BDNF modulates heart contraction force and long-term homeostasis through truncated TrkB.T1 receptor activation

Gianluca Fulgenzi,[1,2] Francesco Tomassoni-Ardori,[1] Lucia Babini,[1] Jodi Becker,[1] Colleen Barrick,[1] Sandrine Puverel,[1] and Lino Tessarollo[1]

[1]Neural Development Section, Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702
[2]Department of Molecular and Clinical Sciences, Marche Polytechnic University, Ancona, 60020, Italy

Brain-derived neurotrophic factor (BDNF) is critical for mammalian development and plasticity of neuronal circuitries affecting memory, mood, anxiety, pain sensitivity, and energy homeostasis. Here we report a novel unexpected role of BDNF in regulating the cardiac contraction force independent of the nervous system innervation. This function is mediated by the truncated TrkB.T1 receptor expressed in cardiomyocytes. Loss of TrkB.T1 in these cells impairs calcium signaling and causes cardiomyopathy. TrkB.T1 is activated by BDNF produced by cardiomyocytes, suggesting an autocrine/paracrine loop. These findings unveil a novel signaling mechanism in the heart that is activated by BDNF and provide evidence for a global role of this neurotrophin in the homeostasis of the organism by signaling through different TrkB receptor isoforms.

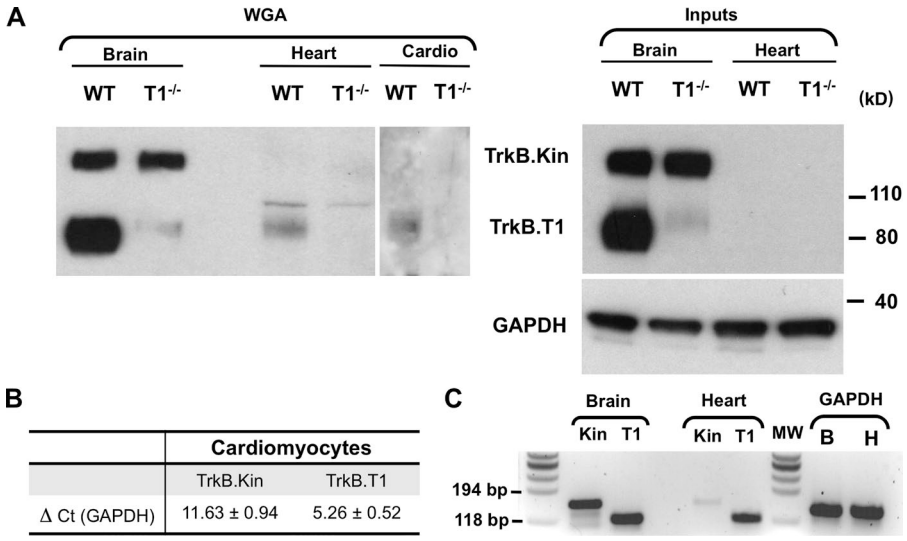
Introduction

Brain-derived neurotrophic factor (BDNF) is a growth factor widely expressed in the nervous system. Changes in its level have been found and correlated to the development of several human diseases including neurodegeneration, depression, psychiatric disorders, and obesity (Chao et al., 2006; Nagahara and Tuszynski, 2011; Lu et al., 2014). The direct relevance of normal BDNF signaling to human fitness has been validated by the use of animal models, which have also allowed the dissection of the molecular mechanism underlying BDNF function in vivo (Rios et al., 2001; Zuccato and Cattaneo, 2009; Baydyuk and Xu, 2014). The TrkB gene encodes BDNF high affinity receptors that are widely expressed in neuronal tissues (Klein et al., 1990; Escandón et al., 1994). This locus generates multiple TrkB isoforms all of which have the same extracellular domain but have different intracellular domains (Stoilov et al., 2002). The two main isoforms include a full-length receptor with a tyrosine kinase domain (TrkB.Kin) used for signaling and a truncated TrkB.T1 receptor lacking kinase activity (Klein et al., 1990; Dorsey et al., 2006). Although a role for TrkB.Kin has been established in neuronal development and function including differentiation, outgrowth, and synaptic plasticity, the physiological significance of TrkB.T1 intrinsic signaling is still unclear despite its high sequence conservation among species and its being the most highly expressed TrkB isoform in the mature animal. Mice lacking TrkB.T1 have increased anxiety-related behavior that is associated with structural alterations in neurites of the amygdala (Carim-Todd et al., 2009). Despite reports of TrkB.T1 signaling in isolated glia cells there are no obvious deficiencies in this cell population in TrkB.T1 mutant mice (Rose et al., 2003; Dorsey et al., 2006; Ohira et al., 2006; Carim-Todd et al., 2009). In the cardiovascular system, BDNF and its receptor TrkB have been described to have an early developmental role in cardiac endothelium formation (Anastasia et al., 2014). Interestingly, in the adult heart only TrkB.T1-specific polyadenylated mRNA has been reported, suggesting protein expression of this particular receptor isoform (Stoilov et al., 2002). Our analysis confirms this finding and we further show that it mediates BDNF inotropic function by regulating Ca^{2+} signaling. We found that specific deletion of TrkB.T1 in cardiomyocytes causes cardiomyopathy and that BDNF is the ligand activating TrkB.T1. We show that BDNF is secreted by cardiomyocytes and its specific deletion in cardiomyocytes causes a cardiomyopathy resembling that caused by TrkB.T1 deficiency. Our data unveil a novel nonneuronal function for BDNF and uncover the first physiologically relevant direct signaling activity of the TrkB.T1 receptor. These findings identify a new pathway regulating cardiac contractility and suggest that perturbation in BDNF and TrkB expression may cause cardiac pathological conditions.

Correspondence to Lino Tessarollo: tessarol@mail.nih.gov

Abbreviations used in this paper: BDNF, Brain-derived neurotrophic factor; bpm, beats per minute; IP_3, inositol-1,4,5-trisphosphate; LVDP, left ventricle developed pressure; PLB, phospholamban; WT, wild type.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 210 No. 6 1003–1012
www.jcb.org/cgi/doi/10.1083/jcb.201502100



	Cardiomyocytes	
	TrkB.Kin	TrkB.T1
Δ Ct (GAPDH)	11.63 ± 0.94	5.26 ± 0.52

Figure 1. **TrkB.T1 receptor isoform is expressed in adult mouse heart and cardiomyocytes.** (A) Western blot analysis of brain, heart, and cardiomyocyte lysates from adult WT and TrkB.T1-deficient ($T1^{-/-}$) mice. Lysates were incubated with wheat germ lectin agarose to enrich for glycoproteins. The wheat germ agglutinated (WGA) precipitates were analyzed by Western blot analysis with an antibody directed against the extracellular domain of TrkB to detect all TrkB isoforms. Note that in whole heart and cardiomyocytes (Cardio) only a truncated TrkB isoform (80–90 Kd) is detected. The absence of the corresponding band in the TrkB.T1 knockout animals verifies the identity of the receptor. Brain lysates were used as a positive control. Right panel, input lysates. (B) Quantification of real-time PCR analysis as expressed by the number of PCR cycles at which full-length TrkB (TrkB.Kin) or TrkB.T1-specific PCR products are equal to GAPDH level (Δ Ct) from total cardiomyocyte RNA. Note that TrkB.T1 is expressed at a much higher level as PCR products appear after only 5 cycles of GADPH detection versus 11 cycles for TrkB.Kin. (C) Ethidium bromide agarose gel visualizing the size of the DNA fragments from heart RT-PCR analysis. Note that the size of the PCR reaction products corresponding to TrkB kinase (Kin) and TrkB.T1 (T1) are the same as those from brain used as a positive control. B, brain; H, heart.

