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# A Chemical-Genetic Approach to Studying Neurotrophin Signaling

Neurotechnique

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## Summary

**Trk tyrosine kinases are receptors for members of the neurotrophin family and are crucial for growth and survival of specific populations of neurons. Yet, the functions of neurotrophin-Trk signaling in postnatal development as well as maintenance and plasticity of the adult nervous system are less clear. We report here the generation of mice harboring *Trk* knockin alleles that allow for pharmacological control of Trk kinase activity. Nanomolar concentrations of either 1NMPP1 or 1NaPP1, derivatives of the general kinase inhibitor PP1, inhibit NGF and BDNF signaling in TrkA$^{F592A}$ and TrkB$^{F616A}$ neurons, respectively, while no such Trk inhibition is observed in wild-type neurons. Moreover, oral administration of 1NMPP1 leads to specific inhibition of TrkA$^{F592A}$, TrkB$^{F616A}$, and TrkC$^{F167A}$ signaling in vivo. Thus, *Trk* knockin mice provide valuable tools for selective, rapid, and reversible inhibition of neurotrophin signaling in vitro and in vivo.**

## Introduction

The neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) control many aspects of mammalian nervous system development. Neurotrophin receptors are members of the Trk receptor tyrosine kinase family; TrkA is a receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3 (Huang and Reichardt, 2003; Segal, 2003). NT-3 also signals through TrkA and TrkB under certain circumstances (Davies et al., 1995; Huang and Reichardt, 2003; Kuruvilla et al., 2004). In addition, each of the neurotrophins binds to the structurally unrelated neurotrophin receptor p75. Many developmental roles of neurotrophins and their receptors have been established. For example, NGF-TrkA signaling is crucial for the survival and growth of sympathetic neurons as well as small-diameter nociceptive sensory neurons (Levi-Montalcini and Booker, 1960; Smeyne et al., 1994). TrkB and TrkC are also critical for the development of specific populations of peripheral nervous system (PNS) neurons (Klein et al., 1994; Klein et al., 1993). Although less well characterized, neurotrophins also appear to control many aspects of central nervous system (CNS) development and modulate synaptic plasticity, metabolism, and neurotransmission in the adult nervous system (for examples, see Guillin et al., 2001; McAllister et al., 1999; Xu et al., 2003).

While standard gene inactivation approaches have been useful for revealing the in vivo roles of neurotrophins and their receptors in the mouse embryo, these strategies are not well suited for the systematic analysis of the functions of neurotrophins in the postnatal or adult nervous system. Indeed, nearly all *neurotrophin* and *Trk* null mice die perinatally. Conditional knockouts, employing the Cre-LoxP strategy, have proven useful for the analysis of at least some postnatal neurotrophin functions, but limitations of this approach include the dependence on a small number of available Cre recombinase strains, the low efficiency of Cre recombinase, the lack of reversibility, and the poor temporal control of target gene excision. Neutralizing antibodies have also been widely used to study neurotrophin functions (Goedert et al., 1978; Levi-Montalcini and Booker, 1960). However, this approach is greatly hampered by issues of antibody specificity, complications associated with nonselective immune responses, the need for repeated injections, and difficulties of antibody delivery to the CNS. Furthermore, large quantities of specific antibodies that selectively neutralize BDNF, NT-3, and NT-4/5 are either limited or not available. Pharmacological inhibitors of Trk kinase, notably the alkaloid K252a, have also been widely used to study Trk function (Koizumi et al., 1988; Ohmichi et al., 1992; Tapley et al., 1992), but interpretations of experiments using inhibitors such as K252a are difficult due to limited drug specificity. Thus, there is a clear need for strategies allowing potent, specific, rapidly acting, and reversible inhibition of Trk signaling in order to study the roles of neurotrophins during embryonic and postnatal development and during the maintenance and plasticity of the adult nervous system. An ideal approach would combine the specificity of gene targeting and the temporal control, reversibility, and rapid-acting properties offered by a pharmacological approach.

Here we report mouse models that combine the benefits of mouse genetics and pharmacology to probe the functions of neurotrophins. We describe the generation and characterization of mice harboring knockin mutations in the murine *Trk* exons that encode regions of the Trk kinase ATP binding pockets. A phenylalanine-to-alanine substitution within kinase subdomain V of each of the Trk receptors renders them sensitive to specific inhibition by membrane-permeable, small-molecule PP1 derivatives, including 1NMPP1 (Bishop et al., 2000). Such inhibition is specific, stable, and reversible in vitro. Furthermore, in vivo administration of 1NMPP1 leads to elimination of specific populations of neurons in Trk knockin mice but not in their wild-type litter-

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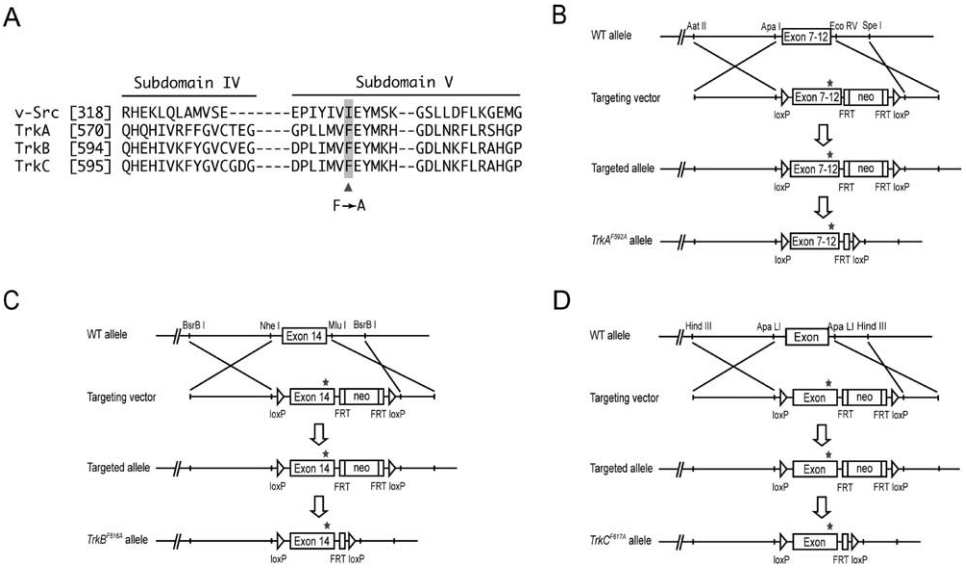


