (500 mg kg^{-1} per 60 h) appeared qualitatively normal and similar to the distribution pattern described previously for drug-free animals.[27] However, morphine-treated animals consistently showed much stronger lacZ reporter activity for the lateral septum and the CA2 region and the dentate gyrus of the hippocampus, in comparison to drug-free littermate controls (Figure 7a–d). The bed nucleus of the stria terminalis (BNST) contained, in areas of immediate vicinity to the lateral septum, a few scattered lacZ+ neurons in morphine-treated animals while drug-free animals had no lacZ reporter activity in that brain region (Figure 7e, f). Noteably, the BNST, the lateral septum and other NT-3 expressing areas such as the cingulate and entorhinal cortex are comparatively rich in noradrenergic fibers.[28]

The expression pattern of NT-3 in the hindbrain did not show qualitative or quantitative differences between morphine-treated and drug-free animals: only few areas of the hindbrain contained significant numbers of lacZ+ neurons. A subgroup of neurons of the nucleus prepositus hypoglossi (NPH) and adjacent vestibular nuclei were among the most heavily stained neurons in any brain region (Figure 7i). The cerebellar cortex, the dorsal cochlear nucleus, and the lateral and rostral portions of the solitary tract nucleus (NTS) were also labeled (Figure 7j). The paragigantocellular nucleus, which together with the NPH and the NTS comprises a major afferent input source to brainstem noradrenergic neurons, contained no NT-3 expressing cells.

Sections from the midbrain that were processed for TH immunoreactivity and lacZ reporter activity contained numerous double-stained sections in the substantia nigra and ventral tegmental area (Figure 7g), as described.[27] However, NT-3 reporter gene expression was, both in newborn and adult brain, strikingly absent in catecholaminergic neurons of the entire hindbrain and pons, including A6 and A2 (Figure 7h). These results are consistent with data obtained from emulsion-dipped sections from wild-type pons hybridized with radiolabeled NT-3 antisense cRNA, showing no specific labeling over noradrenergic cells (Figure 1d).

Discussion

We report genetic evidence for a role of NT-3 in opiate withdrawal and noradrenergic neuron function. Mice with a CNS-wide deletion of NT-3 had attenuated withdrawal reactions which was restored by transgene-derived overexpression of NT-3 in noradrenergic neurons. Noradrenergic neurons of adult conditional NT-3 mutants had alterations in electrical activity both under drug-free conditions and after chronic opiate exposure. The upregulation of TH after chronic opiate exposure was reduced. The NT-3 deficient mice maintained normal numbers of noradrenergic neurons, therefore NT-3 is not essential for noradrenergic neuron survival. These results suggest that loss of NT-3 affects the function of noradrenergic neurons, including the capacity for opiate-induced neurochemical and functional adaptations.

Reduced opiate withdrawal in conditional mutants is accompanied by noradrenergic neuron dysfunction

Brainstem noradrenergic neurons play a key role in the neurobiology of the withdrawal reaction.[2] NT-3 may interfere with the biological effects of opiates by direct effects on noradrenergic neurons: NT-3 opposes, *in vitro*, the observed decline in embryonic A6 neuron numbers after chronic morphine exposure.[29] Furthermore, opiate exposure alters the expression of neurotrophic factors, including NT-3. In rat A6 neurons, NT-3 expression was increased after chronic morphine exposure but fell below baseline levels after acute withdrawal.[5] The high affinity receptor for NT-3, trkC, also shows dynamic changes in expression during chronic opiate exposure and withdrawal.[5] These studies suggested indirectly that NT-3, by affecting noradrenergic neuronal function, modulates opiate addiction and withdrawal behaviors. This hypothesis is supported by the observation that overexpression of NT-3 by noradrenergic neurons restored the withdrawal reaction in the NT-3 conditional mutants.