Results

To address a potential role of BDNF in the mature cardiovascular system we first investigated the pattern of expression of its receptor TrkB in the adult mouse heart (Fig. 1). Although the full-length TrkB tyrosine kinase receptor is expressed in the cardiac endothelium during development (Donovan et al., 2000), we found that in the adult this isoform is virtually undetectable and only trace levels of its mRNA can be found by RT-PCR (Fig. 1, B and C). Instead, truncated TrkB.T1 protein is the dominant cardiac-expressed isoform as previously shown by RNA expression analyses (Fig. 1; Stoilov et al., 2002). The presence of TrkB mRNA and protein in the heart suggests an intrinsic role for this receptor independent of the nervous system cardiac innervation. Thus, we next tested whether BDNF plays a role in adult cardiac function in an ex vivo paradigm by perfusing isolated mouse hearts (Fig. 2). BDNF injected in the fluid streamline of a Langendorff-perfused mouse heart (Broadley, 1979) induced an increase in the cardiac contraction force as shown by an increase in systolic pressure and a decrease of the diastolic pressure (Fig. 2, A–D). The effect of BDNF appeared exclusively inotropic and lusitropic as it did not affect the spontaneous cardiac contraction frequency (Fig. S1). BDNF also did not influence the coronary flow (Fig. S2). Perfusion of the heart paced at 420 beats per minute (bpm) with 1 ng BDNF causes an increase in the left ventricle developed pressure (LVDP; EC_{50} of 0.3 ng BDNF) with a timing similar to that elicited by the control compound caffeine (Fig. 2 E and Fig. S1). To test whether BDNF signaling is mediated by the TrkB tyrosine kinase receptor we attempted to use the TrkBF616A mouse model in which TrkB tyrosine kinase activity can be silenced by the specific phosphatase inhibitor 1-NMPP1 drug (Chen et al., 2005). However, in Langerdorff-perfused wild-type (WT) mouse hearts we found that 100 nM 1-NMPP1 induced a dramatic increase in the cardiac contraction force that blunted the BDNF effect, thus preventing the use of this model for this application (Fig. S3). Nevertheless, pretreatment with the K252a tyrosine kinase inhibitor did not prevent the increase in cardiac force elicited by BDNF, suggesting a function independent of TrkB kinase signaling (Fig. 2, B and D).

Interestingly, pretreatment with isoproterenol, a nonselective β-adrenergic agonist, did not blunt the BDNF inotropic effect but was instead additive, suggesting that BDNF signaling does not overlap with the catecholamine pathways (Fig. 3, A and B). In addition, when we tested whether BDNF changes the phosphorylation level of some of the major molecular players involved in cardiac contraction, including CamKII, phospholamban (PLB), and troponin, we found that in Langendorff-perfused hearts at the peak of BDNF action phosphorylation levels were unchanged contrary to hearts treated with forskolin used as positive control (Fig. 3, C–F). These data suggest that BDNF exerts cardiac function through other pathways or this effect is modulatory and not immediately detectable with conventional biochemical analysis.

The effect on cardiac contraction force suggests that cardiomyocytes may be the cell type responding to BDNF with Ca^{2+} as the possible downstream effector. Therefore, we tested adult cardiomyocytes for TrkB expression and their response to BDNF (Fig. 1 A and Fig. 2, I–K). We found that isolated cardiomyocytes do express TrkB.T1 (Fig. 1 A) and when subjected to direct depolarization they elicited higher Ca^{2+} transients by treatment with BDNF compared with just vehicle (Fig. 2 I). Moreover, the Ca^{2+} transient increase was reversible because it was restored to the basal electrical stimulation level after BDNF removal (Fig. 2 J).

The fast action of BDNF and the finding that K252a does not block this function in heart (Fig. 2 B) and cardiomyocytes (Fig. 2 K) suggest that this role is not mediated by the TrkB ki-