Figure 1. Generation of *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ Knockin Mice

(A) Sequence alignment of mouse TrkA F592, TrkB F616, and TrkC F617 and the corresponding amino acid residues surrounding Src I338 within the ATP binding pocket of protein kinase subdomain V.

(B–D) Targeting strategies for *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ knockin mice. In the targeting vectors, the exons carrying the F-A mutations and FRT-Neo-FRT cassettes are flanked by two LoxP sites. Crosses between the chimeric animals carrying the targeted allele with a mouse line expressing *FlpE* recombinase in the germline leads to excision of the FRT-Neo-FRT cassette. The final *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ targeted alleles contain the targeted exon harboring the F-A mutation, an intron with one FRT site, and flanking introns with LoxP sites.

mates. These *Trk* mutant mice allow specific, potent, and reversible inhibition of Trk signaling and should be useful for both in vivo and in vitro studies addressing neurotrophin functions in the developing and adult nervous system.

## Results

### Generation of *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ Knockin Mice

We sought to develop a chemical-genetic strategy that enables specific, rapid, and reversible inhibition of endogenous Trk kinases. Mutations within the ATP binding pocket of the non-receptor tyrosine kinase v-Src (I338) renders it susceptible to inhibition by small-molecule derivatives of the general kinase inhibitor PP1 (Bishop et al., 2000). At the location corresponding to v-Src I338, TrkA592, TrkB616, and TrkC617, all Trk proteins have a phenylalanine (F) (Figure 1A). Preliminary findings showed that substitution of TrkA F592 with either a glycine (G) or an alanine (A) does not affect NGF-dependent TrkA signaling in PC12 cells, but does render the mutant TrkA receptor susceptible to inhibition by 1NMPP1 and 1NaPP1, derivatives of PP1 (data not shown). Importantly, neither 1NMPP1 nor 1NaPP1 affects the activity of wild-type TrkA. The success of these PC12 cell experiments prompted us to employ a gene-targeting approach to generate knockin mice in which the activities of the endogenous Trk proteins are susceptible to specific inhibition by PP1 derivatives. The gene-targeting strategies used to generate *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ knockin mice are outlined in Figures 1B–1D. Chimeric mice were obtained following blastocyst injection of targeted ES cells, and the neomycin resistant cassettes used for ES cell selection were excised upon crossing the chimeric *Trk* mice with mice expressing *FlpE* recombinase in germ cells. As a result, the *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ knockin mice each harbor a single point mutation, changing F to A within the ATP binding pocket of kinase subdomain V, and contain approximately 100 base pairs of *FRT* and *LoxP* sequences located in adjacent introns. Heterozygous mice harboring the *Trk* F-A knockin mutations were backcrossed to a C57BL/6 background, and homozygous mice were obtained from heterozygous intercrosses.

Unlike each of the *Trk* null mice, which dies perinatally (Klein et al., 1994; Klein et al., 1993; Smeyne et al., 1994), homozygous *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ knockin mice are viable and fertile, with no obvious behavioral abnormality. These findings support the notion that the Trk F-A knockin mutations do not abolish *Trk* gene function. To further address this issue, BDNF-TrkB signaling was assessed using cultured cortical neurons obtained from postnatal day 0.5 (P0.5) *TrkB*$^{F616A}$ pups or wild-type littermates. The BDNF dose-responsiveness for activation of TrkB phosphorylation and downstream effectors, Akt and MAPK, is comparable for wild-type and *TrkB*$^{F616A}$ neurons (Figure 2A). A detailed description of the TrkB$^{F616A}$ kinase kinetics in transfected cells is described elsewhere (N.D. Okerlund, C. Zhang, K.M. Shokat, and P.M. England, submitted). These findings indicate that the F616A mutation has little or no effect on BDNF-mediated TrkB signaling.