Upregulation of TH after chronic morphine exposure was present in wild-type noradrenergic neurons, consistent with previous observations in rats and transgenic mice expressing a lacZ reporter under control of the TH promoter.[18,23] While the functional significance of TH upregulation is not clear, the fact that it is diminished in NT-3 deficient mice suggests impaired capacity to adapt to chronic opiate exposure. Alterations in neuronal activity, including a hypoactive cAMP signaling cascade under drug-free conditions, an

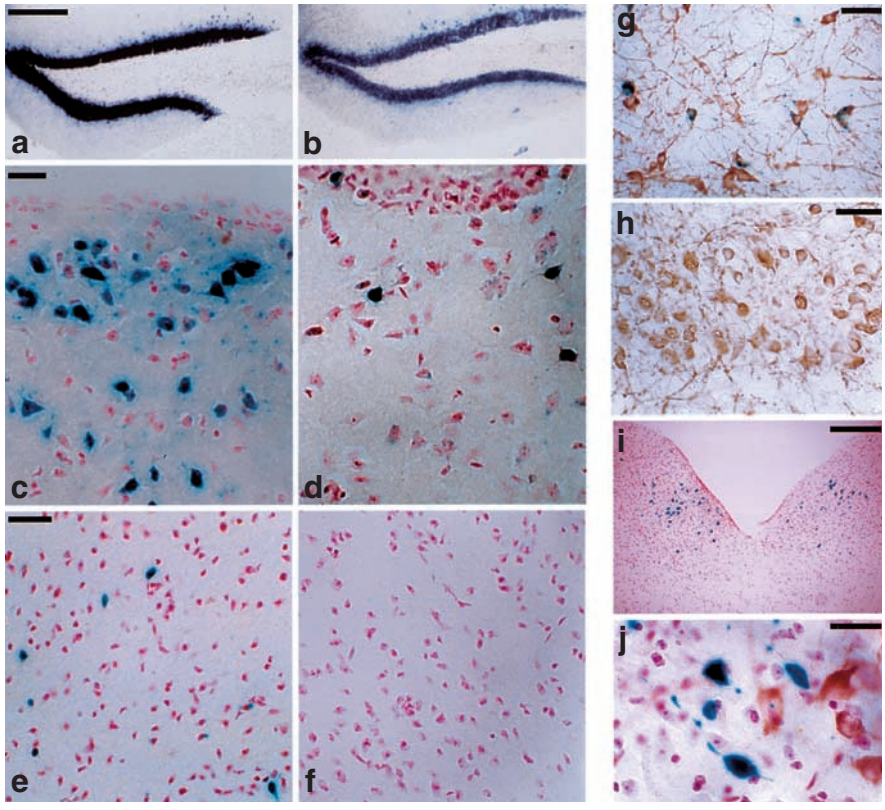


Figure 7 Transcriptional regulation of NT-3 expression. (a–j) Show X-gal stained coronal sections of mice carrying a lacZ reporter knock-in in the endogenous NT-3 locus.[27] (a, b) Show representative examples of labeling in the dorsal dentate gyrus, (c, d) the lateral septum and (e, f) the adjacent bed nucleus of the stria terminalis (BNST) after chronic morphine administration (a, c, e) in comparison to saline-treated littermate (b, d, f). Note increased labeling in morphine-treated animals. (g, h) Sections stained for TH and for lacZ, showing (g) double-labeled dopaminergic neurons in midbrain but (h) no double-labeled neurons in A6 of the same animal. (i) Coronal section of medulla, showing labeled neurons bilateral in area corresponding to the nucleus prepositus hypoglossi (NPH). (j) Medulla section double stained for TH and lacZ, showing co-localizaton of TH+ and lacZ+ neurons in rostral area of the solitary tract nucleus (NTS). Magnification bars in mm: (a, b) 0.5; (c, d) 0.04; (e, f) 0.15; (g) 0.05; (h) 0.05; (i) 0.25; (j) 0.02.

abnormal elevation in baseline firing rate after chronic morphine exposure, provide additional evidence that noradrenergic neurons are dysfunctional in NT-3 conditional mutants. Because total numbers of TH expressing noradrenergic neurons were maintained in adult conditional mutants, NT-3 is not required for neuronal survival and maintenance. Normal numbers of noradrenergic neurons in the mutant brain also argue that the observed alterations in noradrenergic neuron function and opiate withdrawal are due to a physiological role of NT-3 rather than due to a defect in brain development.