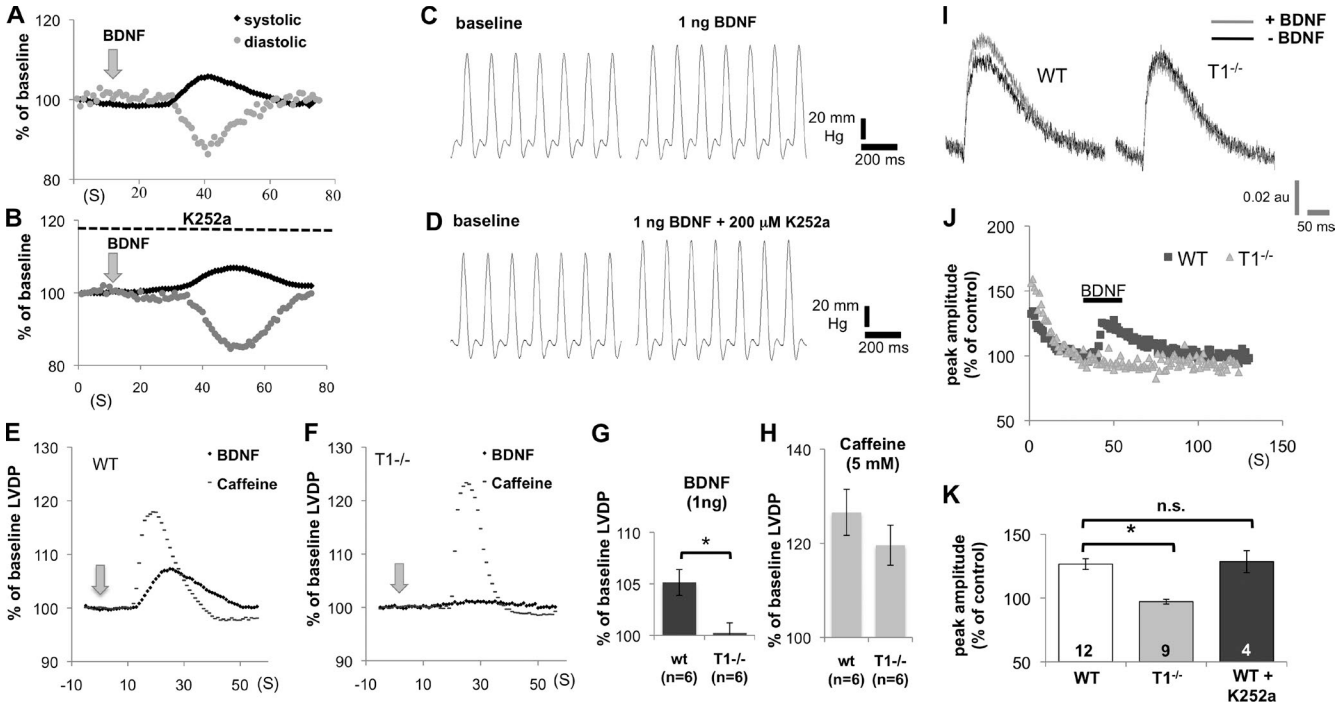


Figure 2. **TrkB.T1 mediates BDNF-induced acute increase in cardiac contraction force and calcium transient increase evoked by direct stimulation in cardiomyocytes.** (A and B) BDNF (1 ng in 50 µl Krebs solution) injected in the fluid streamline of a Langendorff-perfused mouse heart induces an increase in systolic pressure and a consequent decrease of the diastolic pressure (A) that is not affected by the TrkB kinase inhibitor K252a (B), Arrows indicate the time of BDNF injection and the broken line (B) indicates K252a presence throughout the 80-s duration of the experiment. (C) Representative traces showing the changes in LVPD before (baseline) and after BDNF injection. (D) Representative traces showing the changes in LVPD before (baseline) and after BDNF injection in the presence of 200 µM K252A. (E and F) Representative traces showing the increase in LVDP caused by BDNF in WT (E) but not in TrkB.T1 knockout ($T1^{-/-}$; F) hearts. A bolus of 50 µl of 5 mM caffeine in Krebs solution was injected 5 min after BDNF as a positive control. The BDNF and caffeine traces were overlapped using the injection time as the starting point (arrow). Note the lack of LVDP change in response to BDNF in the TrkB.T1 mutant mouse despite normal response to caffeine. (G and H) Quantification of data in E and F showing the percent change of baseline LVPD in response to BDNF (G) and caffeine (H) in WT and $T1^{-/-}$ hearts. (I) Representative traces of Ca^{2+} transients elicited by 2-Hz stimulation in isolated adult cardiomyocytes. Ca^{2+} transient are increased by BDNF application only in WT (BDNF, light gray; no BDNF, black) but not in $T1^{-/-}$ cardiomyocytes. (J) Effect of BDNF on transient amplitude is reversible as shown in typical time course from a WT and a $T1^{-/-}$ cardiomyocyte. BDNF effect on Ca^{2+} release has a rapid onset and is completely reversed in <2 min upon BDNF removal. (K) Quantification of peak amplitude of calcium transient in WT and $T1^{-/-}$ cardiomyocytes. The value for each group represents the transient change in percentage before (considered as 100%) and after BDNF application. 10 transients before and 10 transients after BDNF application were measured for each cardiomyocyte analyzed (*n* is shown within the bar). Values in G–K are indicated as the mean ± SEM. *, $P < 0.05$.

nase receptor but by the TrkB.T1 isoform, which is expressed in cardiomyocytes and has been shown to regulate Ca^{2+} signaling, at least in astrocytes (Rose et al., 2003). This prompted us to test whether this receptor mediates cardiac BDNF actions by using a specific TrkB.T1-deficient mouse model (Dorsey et al., 2006). Injection of BDNF in the perfusion system of TrkB.T1 knockout hearts failed to induce the rapid increase in LVDP observed in control hearts. However, their response to caffeine was normal (Fig. 2, F–H). Similarly, adult cardiomyocytes isolated from TrkB.T1 knockout mice failed to show the increase in Ca^{2+} transient evoked by BDNF in controls (Fig. 2, I–K). These data strongly suggest that the TrkB.T1 receptor isoform mediates the inotropic BDNF function in the adult heart and may have a role in normal cardiac physiology. To investigate the in vivo significance of the findings from the ex vivo and cardiomyocyte experiments we performed histological analysis of adult TrkB.T1-deficient hearts. TrkB.T1 knockout hearts showed a cardiac dilation characterized by reduced thickness of the left ventricular wall (Fig. 4, A and B). Moreover, in vivo echocardiography analysis showed an overall enlarged ventricular volume and thinner posterior wall thickness in $TrkB.T1^{-/-}$ mice compared with WT controls (Fig. 4, C and D). To investigate the significance of the cardiac pathological condition caused by loss of TrkB.T1 we also looked for the presence of mechanisms of cardiac function adaptation including changes in L-type–mediated Ca^{2+} currents (Benitah et al., 2010). Electrophysiological recording of isolated cardiomyocytes detected an increase in L-type–mediated Ca^{2+} current density paired with a higher expression level of L-type Ca^{2+} channels as shown by the biochemical analysis of the CaV1.2 α1c subunit (Fig. 4, E–J). These data combined with the significant impairment in some functional parameters such as a reduced left ventricle-developed pressure measured in Langendorff-perfused mutant hearts suggest that TrkB.T1 is critical for normal cardiac function and its deletion induces secondary changes as a mechanism to compensate for the functional deficits (Table S1). Interestingly, the cardiomyopathy developed by TrkB.T1-deficient mice does not appear to originate during embryonic or early postnatal development as histological analysis of TrkB.T1 mutant mice at postnatal day 12 shows normal posterior wall thickness and no sign of left ventricle dilation (Fig. S4, A and B).

Several studies have reported a putative role for BDNF in central nervous system nuclei controlling cardiac function, peripheral innervation, and the vasculature, suggesting that the phenotype observed in TrkB.T1 mutant mice could be the result of indirect alterations in the microvasculature and/or the sympa-