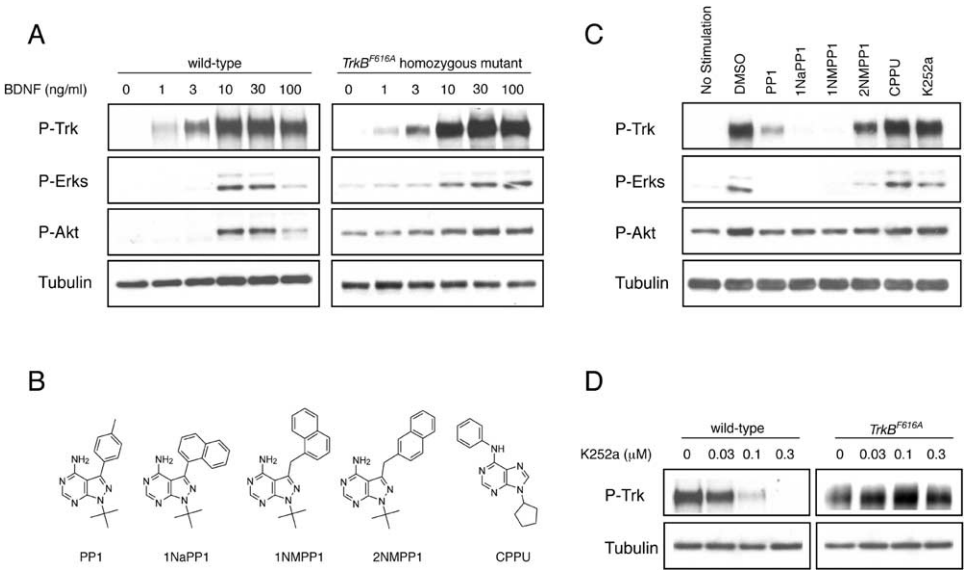


Figure 2. Pharmacological Inhibition of BDNF-TrkB$^{F616A}$ Signaling in Cortical Neurons

(A) Comparable BDNF dose responsiveness for activation of TrkB phosphorylation and its downstream effectors in wild-type and TrkB$^{F616A}$ neurons. Cultured cortical neurons obtained from P0.5 *TrkB$^{F616A}$* homozygous mutant mice or their wild-type littermates were grown in vitro for 5 days, treated with various concentrations of BDNF for 20 min, and P-Trk, P-Akt, and P-Erk signaling was assessed by immunoblot. Differences in basal levels of P-Akt in TrkB$^{F616A}$ and wild-type cultures were not observed in other experiments.

(B) Molecular structures of PP1 and its derivatives.

(C and D) Cultured cortical neurons from *TrkB$^{F616A}$* homozygous mutant mice or their wild-type littermates were treated with BDNF (50 ng/ml) for 20 min in the presence of (C) different PP1 derivatives or K252a or (D) various concentrations of K252a prior to BDNF exposure. Protein extracts were subjected to immunoblot analysis using antibodies against P-Trk(Y490), P-Akt, P-Erks1/2, and α-tubulin.

### Trk F-A Activity Is Specifically and Potently Inhibited by 1NMPP1 and 1NaPP1 In Vitro

To ask if one or more of the PP1 derivatives inhibits the mutant Trk F-A kinases, we tested several PP1 derivatives (100 nM; Figure 2B) for their ability to block BDNF signaling in cortical neurons cultured from *TrkB$^{F616A}$* mice. Both 1NaPP1 and 1NMPP1 achieved complete inhibition of TrkB$^{F616A}$ autophosphorylation and signaling, whereas the same concentration of 2NMPP1 led to partial inhibition, and CPPU had no inhibitory effect (Figure 2C). Interestingly, K252a (100 nM), the commonly used inhibitor of Trk kinases, did not inhibit TrkB$^{F616A}$ signaling, although it did block the activity of wild-type TrkB (Figure 2D). This result indicates that K252a and PP1 derivatives may interact with common residues within the TrkB ATP binding pocket and that the F616A mutation may diminish K252a binding to TrkB.

Side-by-side drug efficacy comparisons were thus carried out with the most potent PP1 derivatives, 1NMPP1 and 1NaPP1. Both drugs were found to potently and specifically inhibit TrkB$^{F616A}$ kinase activity in cortical neurons cultured from either wild-type or *TrkB$^{F616A}$* mutant embryos (Figure 3A; data not shown). The $IC_{50}$ values for 1NaPP1 and 1NMPP1 inhibition of TrkB$^{F616A}$ were both estimated to be approximately 3 nM. In marked contrast, no inhibition of TrkB activity was observed in wild-type cortical neurons treated with either 1NaPP1 or 1NMPP1, even at concentrations as high as 10 μM (Figure 3A; data not shown).

Similar findings were made in experiments designed to test the efficacy and specificity of 1NMPP1 inhibition of TrkA$^{F592A}$ signaling. Since NGF and TrkA are required for survival of sympathetic neurons (Klein et al., 1994; Levi-Montalcini and Booker, 1960), we cultured superior cervical ganglion (SCG) neurons from P0.5 *TrkA$^{F592A}$* mice and their wild-type littermates and assessed their survival after treatment with 1NMPP1. NGF-TrkA$^{F592A}$ signaling was effectively blocked by 100 nM 1NMPP1, as shown by complete inhibition of NGF-dependent survival of sympathetic neurons obtained from *TrkA$^{F592A}$* mice (Figures 3B and 3C). No inhibitory effect was observed for wild-type sympathetic neurons. These findings indicate that 1NMPP1 selectively and potently inhibits the activities of TrkA$^{F592A}$ and TrkB$^{F616A}$, whereas a high concentration of this compound does not affect wild-type Trk receptor signaling.

### 1NMPP1 Inhibition Is Stable and Reversible In Vitro

One of the appealing advantages of using competitive inhibitors to study enzyme function is the reversible nature of their action, which in the case of 1NMPP1 may allow precise temporal control over Trk F-A signaling. To assess the extent of reversibility of 1NMPP1-mediated inhibition of TrkB$^{F616A}$ signaling in cortical neurons, we first exposed TrkB$^{F616A}$ neurons for 2 hr to either 1NMPP1 (100 nM) or DMSO vehicle control, washed away the drug or vehicle, and then reexposed neurons to either DMSO or 1NMPP1. BDNF-TrkB$^{F616A}$ signaling was completely inhibited in 1NMPP1-treated cortical neurons, but is then reestablished after 1NMPP1 washout, indicating that 1NMPP1 inhibition of