Noradrenergic neurons are organized in multiple clusters in the pons and medulla, which may explain the observation that lesions of individual clusters and fiber pathways do not abolish opiate withdrawal reactions.[3,30,31] Pharmacological inhibition of the entire noradrenergic system, on the other hand, is a very effective treatment for opiate withdrawal.[2] Whereas recent evidence points to an important role of the noradrenergic cell groups in the medulla at least for withdrawal-induced aversion,[3] traditionally the A6, which is the largest noradrenergic cell cluster in the brain, has served as a model system for the pharmacology and cellular basis of opiate tolerance and withdrawal.[4] Application of electrical recording and other analytical techniques to noradrenergic cell groups other than the A6 is extremely difficult in the mouse and thus it remains unclear if morphine-induced alterations in A6 neuron electrophysiology and neurochemistry are representative of alterations occurring in other noradrenergic cell groups. However, we found, by TH immunohistochemistry, subtle changes in medullary (A2) noradrenergic neurons in transgenic mice that overexpress NT-3 in these neurons. Therefore, NT-3 may modulate the neurochemistry of several or all noradrenergic populations in the brainstem.

Noradrenergic neurons do not express NT-3

We found no evidence for NT-3 expression in wild-type noradrenergic neurons in postnatal and adult mouse brain. This is in contrast to previous studies in the rat localizing NT-3 mRNA to A6,[5,32] suggesting that related species may differ in regional patterns of neurotrophin gene expression. Because neurotrophins are

subject to retrograde[33] and anterograde transport,[34,35] other NT-3 expressing neuronal populations in adult brain[27,36,37] may supply noradrenergic cells with target-derived NT-3. Furthermore, the observed alterations in electrical activity of A6 neurons and the failure to up-regulate TH after chronic opiate exposure in the conditional mutants may be explained by lack of target-derived NT-3. Alternatively, these changes in mutant A6 neurons could be secondary, with the primary defect being in other neuronal populations that depend on autocrine or paracrine NT-3[38] and that are interconnected with the noradrenergic system.

We found that NT-3 expressing neurons are present in two brainstem nuclei that are interconnected with the noradrenergic system and that provide a major afferent input. These were the nucleus prepositus hypoglossi (Figure 7i) and the rostral portions of the nucleus of the solitary tract (Figure 7j).[39,40] NT-3 expressed in these nuclei may be subject to anterograde axonal transport and may be released from presynaptic terminals to postsynaptic noradrenergic neurons.[35] Alternatively, TrkC or TrkB receptors on axon terminals of noradrenergic neurons may take up NT-3 and transport ligand and receptor in a retrograde direction. In this regard, it is of interest that chronic opiate exposure upregulates endogenous NT-3 gene expression in selected forebrain areas, including the lateral septum (LS), parts of the neighboring bed nucleus of the stria terminalis (BNST) and the dorsal hippocampus (DH). These areas compose part of a circuit mediating avoidance and aversion behavior[41,42] and noradrenergic fibers innervating this particular circuitry are thought to be essential for opiate withdrawal-induced aversion.[3] Therefore, NT-3 produced in these forebrain regions could be important for opiate-induced adaptations of noradrenergic neurons and may explain why withdrawal-induced aversion was reduced in the NT-3 deficient mice of our study. Target-derived NT-3, by modulating noradrenergic neuron function, may affect complex, long-term behavioral sequelae of opiate dependence and withdrawal. This is of particular interest from a clinical point of view. In patients addicted to opiates, the experience of opiate withdrawal may act as strong negative reinforcement, thus maintaining the addicted state.[1] Previous studies suggested that target-derived NT-3 plays an essential role during the developmental period, by modulating neuronal survival in the peripheral nervous system, including proprioceptive and sympathetic neurons.[11,43–45] Our findings suggest, indirectly, that in adult brain, selected neuronal populations, including the noradrenergic system, require target-derived NT-3 for proper function but not necessarily for neuronal maintenance and survival. Future experiments, using genetically engineered mice with a tagged NT-3 coding sequence[46] should clarify if modulation of opiate withdrawal by NT-3 is due to target-derived modulation of noradrenergic signalling and/or additional effects on other neuronal populations of the circuitry that mediates opiate withdrawal and withdrawal-related aversion.

Methods

Genetic engineering

For characterization of the NT-3 conditional knock-out allele and of the Nestin-Cre transgene, see Bates *et al*[14] and Trumpp *et al*.[47] The DBH-NT-3 transgenic mice were generated by pronuclear injection of a plasmid containing sequentially a 5.8-kb fragment of the human dopamine-beta hydroxylase gene promoter as described by Mercer *et al*,[20] a 0.15-kb fragment comprising intron A of the human insulin gene, a 0.96-kb fragment containing the complete translated sequence of a human neurotrophin-3 cDNA, and a 0.27-kb fragment of a mouse protamine I cDNA 3′ UTR, containing the polyadenylation sequence. The line employed for these studies contained 5–10 tandemly integrated copies of the transgene. The genetic background of all mutant and control mice was mixed, but predominantly *129* and *C57/Black6*. Breeding strategies were chosen to obtain wild-type littermates as controls for the NT-3 mutants, in order to minimize the variability in genetic background.