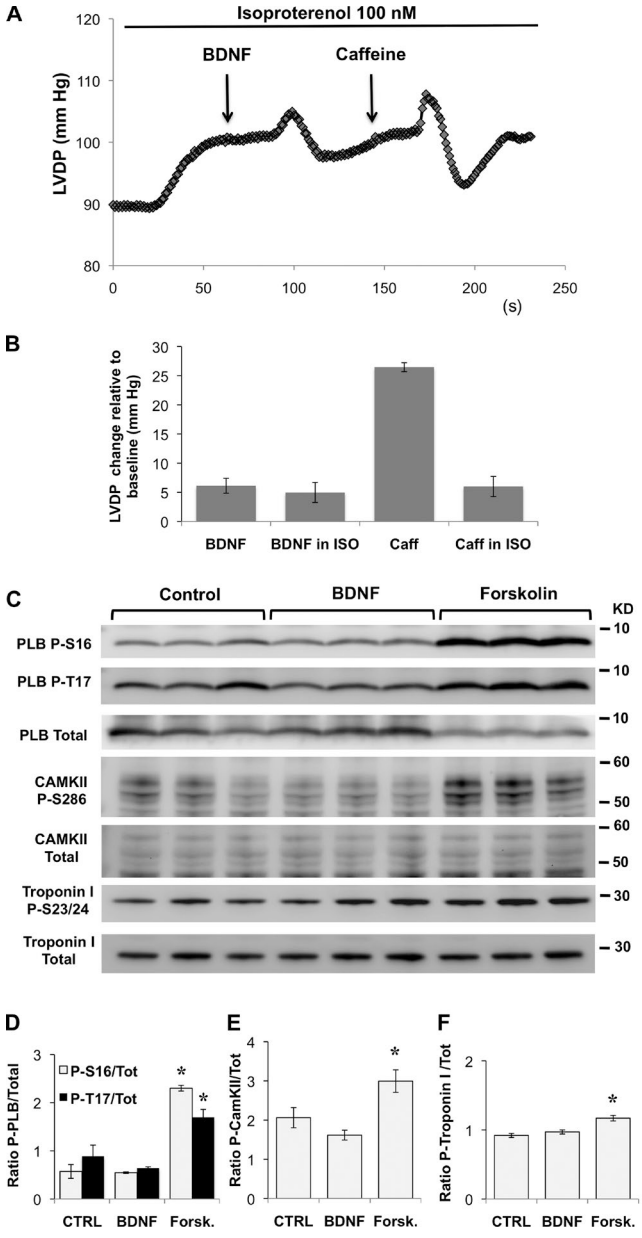


Figure 3. **BDNF signals independently of the catecholaminergic pathway and does not increase cardiac CamKII or PLB phosphorylation at the peak of its inotropic function.** (A) Isoproterenol does not occlude the effect of BDNF in Langendorff-perfused hearts. Representative LVDP trace obtained from application of 100 nM isoproterenol (ISO) to a Langendorff-perfused heart. Once the heart reached stable LVDP a 50 µl (10 ng total) bolus of BDNF was applied followed by a second 50 µl (5 mM) bolus of caffeine as a positive control 2 min later. (B) Quantification of the effect of BDNF and caffeine on baseline LVDP in the absence or presence of ISO. The baseline for the values obtained with ISO is considered LVDP in the presence of ISO. Values are from four independent experiments. (C–F) BDNF does not increase cardiac CamKII or PLB phosphorylation at the peak of its inotropic function. (C) Western blot analysis of PLB phosphorylation at Ser16 (PLB P-S16) and Thr17 (PLB P-T17), CAMKII phosphorylation at Thr 286/287 (CAMKII P-S286), and Troponin I at Ser23/24. Langendorff-perfused hearts were injected with vehicle (negative control), BDNF, or Forskolin (positive control) as described in Material and methods. (D–F) Quantification of the bands intensity (±SEM) from C reported as the ratio between the phosphorylated form over the specific total protein. Means of the three samples ±SEM are shown. *, $P < 0.05$, calculated with two-tail *t* test.

thetic tone (Donovan et al., 2000; Clark et al., 2011; Anastasia et al., 2014; Wan et al., 2014). Although our ex vivo and in vitro analysis support a direct role for BDNF on cardiomyocyte contraction, to address this point we selectively deleted TrkB.T1 in cardiomyocytes by using a transgenic mouse expressing cre under the promoter of the murine α myosin heavy chain polypeptide 6 (Myh6; Fig. S4 C; Agah et al., 1997). As shown in Fig. 5, specific deletion of TrkB.T1 in cardiomyocytes completely abolished the inotropic effect of BDNF in Langendorff-perfused hearts. Interestingly, deletion of TrkB.T1 in endothelial cells using a cadherin5-specific cre mouse line as a control had no impact, as these hearts responded to BDNF as well as WT controls (Fig. 5, C and F). Most importantly, Myh6-cre/TrkB.T1 mutant hearts showed dilated cardiomyopathy with significantly reduced left ventricle posterior wall thickness, confirming an intrinsic cardiomyocyte role for TrkB.T1 in normal cardiac physiology (Fig. 5, G–K). Next we sought to determine whether BDNF is the ligand for TrkB.T1 in cardiomyocytes or whether other neurotrophins or Trk receptors could exert such a cardiac inotropic role. Thus, we evaluated the contraction force of perfused hearts in the presence of NGF or NT3. Whereas NGF had no effect on the heart contractile force, NT3 elicited a response, though slightly reduced compared with BDNF (Fig. S5, A–C). Because NT3 can also bind TrkB this result was not surprising (Coppola et al., 2001). Nevertheless, the lack of an NT3 inotropic effect in hearts lacking TrkB.T1 strongly suggests that TrkB.T1 is the only neurotrophin receptor mediating this function. To further elucidate the source and identity of the neurotrophin activating TrkB.T1 we tested whether cardiomyocytes could secrete a biologically active neurotrophin. Isolated embryonic cardiac myocytes were grown in culture for 7–10 d and the supernatant was tested for its ability to induce phosphorylation of TrkB kinase expressed in a cell line. In fact, although TrkB full length is not present in cardiomyocytes, a ligand that activates it will also activate TrkB.T1 because they have an identical extracellular ligand-binding domain (Fig. 6). This approach was used because of its biological sensitivity and specificity over other assays (Coppola et al., 2001). We found that cardiomyocyte-conditioned medium induced in vitro TrkB phosphorylation (Fig. 6 C, WT). This result suggested that cardiomyocytes produce and secrete a biologically active neurotrophin. To test if BDNF was the neurotrophic factor secreted in the medium we performed an immunoprecipitation experiment from medium conditioned by cardiomyocytes derived from a mouse model with an HA-tagged BDNF gene (Yang et al., 2009). Immunoprecipitation with an anti-HA antibody revealed the presence of BDNF in the medium, suggesting that BDNF can be secreted by cardiomyocytes. Importantly, medium conditioned by BDNF knockout cardiomyocytes was unable to induce TrkB phosphorylation, indicating that although NT3 can cause the inotropic effect in isolated hearts, BDNF is the TrkB.T1 physiological ligand. This result also excluded the possibility that NT4, the other neurotrophin binding TrkB with high affinity, was the neurotrophin secreted by cardiomyocytes in the media, a result further supported by the very low level of NT3- and NT4-specific mRNA detected by RT-PCR in heart and cardiomyocytes (Fig. S5, D–F). Western blot analysis of adult hearts isolated from mice with the HA-tagged BDNF gene also confirmed BDNF expression, again suggesting that this is the physiological ligand source of TrkB.T1 in cardiomyocytes (Fig. 6 A). Therefore, we tested whether specific deletion of BDNF in cardiomyocytes caused cardiomyopathy. Hearts