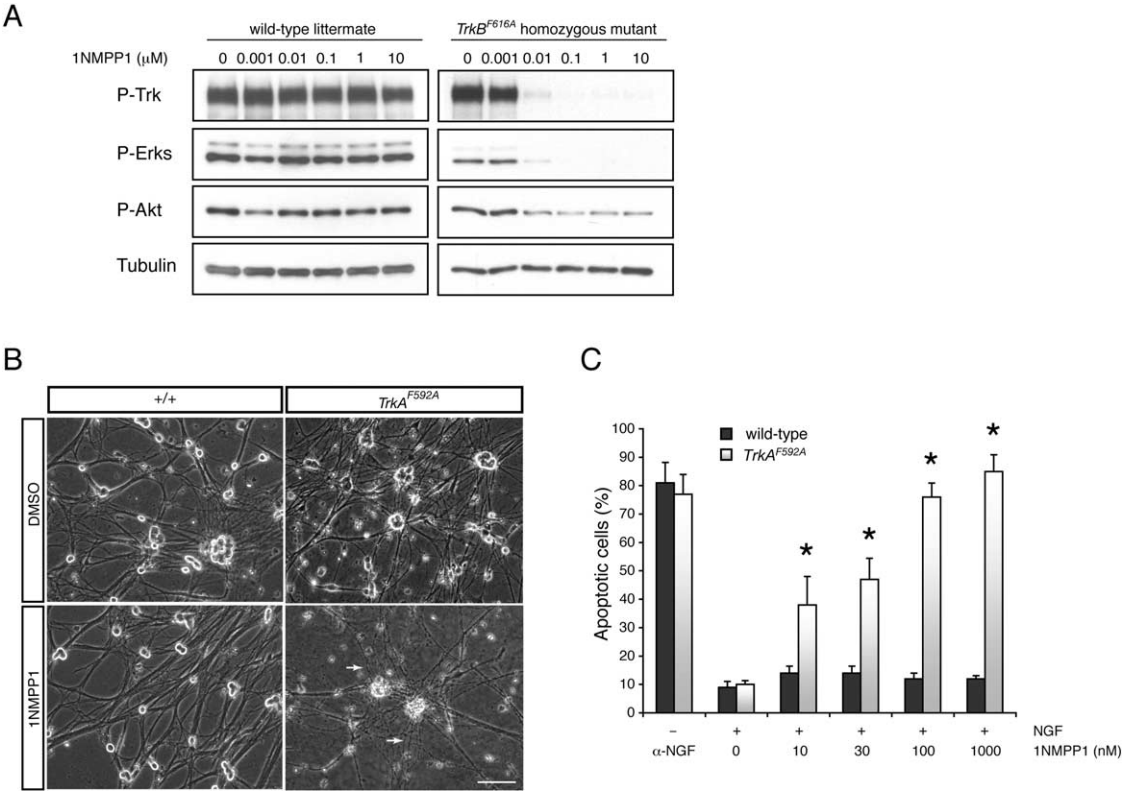


Figure 3. 1NMPP1 Is a Potent and Specific Inhibitor of TrkB$^{F616A}$ and TrkA$^{F592A}$ Signaling

(A) 1NMPP1 specifically inhibits BDNF signaling in TrkB$^{F616A}$ cortical neurons but not wild-type neurons. Cultured cortical neurons from P0.5 *TrkB$^{F616A}$* homozygous mutant mice or their wild-type littermates were treated with the indicated concentrations of 1NMPP1 (2 hr) prior to BDNF exposure (50 ng/ml; 20 min).

(B and C) 1NMPP1 specifically inhibits TrkA$^{F592A}$ but not wild-type TrkA signaling. (B) Phase-contrast images of wild-type (left) and TrkA$^{F592A}$ (right) neurons treated with NGF (50 ng/mL) + vehicle (DMSO) or NGF (50 ng/mL) + 1NMPP1 (100 nM and 1000 nM concentrations for TrkA$^{F592A}$ and wild-type neurons, respectively). Arrows indicate degenerating neurites in TrkA$^{F592A}$ neuronal cultures treated with 1NMPP1. (C) Cultures of sympathetic neurons established from wild-type and TrkA$^{F592A}$ mice were grown in media containing 50 ng/mL NGF for 3–5 days in vitro and then treated with anti-NGF, NGF (50 ng/mL) alone, or various concentrations of 1NMPP1 for 24 hr. After treatment, cells were fixed and stained with Hoechst 33258. Apoptotic cells were scored based on nuclear morphology. The amount of neuronal cell death is presented as mean ± SEM from three independent experiments. *$p < 0.05$, significantly different as ascertained by one-way ANOVA followed by a Tukey's Multiple Comparision test. Scale bar, 100 μM.

TrkB$^{F616A}$ is reversible (Figure 4A). Additionally, in contrast to the commonly used nonselective Trk inhibitor K252a, which requires replenishment every 12 to 24 hr, in vitro stability experiments indicate that a single application of 1NMPP1 (100 nM) to cultured neurons led to sustained inhibition of TrkB$^{F616A}$ signaling for at least 3 days, the duration of the experiment (Figure 4B). These results, taken together, indicate that 1NMPP1 is a potent, specific, stable, and reversible inhibitor of TrkA$^{F592A}$ and TrkB$^{F616A}$ signaling in neurons.

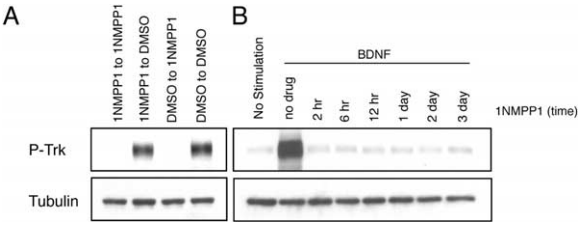


Figure 4. 1NMPP1 Is a Stable and Reversible Inhibitor of TrkB$^{F616A}$ Signaling In Vitro

Cultured cortical neurons from *TrkB$^{F616A}$* homozygous mutant mice were treated with 100 nM 1NMPP1 for 2 hr and then with either DMSO or 100 nM 1NMPP1 for 2 hr (A) or the indicated times (B) and then BDNF (50 ng/ml) for 20 min. Note that removal of 1NMPP1 for 2 hr restores BDNF activation of TrkB$^{F616A}$.