Morphine injection protocol

Procedures were similar to Taylor *et al*[48] and Punch *et al*.[49] Opiate dependence was induced by a progressive treatment schedule of morphine sulfate i.p. spaced every 6–10 h (day 1: 20–40–60 mg kg^{-1}, day 2: 80–100–100 mg kg^{-1}, day 3: 100 mg kg^{-1}). Thus, the total cumulative morphine dose was for each animal 500 mg kg^{-1} per 60 h. These protocols was used for the Western blot analysis, the histological studies, the electrophysiological recordings, the analgesia experiments and the observer-based ratings for somatic withdrawal. For the conditioned place aversion experiments, 50% of animals were subject to a 'low dose morphine' progressive treatment schedule (340 mg kg^{-1} total over 60 h) and 50% of animals were subject to a 'high dose morphine' progressive treatment schedule (720 mg kg^{-1} total over 72 h) schedule. For the rating and conditioning experiments, withdrawal was precipitated 2 h after the last morphine injection with naloxone.HCl s.c. 1 mg kg^{-1}).

In situ hybridization

Brains were perfusion-fixed with phosphate-buffered 4% paraformaldehyde, postfixed overnight in the same fixative, cryoprotected in 30% phosphate-buffered sucrose, frozen and cut on a cryostat in series of 20-μm thick coronal sections which were stored in above fixative for another 2 h, then mounted on Vectabond (Vector, Burlingame, CA, USA) coated slides and air-dried. Sections were washed twice in 0.1 M phosphate-buffered glycine and once in phosphate buffer (pH 7.4), then digested with proteinase K (1 μg ml^{-1} in 0.1 M Tris pH 8.0, 50 mM EDTA) for 30 min at 37°C, then washed for 10 min at RT in 0.25% acetic anhydride in 0.1 M TEA (pH 8.0), then washed twice in 2 × SSC (15 min, RT). They were then dehydrated, defatted in 100% chloroform for 10 min, rehydrated and airdried, then incubated in a humidified chamber for 24 h at 60°C in hybrization buffer (10% dextrane, 50× dionized

formamide, 50× Denhardts, 300 μg ml^{-1} herring sperm DNA, 0.15 mg ml^{-1} yeast t-RNA, 20 mM DTT) containing 10 000 cpm μl^{-1} of a P^{33} or S^{35}-labeled NT-3 full length cRNA or a 250-bp fragment of protamine cRNA. Sections were washed in 4 × SSC (twice for 30 min at 60°C), then digested with RNAse A (20 μg ml^{-1}) in RNAse buffer (0.5 M NaCl, 10 mM Tris pH 8.0/1 mM EDTA), then washed twice in 2 × SSC, twice in 0.5 × SSC, once at 0.1 × SSC (30 min each, 60°C), dipped in water, airdried and either exposed to beta max hyperfilm (Amersham, Piscataway, NJ, USA) for 10 days or dipped in Kodak (Rochester, NY, USA) NTB2 emulsion and developed after 28 days, then counterstained and coverslipped.

Western blot analysis

Brains of 10 control mice and 10 conditional mutants (5 per group were untreated, 5 per group received chronic morphine; 500 mg per 60 h) were rapidly removed after decapitation, and A6 was excised using a 15-gauge syringe as previously described for the mouse.[18] Samples were homogenized in 2% SDS and aliquots of A6 containing 20 μg of protein were loaded on 7.5% SDS polyacrylamide gels, electrophoresed and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, USA). Blots were immunolabeleled for TH exactly as described.[23] Equal loading and transfer was controlled by immunolabeling for tubulin. Differences between mutants and controls were quantified using optical densitometry of film autoradiograms and NIH imaging software.