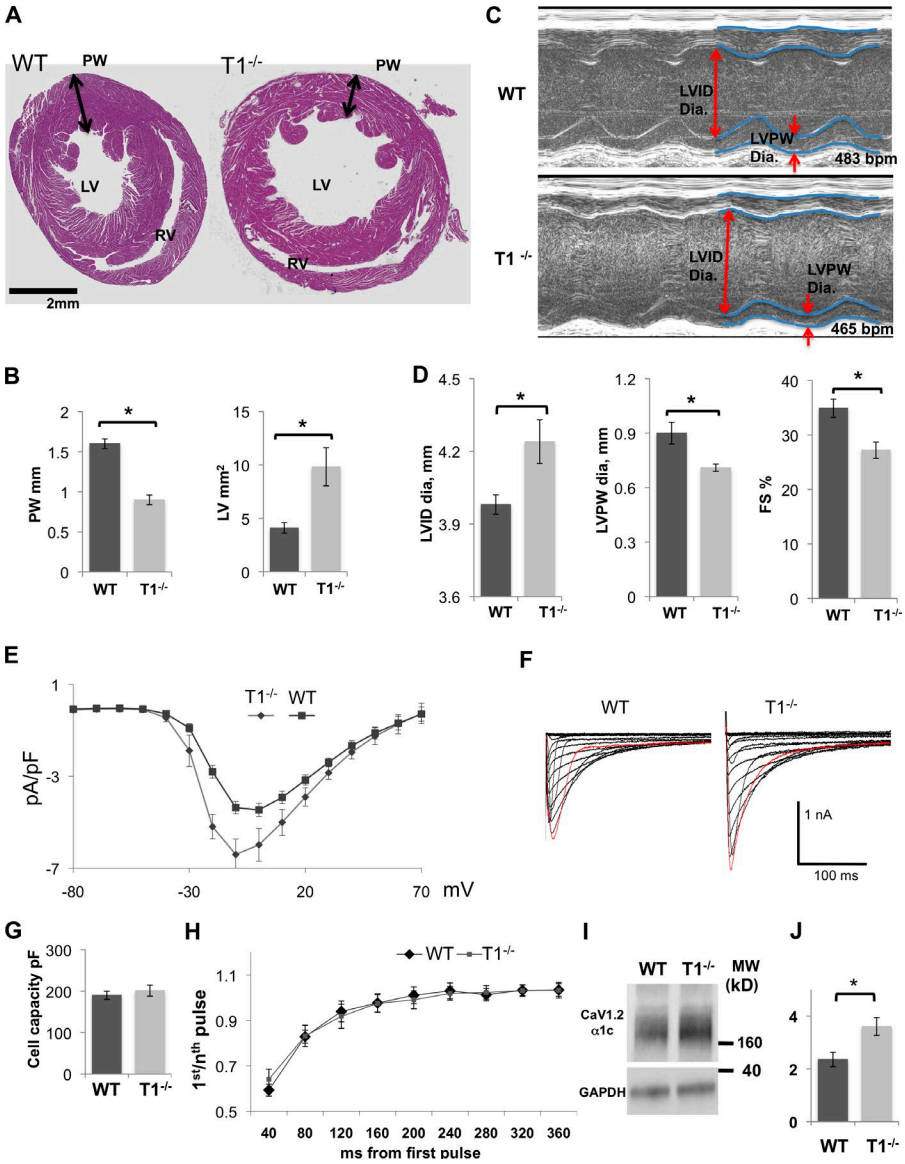


Figure 4. **TrkB.T1 deletion induces cardiac pathological alterations coupled with an increase in L-type Ca^{2+} channel level and currents.** (A) WT and TrkB.T1$^{-/-}$ hematoxylin and eosin–stained transversal heart sections showing left ventricle dilation and reduced thickness of left ventricle posterior wall apparent in TrkB.T1-deficient animals. (B) Quantification of the thickness (arrow) of the posterior wall (PW) and left ventricle area (LV); RV, right ventricle; $n = 4$ for each genotype. (C) Representative echocardiography recording in M-mode obtained in WT and T1$^{-/-}$ mice showing the left ventricle internal dimension at diastole (double head red arrows, LVID) and the left ventricle posterior wall (LVPW) thickness delineated by blue lines. (D) Quantification of LVID and LVPW thickness measured at diastole (dia), and fractional shortenings (FS) values obtained from the echocardiography recordings ($P < 0.05$). WT, $n = 4$; T1$^{-/-}$, $n = 5$. (E–J) TrkB.T1 deletion increases L-type calcium levels and currents in adult isolated cardiomyocytes. (E) I/V curves obtained in whole cell patch-clamp isolated adult cardiomyocytes showing similar activation of L-type calcium channels but higher current density in T1$^{-/-}$ versus WT cardiomyocytes. (F) Channel kinetics and current decay recordings are comparable in T1$^{-/-}$ and WT cardiomyocytes. (G) Measurements of plasma membrane capacitance are similar between WT and T1$^{-/-}$ cardiomyocytes, suggesting no difference in cell size. (H) Graph showing similar calcium-induced L-type current inactivation calculated by paired pulse protocol in WT and T1$^{-/-}$ cardiomyocytes. (I) Representative Western blot analysis of cardiac lysates from WT and T1$^{-/-}$ hearts using an antibody specific for the α 1c subunit of the Cav1.2 channel. (J) Quantification of Western blot CaV1.2 a1c band intensity in relation to GAPDH; $n = 6$. Data are indicated as the mean ± SEM. *, $P < 0.05$.

from two- to three-month-old Myh6-cre-BDNF knockout mice showed dilated cardiomyopathy with significantly reduced posterior wall thickness compared with littermate controls (Fig. 5, G–K). Strikingly, the similarity between the phenotype observed in Myh6-cre-BDNF– and Myh6-cre-TrkB.T1–deficient mice suggests that BDNF-TrkB.T1 signal is intrinsic to cardiomyocytes and functions by an autocrine/paracrine mechanism.

Discussion

We have identified a new unexpected role of BDNF in adult cardiac physiology. This action is mediated by truncated TrkB.T1 receptors expressed in cardiomyocytes and is independent of BDNF function in the nervous system. Although truncated TrkB.T1 has an intracellular domain that is extremely conserved among species, so far there is no data suggesting physiological relevance of its direct signaling in vivo. In isolated glial cells, TrkB.T1 evokes Ca^{2+} signal, but mice lacking this receptor do not show any obvious defect in this cell population (Carim-Todd et al., 2009). The lack of a phenotype could be a result of compensatory mechanisms, redundancy of TrkB.T1 -mediated Ca^{2+} signaling in this cell type, or a function required during specific physiological challenges. However, we found that in cardiomyocytes Ca^{2+} regulation by TrkB.T1 is critical as deletion of this receptor causes dilated cardiomyopathy. BDNF activation of TrkB.T1 appears modulatory of cardiac function as in a Langendorff-perfused heart it causes only a 5% increase in cardiac contraction force; yet loss of this pathway leads to cardiomyopathy over the life of the animal. Indeed, it is not surprising that even small perturbations in the proper control of Ca^{2+} levels are deleterious to normal cardiac function as Ca^{2+} regulation is central to the function of cardiomyocytes. Moreover, we provide evidence that BDNF is the ligand activating TrkB.T1 in an autocrine/paracrine fashion as it is expressed and released by cardiomyocytes and its deletion in this cell type causes a cardiomyopathy resembling that caused by TrkB.T1 knockout. Most recently, a parallel study has suggested that TrkB is required for normal cardiac contraction and relaxation (Feng et al., 2015). Contrary to our study that shows that TrkB.T1 is the receptor for BDNF in the heart, Feng et al. (2015) suggested that instead this function is mediated by the TrkB kinase