### 1NMPP1 Inhibits Neurotrophin Signaling in Trk F-A Mice In Vivo

We next sought to determine the efficacy and specificity of 1NMPP1 inhibition of Trk F-A signaling in vivo. We reasoned that 1NMPP1 application to *TrkA$^{F592A}$*, *TrkB$^{F616A}$*, and *TrkC$^{F617A}$* embryos, but not to their wild-type littermates, should result in phenotypes similar to those observed in *Trk* null mice. The dependence of sympathetic neurons on TrkA, nodose ganglion neurons on TrkB, and parvalbumin (PV)-positive dorsal root ganglion (DRG) neurons on TrkC is evident from the dramatic cell loss in mice harboring null mutations in the

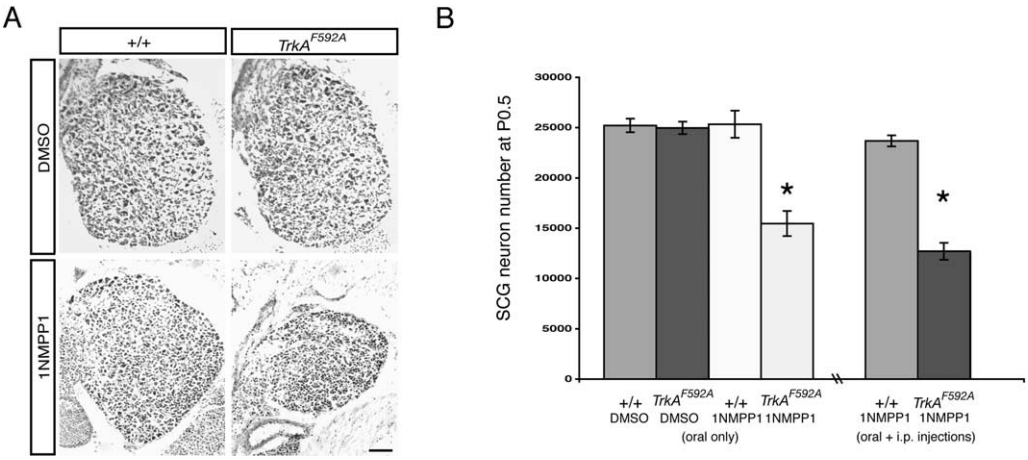


Figure 5. 1NMPP1 Specifically Inhibits TrkA$^{F592A}$ Signaling In Vivo

(A) Nissl staining of superior cervical ganglia.

(B) Cell counts of superior cervical ganglia at P0.5. Heterozygous females that were mated with heterozygous males received either vehicle DMSO or 25 μM 1NMPP1 in drinking water beginning 1 day PC and continued for the duration of the pregnancy. Some animals also received two boosting i.p. injections (16.6 ng/g body weight) at E15 and E17. Results are presented as mean ± SEM from four independent experiments. *$p < 0.05$; one-way ANOVA followed by a Bonferroni post hoc test. Scale bar, 100 μm.

respective *Trk* and *neurotrophin* genes (Klein et al., 1994; Klein et al., 1993; Smeyne et al.,1994). We therefore assessed the integrity of SCG, nodose ganglia, and PV-positive DRG neurons in the Trk F-A knockin mice. Cell counts revealed no significant differences in SCG, nodose ganglion, and PV-positive DRG neuron numbers between Trk F-A knockin pups and their wild-type littermates at P0.5 (Figures 5–7). Therefore, consistent with the aforementioned results of in vitro experiments, the F-A knockin mutations do not adversely affect neurotrophin signaling in vivo. To assess the extent of 1NMPP1 inhibition of NGF-TrkA$^{F592A}$ signaling in vivo, we administered 1NMPP1 in drinking water (25 μM) to *TrkA$^{F592A}$* heterozygous mice throughout their pregnancies after they were mated to mice of the same genotype. In parallel experiments, 1NMPP1 was administered in the drinking water together with two intraperitoneal boosting injections (16.6 ng/g body weight) at E15 and E17. In both sets of experiments, neuronal counts of SCG and nodose ganglion were done in the P0.5 progeny of 1NMPP1 or vehicle-treated *TrkA$^{F592A}$* mice. SCG counts revealed approximately 50% neuronal loss in *TrkA$^{F592A}$* homozygous offspring whose mothers received 1NMPP1, whereas no effect was observed in wild-type littermates (Figure 5). This degree of cell loss is comparable to that observed in P0 *TrkA* null mice (Fagan et al., 1996). As expected, nodose ganglion neurons, which depend on TrkB signaling for survival, were not affected in these experiments, indicating that TrkB signaling is not affected by 1NMPP1 treatment of *TrkA$^{F592A}$* mice (data not shown).