Immunohistochemistry

For immunohistochemical experiments, drug-naive mice were compared with mice exposed to chronic morphine (see above). c-fos immunoreactivity in noradrenergic neurons was examined in drug-naive mice and in mice that were exposed to chronic morphine and that received naloxone.HCl s.c. 1 mg kg^{-1} after the last morphine injection. Withdrawal reactions (as described above) were verified by a trained observer, and 110 min after the naloxone injection, the animals were killed. All mutant and control brains were perfusion-fixed with phosphate-buffered 4% paraformaldehyde, postfixed in the same fixative for 2 h, cryoprotected in 30% phosphate-buffered sucrose, frozen and cut in series of 20-μm thick coronal sections on a cryostat (Reichert-Jung, Vienna, Austria). Sections were incubated free floating at room temperature for 20 h with a monoclonal antibody for tyrosine hydroxylase (1:1000) (Incstar, Stillwater, MN, USA), and for double-labeling, an antiserum for the 43-kDa phosphorylated form of CREB (1:500),[21] Upstate Biotech (Lake Placid, NY, USA), or an antiserum recognizing c-fos (1:500) (Oncogene, Boston, MA, USA) in phosphate-buffered 0.3% Triton X-100 with 4% horse or goat serum. After washing, sections were incubated either in biotinylated secondary antibodies processed for DAB staining (Vectastain, Burlingame, CA, USA), according to the manufacturer's instruction or incubated in FITC or Texas Red conjugated secondary antibodies (1:200, Vector) for 60 min. For all immunohistochemical experiments, thionine-stained parallel sections were used for cytoarchitectonic studies and anatomical localization. A separate set of sections, obtained from mice carrying a lacZ reporter in the endogenous NT-3 locus[27] were first stained free floating for beta-galactosidase activity: sections were rinsed twice (5 min and 20 min, RT) in buffer A (0.1 M phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 5 mM EGTA), then rinsed twice (5 min each) in buffer B (0.1 M phosphate buffer pH 7.4, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40) and then transferred into the staining solution (buffer B, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg ml^{-1} X-gal), incubated for 16 h at 37°C, then washed in phosphate buffer and then were either processed for TH immunohistochemistry as above or mounted on gelatin/chrom alum coated slides, counterstained with Neutralred, dehydrated and coverslipped.

Cell counting studies

Drug-naive mutant and control animals were used for cell counting studies. Total numbers of bilateral A6 and A2 neurons were determined in series of 20-μm thick sections that were processed for DAB-stained TH immunoreactivity and then counterstained with cresyl violet. Every second section was used for counting. Because both A6 and A2 showed in all mutants and controls considerable variability in neuronal distribution and density along the rostrocaudal axis, optical dissector and volume-based cell counting methods that rely on constant cell densities in a given volume were inaccurate for the present study. Instead, TH immunoreactive neurons (defined by brown cytoplasm in DAB-stained material) cut through the level of the nucleus were counted in each section and total neuronal numbers were calculated by adding counts of single sections. The noradrenergic cell group A2 was localized using the detailed cytoarchitectonic descriptions of Kalia *et al*.[17] Three-month-old NT-3 conditional knockout mice and littermate controls and DBH NT-3 transgenic mice and littermate controls of the same age groups were used, with a minimum of five animals for each age group and genotype. Data from the two age groups were taken together and differences between genotypes expressed as means ± SE.

Electrophysiology

Electrophysiological recordings from A6 neurons were performed on a series of 3-week, 10- and 15–16-week-old mutant mice and controls, as previously described.[23] Briefly, brain slices containing the A6 were transferred onto the stage of a gas–liquid interface brain slice chamber under a constant flow of humidified 95% O_2:5% CO_2 and physiological buffer. The A6 were visually identified and single-unit extracellular potentials were recorded with glass microelectrodes filled with 2 M NaCl. Consecutive cells were sampled by multiple electrode tracts randomly positioned within the A6 and recorded for a minimum of 3–5 min to ensure that the firing rates were stable. Recordings were conducted on drug-naive untreated animals and

on mice exposed to chronic morphine. Recordings began not earlier than 90 min after setting up the slice preparation. This waiting period ensured complete wash-out of morphine in the drug-treated animals, as described.[25] Transient electrical silencing after brief application of short-lived enkephalin was used to confirm that recording was done from LC neurons. Firing rates of A6 neurons were recorded after bath application (for at least 10 min) of forskolin (10 μmol). At this concentration, maximal electrophysiological responses from A6 neurons are elicited. In addition, μ (opioid) receptor inhibition was examined by dose-response curves with the synthetic agonist DAMGO. For statistical analysis, the firing rates of neurons (typically 10–14 from a single A6) were averaged and considered as a single data point. A minimum of 12 animals was used for each genotype and treatment.