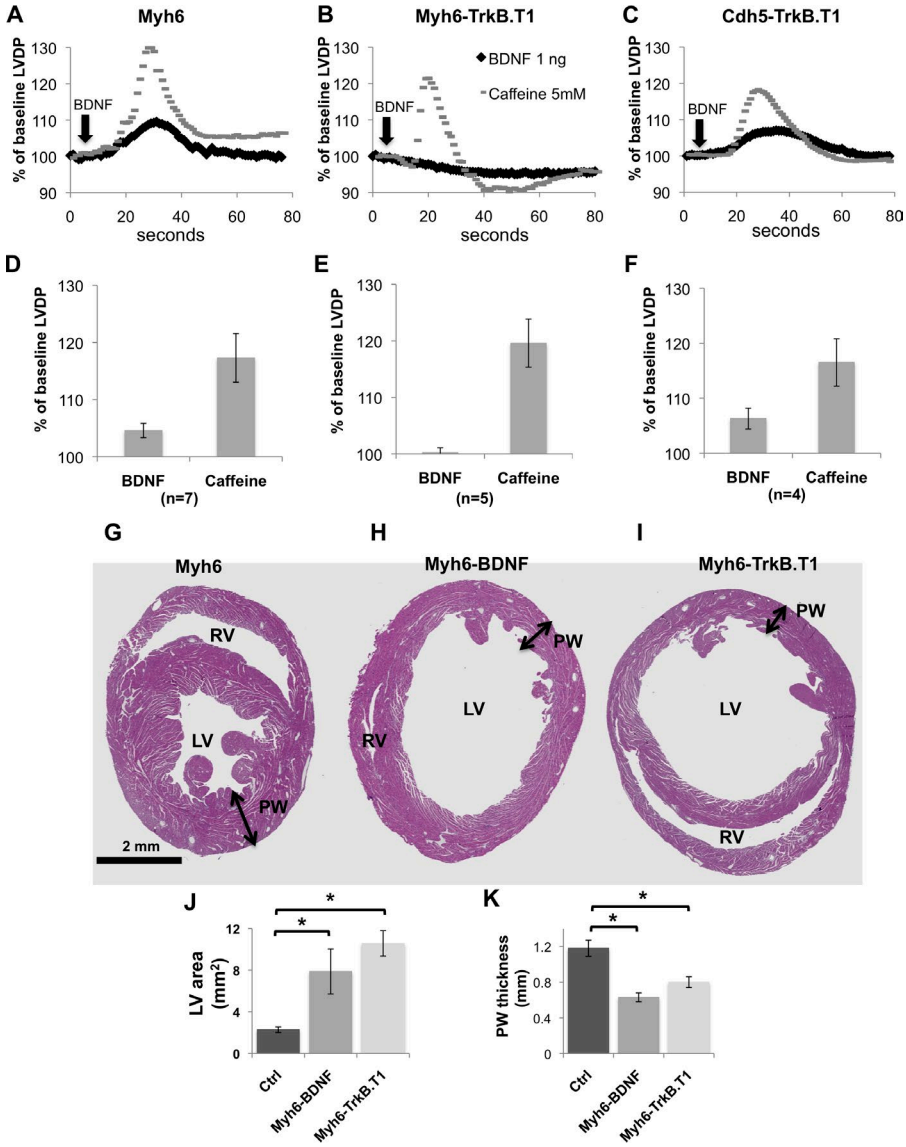


Figure 5. **Specific deletion of TrkB.T1 in cardiomyocytes abolishes BDNF inotropic effect and leads to cardiomyopathy.** (A–C) Representative traces of LVDP before (baseline considered as 100%) and after BDNF or caffeine injection (arrows) in control myosin 6–specific cre transgenic (myh6-cre) mice used as controls (A), double Myh6-cre, TrkB.T1 conditional (Myh6-cre/TrkB.T1^{loxP}; B), and endothelial-specific cre (Cdh5-cre) TrkB.T1 conditional (Cdh5-cre/TrkB.T1^{loxP}; C) mutant mice. Caffeine was injected 5 min after BDNF as a positive control as in Fig. 2. The BDNF and caffeine traces were overlapped using the injection time (arrow) as a starting point. (D–F) Quantification of LVDP recorded from the mice in A (D), B (E), and C (F). (G–I) Cardiomyocyte-specific deletion of TrkB.T1 or BDNF causes dilated cardiomyopathy. Representative hematoxylin and eosin–stained sections from 2–3-mo-old control Myh6-cre transgenic (G; myh6; $n = 4$), Myh6-cre/BDNF$^{loxP/loxP}$ (H; Myh6-BDNF; $n = 5$), or Myh6-cre/TrkB.T1$^{loxP/loxP}$ (I; Myh6-TrkB.T1; $n = 5$) mouse hearts showing dilated left ventricle and reduced left ventricle posterior wall thickness caused by BDNF or TrkB.T1 deletion. (J and K) Quantification of left ventricle (LV) area and posterior wall (PW) thickness was measured in heart transverse sections at the level and in between the papillary muscles (arrow). RV, right ventricle. Data are indicated as the mean ± SEM. *, $P < 0.05$.

receptor isoform. This finding is somewhat surprising because we and others have found that in the heart TrkB.T1-encoding mRNA is highest, whereas the TrkB kinase messenger RNA is virtually undetectable (Stoilov et al., 2002). The discrepancies in protein analysis data can be explained by the use of different antibodies and appropriate controls. Nevertheless, the most important difference relates to the cardiac phenotype observed by Feng et al. (2015) in mouse models with specific inactivation of the kinase domain either by a chemical genetic (i.e., the TrkBF616A/1-NMPP1 model) or by a conditional deletion approach. We found that in Langendorff-perfused WT mouse hearts 100 nM 1-NMPP1 induced a dramatic increase in the cardiac contraction force and blunted the BDNF effect. Although Feng et al. (2015) reported that 1-NMPP1 did not alter basal cardiomyocyte function they did not include a control to test whether 1-NMPP1 blunts BDNF action as we found (Fig. S3). Thus, it is possible that such an omission leads to misinterpreting the lack of BDNF inotropic function in the TrkB-F6161A/1-NMPP1 model as a specific phenotype caused by loss of TrkB kinase activity.

Although, we do not have a definitive explanation for the results obtained with a conditional deletion of a TrkB tyrosine kinase–specific exon, it has been reported that targeting the kinase domain of TrkB causes a dramatic down-regulation of the TrkB.T1 isoform protein (Klein et al., 1993). Although Klein et al. (1993) had no explanation for the disregulation, this finding suggests the possibility that a similar targeting of the kinase region by Feng et al. (2015) generates a TrkB.T1 hypomorph. In support of this scenario is the fact that targeting of the kinase domain causes only reduced systolic function insufficient to trigger chamber dilation (Feng et al., 2015), whereas the complete deletion of TrkB.T1 achieved in our study causes a more severe pathology that includes cardiac dilation developed as a consequence of impaired Ca^{2+} signaling. Importantly, targeting of the TrkB.T1-specific exon does not cause changes in the expression of the TrkB kinase receptor, again suggesting specificity of the phenotype (Fig. 1; Dorsey et al., 2006). Nevertheless, the study by Feng et al. (2015) is important because it supports the finding that TrkB has an intrinsic role in cardiomyocyte function that is independent of cardiac innervation. Moreover, despite the possible explanations for the discrepancies between the two analyses, we cannot entirely exclude that a very small level of the TrkB tyrosine kinase receptor may in part be responsible for the BDNF effect in the heart.