Comparable experiments were carried out to assess the efficacy and specificity of 1NMPP1 inhibition of TrkB$^{F616A}$ and TrkC$^{F617A}$ signaling in vivo. Nodose ganglion neuron counts revealed 60% neuronal loss in *TrkB$^{F616A}$* homozygous offspring whose mothers were treated with 1NMPP1, while no cell loss was observed in wild-type littermates. Drinking water administration of 1NMPP1 was sufficient to block TrkB$^{F616A}$ signaling in nodose ganglion neurons; a comparable amount of nodose ganglion cell loss was observed in experiments with and without the boosting injections (Figure 6). As expected, SCG neuron viability was not affected in either group (data not shown). Moreover, we observed a dramatic decrease of PV-positive fibers in the lumbar spinal cord and PV-positive DRG neurons in 1NMPP1-treated *TrkC$^{F617A}$* mice, but not in their wild-type littermates (Figure 7). This degree of proprioceptive neuron loss is comparable to that observed in *Nt3* null mice (Ernfors et al., 1994). Taken together, these results indicate that 1NMPP1, administered orally, effectively and specifically inhibits TrkA$^{F592A}$, TrkB$^{F616A}$, and TrkC$^{F617A}$ signaling in vivo.

## Discussion

Here we describe mouse models that allow for potent, specific, and reversible inhibition of Trk kinase activity in vitro and in vivo. Our chemical-genetic approach for studying neurotrophin-Trk receptor signaling combines the specificity of gene targeting and the convenience, rapid temporal control, and reversibility of competitive pharmacology. A similar strategy should prove useful for studying the neural functions of other growth factor receptors, including c-Ret, FGF receptors, EGF receptors, and Eph receptors.

Major advantages of the chemical-genetic approach to studying neurotrophin signaling include the specificity of inhibition and the availability of a compelling control, the wild-type littermate. These benefits circumvent the concerns of typical pharmacological experiments. There are more than 500 kinases in the protein kinase superfamily, and they exhibit a remarkable degree of conservation within their ATP binding domains. The most commonly used protein kinase inhibitors compete with ATP binding, but the interpretations of such experi-

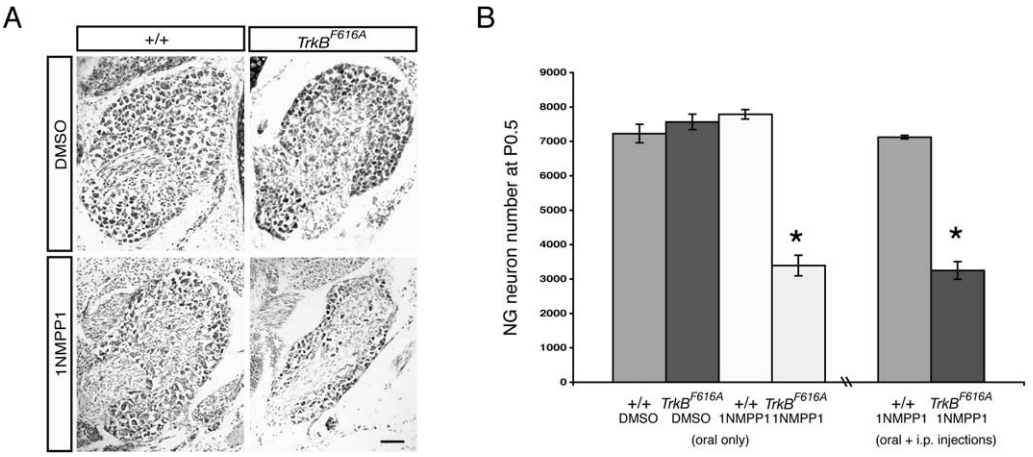


Figure 6. 1NMPP1 Specifically Inhibits TrkB$^{F616A}$ Signaling In Vivo

(A) Nissl staining of nodose ganglia.

(B) Cell counts of nodose ganglia at P0.5. Heterozygous females that were mated with heterozygous males received either vehicle DMSO or 25 μM 1NMPP1 in drinking water beginning 1 day PC and continued for the duration of the pregnancy. Some animals also received two boosting i.p. injections (16.6 ng/g body weight) at E15 and E17. Results are presented as mean ± SEM from four independent experiments. *p < 0.05; one-way ANOVA followed by a Bonferroni post hoc test. Scale bar, 100 μm.

ments are hampered by the uncertainty of inhibitor specificity. Indeed, the most commonly used Trk inhibitor, K252a, also blocks the activities of other tyrosine kinases as well as a number of serine/threonine kinases, including MLCK and PKC (Kase et al., 1987; Nakanishi et al., 1988). Paradoxically, high concentrations of K252a can actually support survival of neurotrophin-dependent neurons, consistent with the existence of multiple unidentified targets of this drug (Borasio, 1990; Roux et al., 2002). Furthermore, it is difficult to design control experiments that provide a measure of specificity of K252a or the other ATP competitive inhibitors. For experiments employing the Trk F-A mice described here, an excellent control of 1NMPP1 specificity is provided by wild-type littermates. The sole difference between experimental and control neurons is the F-A substitution, which is by itself functionally silent. The Trk F-A mutants reported here are potently and specifically inhibited by the PP1 derivatives. Indeed, the $IC_{50}$ of 1NMPP1 and 1NaPP1 inhibition of Trk F-A neurons is ~3 nM, whereas no inhibition of wild-type TrkA and TrkB is observed with drug concentrations as high as