Somatic withdrawal
Behavioral ratings were calculated over a 15-min period by a trained observer blind to experimental treatment and genotype. Three different groups of mice were tested (minimum 10 animals per group): (1) 3-month-old NT-3 conditional mutant mice; (2) 3-month-old conditional NT-3 mutant mice carrying the DBH NT-3 transgene; (3) wild-type controls. Additional experiments were conducted with NT-3 conditional mutants and controls that were $\geq$4 months of age. The overall withdrawal score was determined by the total number of checked signs (diarrhea, rhinorhea, lacrimation, abnormal posture, irritability, ptosis) and the frequency of each counted sign (wet dog shakes, freezing, grooming, rear, dig, jumping, tremor) as described.[48] Analgesic effects of morphine were evaluated by the warm-water tail-flick test. Conditional mutants, in comparison to controls, did not show significant differences in tail flick latencies after receiving a single dose of 20 mg kg^{-1} morphine (mutants: 11.3 $\pm$ 6.4 ms; controls 10.4 $\pm$ 3.4 ms) or at the 100 mg kg^{-1} endpoint of the progressive morphine treatment regimen (mutants 9.5 $\pm$ 6.3 ms; controls 11.8 $\pm$ 7.0 ms), suggesting that analgesic effects and development of tolerance is preserved in the mutant.

Conditioned place aversion
A total of 20 mutant and 20 age- and gender-matched control mice (age range 10–16 weeks) were utilized, under blind conditions, for the conditioned place aversion (CPA) test. Animals were housed individually in a temperature and humidity-controlled room on a 12/12 h light-dark cycle with food and water *ad libitum*. Conditioning and testing occurred in custom built mouse CPA chambers (MED Associates, Ludlow, VT, USA). During pre- and post-conditioning (day 1 and day 6, respectively), animals explored the two chambers and the middle compartment freely for 900 s. Subjects spending more than 540 s in either chamber or preferring the middle compartment during pre-conditioning were discarded. A progressive treatment schedule of 'low dose morphine' (340 mg kg^{-1} total over 60 h) and 'high dose morphine' (720 mg kg^{-1} over 72 h) was administered. On the last day of conditioning (day 5) animals underwent pairing of one side of the chamber with saline, the other with naloxone (1 mg kg^{-1}). Test performance was defined as the difference in the time spent in each compartment before *vs* after conditioning. Simple conditioning with electric foot shocks was performed as described,[50] including analysis by MATLAB software (see also http://web.mit.edu/linus/www/meteor/).

Data analysis
In order to examine significance of differences between mutants and controls, unpaired two-tailed *t*-tests were conducted for cell counting, histology, electrophysiology data and the simple conditioning (footshock) experiments. ANOVA and Fisher's PLSD for post hoc comparison was used for the ratings of somatic withdrawal. The data of the conditioned place aversion experiments were first subjected to a mixed factor ANOVA to evaluate the effect of genotype, sex and morphine dose regimen and then pairwise comparisons were performed with the Neuman–Keuls test.

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References

1 Koob GF, Le Moal M. Drug abuse: hedonic homeostatic dysregulation. *Science* 1997; **278**: 52–58.
2 Maldonado R. Participation of noradrenergic pathways in the expression of opiate withdrawal: biochemical and pharmacological evidence. *Neurosci Biobehav Rev* 1997; **21**: 91–104.
3 Delfs JM, Zhu Y, Druhan JP, Aston-Jones G. Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature* 2000; **403**: 430–434.
4 Nestler EJ, Aghajanian GK. Molecular and cellular basis of addiction. *Science* 1997; **278**: 58–63.
5 Numan S, Lane-Lad SB, Zhang L, Lundgren KH, Russell DS, Seroog KB *et al*. Differential regulation of neurotrophin and trk receptor mRNA in catecholaminergic nuclei during chronic opiate treatment and withdrawal. *J Neurosci* 1998; **18**: 10700–10708.
6 Sandell JH, Baker LS Jr, Davidov T. The distribution of neurotrophin receptor trkC like immunoreactive fibers and varicosities in the rhesus moneky brain. *Neuroscience* 1998; **86**: 1181–1194.
7 King VR, Michael GJ, Joshi RK, Priestley JV. trkA, trkB and trkC messenger RNA expression by bulbospinal cells of the rat. *Neuroscience* 1999; **92**: 935–944.
8 Davies AM, Minichiello L, Klein R. Developmental changes in NT-3 signalling via TrkA and TrkB in embryonic neurons. *EMBO J* 1995; **14**: 4482–4489.
9 Tessarollo L, Tsoulfas P, Donovan MJ, Palko ME, Blair-Flynn J, Hempstead BL *et al*. Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis. *Proc Natl Acad Sci USA* 1997; **94**: 14776–14781.
10 Huang EJ, Wilkinson GA, Farinas I, Backus C, Zang K, Wongl SL *et*