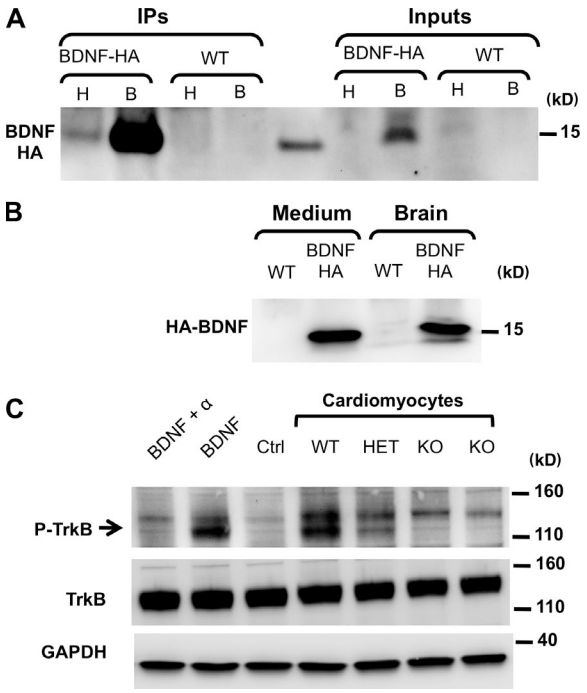


Figure 6. **BDNF is expressed in the adult heart and is secreted by cardiomyocytes.** (A) Heart (H) and brain (B) lysates obtained from a knock-in BDNF HA tagged and a WT mouse used as a control were immunoprecipitated and blotted with anti-HA antibodies. 15-kD molecular mass marker. Lysate inputs are on the right of the panel. (B and C) Embryonic cardiomyocytes secrete biologically active BDNF. (B) Culture medium from WT and BDNF-HA cultured embryonic cardiomyocytes was immunoprecipitated as in A. WT and BDNF-HA brains were used as negative and positive controls, respectively. (C) Cells stably expressing TrkB tyrosine kinase were used as a sensitive biological assay to test for the presence of secreted biologically active BDNF in the medium of cultured cardiomyocytes. After a 10-min incubation with the medium from different cardiomyocyte cultures, cells expressing TrkB were lysed and subjected to Western analysis with an anti–phospho-TrkB antibody. Anti-TrkB and GAPDH antibodies were used as controls for loading. The tested supernatants were from WT, BDNF heterozygous (HET), and BDNF knockout cultured cardiomyocytes. Unconditioned media supplemented with 1 ng/ml of recombinant BDNF (BDNF)F and 1 µg/ml TrkB-Fc BDNF blocking antibody (BDNF+α) were used as positive and negative controls, respectively. Ctrl, unconditioned media (DMEM). The molecular mass is shown on the right.

An important new contribution of our study relates to the fact that we have identified the source and identity of the neurotrophin activating TrkB.T1 in the heart. This is relevant because other neurotrophins expressed in the heart could be responsible for this function (Scarisbrick et al., 1993; Emanueli et al., 2014). Moreover, our data suggest that BDNF produced in loco is a significant determinant of TrkB.T1 cardiac function. This was even more surprising considering the relatively low level of BDNF present in the heart compared with the brain (only ∼5% per gram of tissue; Fig. 6 A). In the future it will be of interest to investigate whether BDNF produced by peripheral districts (i.e., striatal muscle) can affect cardiac function and whether loss of either cardiac or peripheral BDNF is sufficient to cause cardiomyopathy.

A major question still unresolved relates to how TrkB.T1 transduces BDNF inotropic function. The fact that the BDNF effect is additive to that of isoproterenol, a nonselective β-adrenergic agonist, suggests that BDNF signaling does not overlap with the catecholamine-activated pathway. Moreover, in an initial analysis of some of the key players in cardiac excitation–contraction coupling, including CamKII, PLB, and troponin I, we found no changes in their level of activation in response to BDNF treatment. This result appears to contrast the finding by Feng et al. (2015) that BDNF increases PLB, CamKII, and RyR receptor phosphorylation. However, their result was obtained after a prolonged treatment (10 or 20 min) of cardiomyocytes with BDNF, raising the possibility that the increase in the phosphorylation of these excitation–contraction coupling players may be secondary because it is detected after BDNF inotropic effect has already subsided. In our paradigm we found that at the peak of BDNF inotropic function in Langendorff-perfused hearts there was no increase of PLB or CamKII phosphorylation, suggesting that they are not directly involved in BDNF contraction in cardiomyocytes.

In glia cells TrkB.T1 mediates inositol-1,4,5-trisphosphate (IP_3)–dependent calcium release from intracellular stores. It was proposed that this mechanism involves an as yet unidentified G protein that stimulates PLC production of IP_3, Ca^{2+} release from stores, and Ca^{2+} entry from the extracellular space. It is tempting to speculate that a similar mechanism is at work in cardiomyocytes where TrkB.T1 induces Ca^{2+} release from the intracellular stores and increases systolic Ca^{2+} transients (Fig. 2). Indeed, IP_3 can contribute to systolic Ca^{2+} transients through a mechanism by which IP_3 receptors are opened by a combination of IP_3 and Ca^{2+} binding (Missiaen et al., 1994; Bootman et al., 1995). According to such a model, IP_3 receptors can sit with IP_3 bound and wait for an activating Ca^{2+} signal to derive from voltage-activated channels or neighboring RyRs (Kockskämper et al., 2008). In turn, activated IP_3 receptors would cause further release of Ca^{2+} from the ER, increasing the systolic Ca^{2+} transients. Importantly, several studies point toward a relevant role of PLC–IP_3 signaling in the development and progression of cardiac hypertrophy and dilation, further supporting a link of BDNF to this pathway (Mende et al., 1998; Kockskämper et al., 2008). In the future, it will be important to investigate through pharmacological and genetics approaches whether this is indeed the TrkB.T1-activated pathway in cardiomyocytes.

Lastly, considering the importance of BDNF/TrkB signaling in the control of food intake, mood disorders, and neurodegenerative diseases there is a significant effort to develop pharmacological agents to harness the activation of this pathway (Nagahara and Tuszynski, 2011; Lu et al., 2014). Although most studies have been focused on the generation of BDNF mimetic molecules, which can activate all TrkB receptors (Longo and Massa, 2013; Rosenthal and Lin, 2014), the present results suggest that any such effort should consider possible effects on the function of the cardiovascular system. Nevertheless, our data indicate that pharmacological activation of intracellularly activated TrkB kinase pathways such as by a transactivation mechanism may achieve central nervous system activation of TrkB kinase without causing cardiac toxicity (Wiese et al., 2007; Yanpallewar et al., 2012).

Materials and methods

Mouse models

TrkB.T1 knockout and conditional mutant mice were generated as described previously (Dorsey et al., 2006). BDNF-HA mice (Yang et al., 2009) were a gift of B. Hempstead (Cornell University, New York, NY). BDNF conditional (Rios et al., 2001) mutants as well as Myh6-cre (Agah et al., 1997) and Cadherin5-cre (Alva et al., 2006) transgenic

mice were obtained from the Jackson Laboratory. The Rosa26floxed-LacZ mouse (strain 003474; The Jackson Laboratory) was used as a Cre reporter strain to test the tissue/cellular expression pattern of *cre* transgenic mice. Animals were bred in a specific, pathogen-free facility with food and water ad libitum. All experimental procedures followed the National Institutes of Health guidelines for animal care and use and were approved by the National Cancer Institute at Frederick Animal Care and Use Committee.