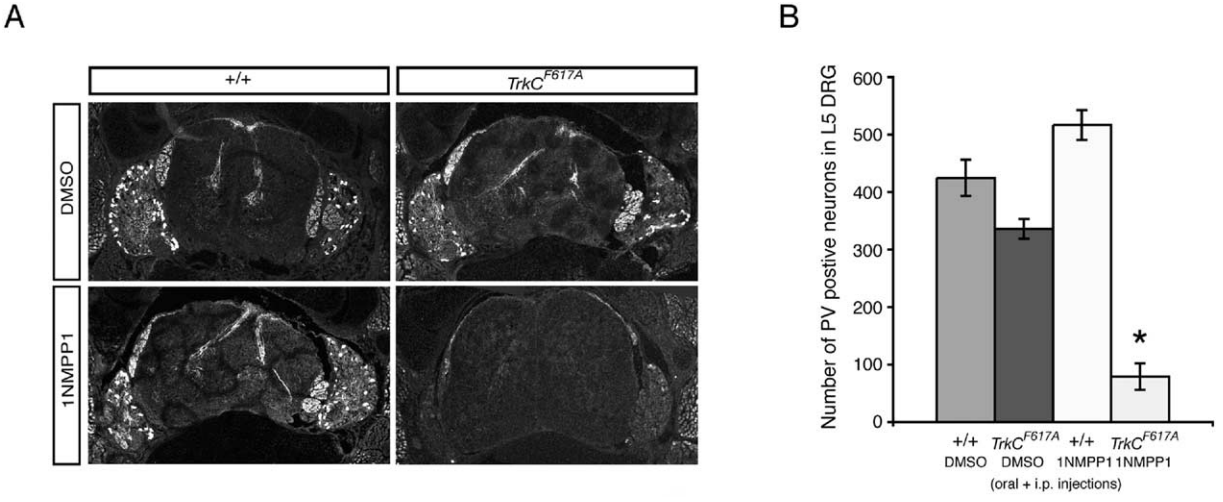


Figure 7. 1NMPP1 Specifically Inhibits TrkC$^{F617A}$ Signaling In Vivo

(A) Parvalbumin staining of lumber spinal cord and dorsal root ganglia.

(B) Cell counts of parvalbumin-positive neurons in L5 dorsal root ganglia at P0.5. Heterozygous females that were mated with heterozygous males received either vehicle DMSO or 25μM 1NMPP1 in drinking water beginning 1 day PC and continued for the duration of the pregnancy. These animals also received two boosting i.p. injections (16.6 ng/g body weight) at E13 and E15. Results are presented as mean ± SEM from four independent experiments. *p < 0.05; one-way ANOVA followed by a Bonferroni post hoc test. Scale bar, 100 μm.

10 μM. This high degree of inhibitor potency compares quite favorably to the most potent kinase inhibitors, including the heat-stable inhibitor of PKA, PKI (Mitchell et al., 1995). Furthermore, the PP1 derivatives are extremely stable in vitro, as compared to other inhibitors. While K252a experiments require drug replenishment every 12 to 24 hr, a single dose of 1NMPP1 leads to potent inhibition of Trk F-A signaling for at least 3 days. Finally, 1NMPP1 inhibition of Trk F-A mutants is both fast acting and reversible. Along with inhibitor specificity, these convenient features of 1NMPP1 pharmacology should enable a wide range of future experiments addressing Trk receptor function.

Another advantage of the Trk F-A knockin mice is the convenient mode of drug administration. PP1 inhibitors have proven effective when administered through IP injection or drinking water. Drinking water administration of 1NMPP1 effectively blocks Trk F-A signaling in vivo, as shown by our findings that oral administration of 1NMPP1 to pregnant *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ heterozygous mice leads to loss of selective populations of neurons in homozygous Trk F-A embryos. 1NMPP1 inhibition of Trk F-A signaling in vivo is robust. The SCG of 1NMPP1-treated *TrkA*$^{F592A}$ mice exhibit ~50% of the normal complement of sympathetic neurons. This result compares favorably to the extent of cell loss observed in *TrkA* null mice at P0.5 (Fagan et al., 1996). Similarly, comparable to that observed in *Nt3* null mice (Ernfors et al., 1994), PV-positive DRG neurons are almost completely lost in 1NMPP1-treated *TrkC*$^{F617A}$ mice. The destruction of specific populations of neurons in Trk F-A mice but not control littermates whose mothers were treated with 1NMPP1 indicates that this inhibitor can pass the placental barrier to selectively inhibit Trk F-A signaling in embryos in utero. Thus, the oral route of 1NMPP1 delivery will likely prove useful for experiments designed to establish the precise developmental times at which Trk signaling events are required for aspects of neuronal development. Importantly, 1NMPP1 can also pass the blood-brain barrier. Indeed, Wang et al. showed that 1NMPP1 effectively inhibits the activity of a mutant CaMKII produced by transgenic overexpression in the adult brain (Wang et al., 2003). Thus, low molecular weight, membrane-permeable PP1 derivatives are well suited for in vivo functional experiments requiring precise temporal control over Trk F-A receptor activity.

Much remains to be learned about the postnatal functions of neurotrophin signaling, since the lethality of *neurotrophin* and *Trk* null mice has severely limited their usefulness in addressing neurotrophin function during much of CNS development. The Trk F-A knockin mice, unlike *Trk* null mice, are healthy and have no observable behavioral or physiological phenotype. We therefore anticipate that the Trk F-A mice will be valuable for assessing the requirement of Trks in the postnatal development of the nervous system. Pharmacological inhibition of TrkA$^{F592A}$ in postnatal mice should allow for a determination of the role of NGF-TrkA signaling during development of basal forebrain cholinergic neurons, which express high levels of TrkA. Similarly, inhibition of TrkB$^{F616A}$ and TrkC$^{F617A}$ in double knockin mice should provide insights into the potentially redundant developmental roles of TrkB and TrkC, which are coexpressed in many populations of postnatal CNS neurons. The Trk F-A mice should also prove useful for addressing adult functions of neurotrophins, including their potential roles in neuronal survival, axon and dendrite maintenance, neural regeneration, adult stem cell differentiation and growth, neurotransmission, neural plasticity, and behavior. Finally, the rapid and reversible nature of 1NMPP1 inhibition of Trk F-A signaling should enable studies that define the precise temporal contributions of neurotrophin-Trk signaling events during development, plasticity, and behavior.