al. Expression of Trk receptors in the developing mouse trigeminal ganglion: *in vivo* evidence for NT-3 activation of trk A and trkB in addition to trkC. *Development* 1999; **126**: 2191–21203.
11 Farinas I, Jones KR, Backus C, Wang XY, Reichardt LF. Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* 1994; **369**: 658–661.
12 Ernfors P, Lee K-F, Kucera J, Jaenisch R. Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* 1994; **77**: 503–512.
13 Donovan MJ, Hahn R, Tessarollo L, Hempstead BL. Identification of an essential non-neuronal function of Neurotrophin-3 in mammalian cardiac development. *Nat Genet* 1996; **14**: 210–213.
14 Bates B, Rios M, Trumpp A, Chen C, Fan G, Bishop JM *et al*. Neurotrophin-3 is required for proper cerebellar development. *Nat Neurosci* 1999; **2**: 115–117.
15 Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; **21**: 70–71.
16 Arenas E, Persson H. Neurotrophin-3 prevents the death of adult central noradrenergic neurons in vivo. *Nature* 1994; **376**: 368–371.
17 Kalia M, Fuxe K, Goldstein M. Rat medulla oblongata. II. Dopaminergic, noradrenergic (A1 and A2) and adrenergic neurons, nerve fibers, and presumptive terminal processes. *J Comp Neurol* 1985; **233**: 308–332.
18 Boundy VA, Gould SJ, Messer CJ, Chen J, Son JH, Joh TH *et al*. Regulation of tyrosine hydroxylase promoter activity by chronic morphine in TH9.0-LacZ transgenic mice. *J Neurosci* 1998; **18**: 9989–9995.
19 Tinti C, Conti B, Cubells JF, Kim KS, Baker H, Joh TH. Inducible cAMP early repressor can modulate tyrosine hydroxylase gene expression after stimulation of camp synthesis. *J Biol Chem* 1996; **271**: 25375–25381.
20 Mercer EH, Hoyle GW, Kapur RP, Brinster RL, Palmiter RD. The dopamine beta hydroxylase gene promoter directs expression of *E. coli* lacZ to sympathetic and other neurons in adult transgenic mice. *Neuron* 1991; **7**: 703–716.
21 Ginty DD, Bonni A, Greenberg ME. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* 1994; **77**: 713–725.
22 Maldonado R, Blendy JA, Tzavara E, Gass P, Roques BP, Hanoune J *et al*. Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 1996; **273**: 657–659.
23 Lane-Ladd SB, Pineda J, Boundy V, Pfeuffer T, Krupinski J, Aghajanian GK *et al*. CREB in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J Neurosci* 1997; **17**: 7890–7901.
24 Hayward MD, Duman RS, Nestler EJ. Induction of the c-fos proto oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. *Brain Res* 1990; **525**: 256–266.
25 Kogan JH, Nestler EJ, Aghajanian GK. Elevated basal firing rates and enhanced responses to 8-Br-cAMP in locus coeruleus neurons in brain slices from opiate-dependent rats. *Eur J Pharmacol* 1992; **211**: 47–53.
26 Zhang J, Ferguson SS, Barak LS, Boddulir SR, Laporte SA, Law PY *et al*. Role for G protein-coupled receptor kinase in agonist-specific regulation of responsiveness. *Proc Natl Acad Sci USA* 1998; **95**: 7157–7162.
27 Vigers AJ, Baquet ZC, Jones KR. Expression of neurotrophin-3 in the mouse forebrain: insight from a targeted lacZ reporter. *J Comp Neurol* 2000; **416**: 398–415.
28 Gehlert DR, Schober DA, Gackenheimer SL. Comparison of (R)–[3H] tomoxetine and (R/S)–[3H] nisoxetine binding in rat brain. *J Neurochem* 1995; **64**: 2792–2800.
29 Sklair-Tavron L, Nestler EJ. Opposing effects of morphine and the neurotrophins, NT-3, NT-4 and BDNF, on locus coeruleus neurons *in vitro*. *Brain Res* 1995; **702**: 117–125.
30 Christie MJ, Williams JT, Osborne PB, Bellchambers CE. Where is the locus in opioid withdrawal? *Trends Pharmacol Sci* 1997; **18**: 134–140.