In summary, we have developed mouse models that allow for specific, temporal, and reversible inhibition of neurotrophin-Trk signaling in vitro and in vivo. The *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ knockin mice should prove useful for elucidation of the functions of neurotrophins during embryonic and postnatal development as well as during maintenance and plasticity of the adult nervous system.

## Experimental Procedures

### Generation of *TrkA*$^{F592A}$, *TrkB*$^{F616A}$, and *TrkC*$^{F617A}$ Mutant Mice

Murine TrkA F592, TrkB F616, and TrkC F617 are the residues corresponding to Src I338 used previously to establish specific chemical inhibition of Src kinase activity (Bishop et al., 2000). The *trk* exons encoding these amino acids were altered by homologous recombination. To construct the targeting vector for *TrkA*$^{F592A}$, a 12.3 kb Aat II-Spe I fragment containing exon 12 of *TrkA* was isolated from a 129J mouse genomic BAC library (RPCI-22). The targeting vector was comprised of a 1.0 kb short arm, a 7.6 kb long arm, a 3.7 kb targeted sequence carrying the F592A mutation, and the FRT-Neo-FRT cassette flanked by two loxP sites. To construct the targeting vector for *TrkB*$^{F616A}$, a 11.2 kb Bsr BI fragment containing exon 14 of *TrkB* was isolated from a 129J mouse genomic BAC library (RPCI-22). The targeting vector was comprised of a 0.7 kb short arm, a 7.3 kb long arm, 3.2 kb targeted sequence carrying the F616A mutation, and the FRT-Neo-FRT cassette flanked by two loxP sites. To construct the targeting vector for *TrkC*$^{F617A}$, a 11.7 kb Hind III fragment containing the target exon of *TrkC* was isolated from a 129J mouse genomic BAC library (RPCI-22). The targeting vector was comprised of a 3.8 kb short arm, a 7.0 kb long arm, a 0.9 kb targeted sequence carrying the F617A mutation, and the *FRT-Neo-FRT* cassette flanked by two loxP sites. Mutagenesis was done using a site-directed PCR mutagenesis strategy (Stratagene). Linearized targeting vectors were electroporated into 129.1 mouse strain embryonic stem (ES) cells, and ES cell clones were selected with G418 (300 μg/ml). ES cell clones were screened by PCR, and results were confirmed by Southern blotting. Four positive ES clones for each construct were injected into C57BL/6 blastocysts, which were then introduced into pseudopregnant females. Chimeric animals were mated with C57BL/6 to produce heterozygous animals, and these mice were subsequently crossed with mice expressing FlpE recombinase (Rodriguez et al., 2000) in germ cells (kindly provided by Susan Dymecki, Harvard University) to excise the *neo* cassettes. All analyses were performed on mice with a 129J and C57BL/6 hybrid genetic background. All procedures relating to animal care and treatment conformed to institutional and NIH guidelines.

### Chemical Synthesis of 1NMPP1 and 1NaPP1

1NMPP1 and 1NaPP1 were synthesized as described previously (Benjamin et al., 2003; Wang et al., 2003).

### Sympathetic Neuron Survival Assays

Sympathetic neurons were prepared from superior cervival ganglia from postnatal day 0.5 (P0.5) mice as previously described (Kuruvilla et al., 2000). Cells were washed three times with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) and then subjected to the various treatments as indicated in the figure legends. Cell sur-

vival assays were performed as previously described (Kuruvilla et al., 2000).

### Cortical Neuron Culture

High-density cortical neuron cultures were prepared as previously described (Rosen et al., 1994), except that the cells were maintained in Neurobasal-SFM medium supplemented with B-27 and 0.5 mM Glutamine (Invitrogen). Cortical neurons were used 5 days after plating for treatments and immunoblot experiments as indicated in the figure legends. Cells were lysed with boiling Laemmli buffer, and samples were resolved by SDS-PAGE, followed by immunoblotting.

### Immunoblotting and Antibodies

Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against P-TrkA(Y490), P-Akt (Ser473), or P-Erk1/2 (Cell Signaling). Normalization for protein amounts was done by reprobing the immunoblots with an antibody against $\alpha$-tubulin (Santa Cruz Biotechnology). All blocking and washing solutions for immunoblotting were using TBS (50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) containing 5% milk and 0.1% Tween-20.

### Immunostaining Procedures

Embryonic tissues were fixed in PBS containing paraformaldehyde (PFA, 4%), cryoprotected overnight in PBS containing 30% sucrose, and 10 $\mu$m sections were prepared using a cryostat. For parvalbumin immunostaining, sections were blocked in PBS containing BSA (1%) and Triton X-100 (0.1%) for 1.5 hr. Primary antibody (PV28, SWANT) was diluted 1:1500 in blocking solution and incubated overnight at 4°C. Fluorescent secondary antibody (Molecular Probes) was diluted 1:750 in blocking solution.

### Neuronal Counts

Tissues were fixed in PBS containing 4% paraformaldehyde (PFA), cryoprotected overnight in PBS containing 30% sucrose, and frozen. Cyrostat sections (10 $\mu$m) of superior cervical ganglion (SCG) and nodose ganglion were prepared and stained with a solution containing 0.5% cresyl violet, and cells with visible nucleoli were counted as neurons (Lonze et al., 2002). For SCG cell count, every fifth section was collected, and nissl staining was performed. Area was measured on all sections, and three representative sections were counted for SCG. For nodose ganglion cell counts, every fifth section was collected, nissl staining was performed, and every section was counted. For dorsal root ganglion (DRG) cell counts, every fourth cryostat section (10 $\mu$m) was collected. Parvalbumin immunostaining was performed on all sections, and every section was counted. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni test.

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