31 Caille S, Espejo EF, Reneric JP, Cador M, Koob GF, Stinus L. Total neurochemical lesion of noradrenergic neurons of the locus coeruleus does not alter either naloxone-precipitated or spontaneous opiate withdrawal nor does it influence ability of clonidine to reverse opiate withdrawal. *J Pharmacol Exp Ther* 1999; **290**: 881–892.
32 Smith MA, Makino S, Altemus M, Michelson D, Hong SK, Kvetnansky R *et al*. Stress and antidepressants differentially regulate neurotrophin 3 mRNA expression in the locus coeruleus. *Proc Natl Acad Sci USA* 1995; **92**: 8788–8792.
33 DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM *et al*. The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron* 1992; **8**: 983–993.
34 Altar CA, DiStefano PS. Neurotrophin trafficking by anterograde transport. *Trends Neurosci* 1998; **21**: 433–437.
35 Conner JM, Lauterborn JC, Gall CM. Anterograde transport of neurotrophin proteins in the CNS—a reassessment of the neurotrophic hypothesis. *Rev Neurosci* 1998; **9**: 91–103.
36 Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME *et al*. NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* 1990; **5**: 501–509.
37 Lauterborn JC, Isackson PJ, Gall CM. Cellular localization of NGF and NT-3 mRNAs in postnatal rat forebrain. *Mol Cell Neurosci* 1994; **5**: 46–62.
38 Acheson A, Lindsay RM. Non-target derived roles of the neurotrophins. *Philos Trans R Soc Lond Biol Sci* 1996; **351**: 417–422.
39 Drolet G, Van Bockstaele EJ, Aston-Jones G. Robust enkephalin innervation of the locus coeruleus from the rostral medulla. *J Neurosci* 1992; **12**: 3162–3174.
40 Van Bockstaele EJ, Peoples J, Telegan P. Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. *J Comp Neurol* 1999; **412**: 410–428.
41 Duncan GE, Knapp DJ, Breese GR. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. *Brain Res* 1996; **713**: 79–81.
42 Desmedt A, Garcia R, Jaffard R. Vasopressin in the lateral septum promotes elemental conditoning to the detriment of contextual fear conditioning in mice. *Eur J Neurosci* 1999; **11**: 3913–3921.
43 DiCicco-Bloom E, Friedman WJ, Black IB. NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. *Neuron* 1993; **11**: 1101–1111.
44 Albers KM, Perrone TM, Goodness TP, Jones ME, Green MA, Davis BM. Cutaneous overexpression of NT-3 increases sensory and sympathetic neuron number and enhances touch dome and hair follicle innervation. *J Cell Biol* 1996; **134**: 487–497.
45 Francis N, Farinas I, Brennan C, Rivas-Plata K, Backus C, Reichardt L *et al*. NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors. *Dev Biol* 1999; **210**: 411–427.
46 Moller JC, Kruttgen A, Heymach JV Jr, Ghori N, Shooter EM. Subcellular localization of epitope-tagged neurotrophins in neuroendocrine cells. *J Neurosci Res* 1998; **51**: 463–472.
47 Trumpp A, Depew MJ, Rubenstein JL, Bishop JM, Martin GR. Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev* 1999; **13**: 3136–3148.
48 Taylor JR, Elsworth JD, Garcia EJ, Grant SJ, Roth RH, Redmond DE Jr. Clonidine infusions into the locus coeruleus attenuate behavioral and neurochemical changes associated with naloxone-precipitated withdrawal. *Psychopharmacology* 1988; **96**: 121–134.
49 Punch LJ, Self DW, Nestler EJ, Taylor JR. Opposite modulation of opiate withdrawal behaviors on microinfusion of a protein kinase A inhibitor versus activator into the locus coeruleus or periaqueductal gray. *J Neurosci* 1997; **17**: 8520–8527.
50 Huerta PT, Sun LD, Wilson MA, Tonegawa ST. Formation of temporal memory requires NMDA receptors within CAI pyramidal neurons. *Neuron* 2000; **25**: 473–480.

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