Western blot analysis

Mouse hearts were quickly dissected and washed in cold PBS. After removal of the atria, ventricles were lysed in a Precellys ceramic lysing kit tube with 1 ml RIPA buffer by two 30-s cycles at 5,000 rpm in PRECELLYS 24 (Bertin Technologies). Ventricle or adult cardiomyocyte lysates (see the Adult mouse cardiomyocyte isolation section) were then incubated with wheat germ agglutinin–agarose beads in RIPA buffer for 4 h at 4°C. Beads were washed three times with RIPA buffer, resuspended in Laemmli sample buffer, and boiled for 5 min before loading in 4–12% NuPAGE (Life Technologies) precast gels for Western analysis. For the analysis of CamKII, PLB, and Troponin I, hearts were perfused in a Langendorff apparatus as described in the Langendorff heart preparation section with a modified Krebs solution with or without 20 ng/ml BDNF (PeproTech) or 5 µM Forskolin (Sigma-Aldrich) used as a positive control. Heart contractility was monitored during the perfusion process and at the peak of cardiac pressure induced by BDNF or Forskolin the hearts were snap frozen with stainless steel clamps cooled in liquid nitrogen and stored frozen before homogenization in RIPA buffer supplemented with proteases (complete mini; Roche) and phosphatase inhibitors (PhosSTOP; Roche). Lysates were centrifuged at 15,000 *g* for 15 min at 4°C and the supernatants were diluted in 2× Laemmli sample buffer, boiled, and loaded in a gel for Western analysis. Membranes (PVDF; Life Technologies) were blocked in 5% nonfat milk or 5% BSA in TBS-Tween before incubation with a specific antibody. Antibodies were as follows: anti-TrkB (against the extracellular domain of TrkB and therefore recognizing all TrkB isoforms; EMD Millipore), anti-TrkB.T1 (Santa Cruz Biotechnology, Inc.), anti-GAPDH (EMD Millipore), anti-CaV 1.2a1c (L-Type calcium channel subunit a1c,; Alomone Lab), anti-HA (for Western, Covance; for immunoprecipitation, Sigma-Aldrich), anti–Phospho-TrkB raised against phosphorylated TrkB-Tyr516 (Cell Signaling Technology), anti–total CAMKII (Cell Signaling Technology), anti–CAMKII P-T286/T287 (Cell Signaling Technology), anti–total Phospholamban (Thermo Fisher Scientific), anti-Phospholamban P-S16 (Santa Cruz Biotechnology, Inc.), anti-Phospholamban P-T17 (Badrilla), and anti–total and P-Ser 23/24 Troponin I (Cell Signaling Technology). After incubation with the appropriate HRP-conjugated secondary antibody, membranes were incubated with ECL substrate for detection of HRP enzyme activity (Thermo Fisher Scientific) and visualized in a Syngene gel documentation system. Images were splined (Arganda-Carreras et al., 2006) as needed and quantified by ImageJ analysis (National Institutes of Health). Student's *t* test was used for statistical significance assessment.

RT-PCR

Total RNA was extracted from isolated cardiomyocytes, heart, or cultured cells using the RNeasy Mini kit (QIAGEN). Following the manufacturer's procedure, cDNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Life Technologies). Real-time PCR was performed with iTaq Universal SYBR-green Supermix (Bio-Rad Laboratories) in a MX3000P (Agilent Technologies) apparatus with the following program: 95°C for 3 min; 95°C for 10 s, 60°C for 20 s for 40 cycles; 95°C for 1 min, and down to 55°C (gradient of 1°C) for 41 cycles (melting curve step). Values were expressed in Δ Ct using GAPDH as a reference.

Primers were as follows: TrkB common forward, 5′-AGCAATCGGGAGCATCTCT-3′; TrkB.FL reverse, 5′-CTGGCAGAGTCATCGTCGT-3′; TrkB.T1 reverse, 5′-TACCCATCCAGTGGGATCTT-3′; GAPDH forward, 5′-TGCGACTTCAACAGCAACTC-3′; GAPDH reverse, 5′-ATGTAGGCCATGAGGTCCAC-3′; BDNF forward, 5′-CGACATCACTGGCTGACACT-3′; BDNF reverse, 5′-CAAAGGCACTTGACTGCTGA-3′; NT3 forward, 5′-CCCCGTCAGCCAGGATAATG-3′; NT3 reverse, 5′-CGCCTGGATCAGCTTGATGA-3′; NT4 forward, 5′-TATCCTACAAAGGGGCCCCA-3′; NT4 reverse, 5′-CTGGGGAGGAGGAAGAGGAA-3′. RT-PCR products were separated in a 2% agarose gel stained with ethium bromide.

Langendorff heart preparation

Male mice (2 mo, 19–25 g) were injected with heparin (5,000 U/kg i.p.) and after 10 min were anesthetized with Avertin (150 mg/kg i.p.). Hearts were quickly removed from the chest and put into 16–18°C calcium-free modified Krebs solution containing 118 mmol NaCl, 23 mmol $NaHCO_3$, 3.2 mmol KCl, 1.2 mmol KH_2PO_4, 1.2 mmol $MgSO_4$, 11 mmol glucose, and 2 mmol sodium pyruvate before being mounted in a Langendorff perfusion system (Broadley, 1979). Hearts were perfused with modified Krebs solution containing 1.5 mM $CaCl_2$ (37.5°C) via the aorta. All solutions were bubbled with 95% O_2–5% CO_2, pH 7.40, and kept at 37°C. An inflatable water-filled balloon was inserted into the left ventricle via a small incision in the left atrium for measurement of the left ventricle pressure (BIOPAC Systems Inc.). Perfusion pressure and coronary flow were also continuously monitored via a differential pressure transducer. All channels were fed to a digidata 1322a (Axon) for digitalization storage and analysis using Igor 4.0 (WaveMetrics) or Clampfit (Axon) software.

After mounting, the hearts were equilibrated for 10 min at a perfusion pressure of 80 mmHg and at an end-diastolic pressure of 5–10 mmHg. Only hearts with a sinus heart rate ≥300 bpm and a left ventricle systolic pressure ≥70 mmHg at the end of the equilibration period were included in the studies. After the equilibration period, hearts were paced by platinum electrode at 420 bpm and the baseline contractile parameters and coronary flow were recorded for an additional 10 min. Neurotrophins were added to the Krebs solution immediately before use from frozen stocks and injected into the flow stream in a T tube placed before the temperature control unit. Caffeine (5 mM in Krebs) and denatured BDNF (10 ng in Krebs; 95°C for 5 min) were used, respectively, as positive and negative control. The total volume from the injection point to the tip of the cannula was 1.4 ml and drugs were always injected in a 50-µl volume. The effect of any substance on heart parameters was calculated as a percentage of the mean value from 20 s before the injection. 1-NMPP1 was purchased from EMD Millipore and NT3 and NGF were purchased from Alomone Laboratories.

Adult mouse cardiomyocyte isolation

2-mo-old male mice were injected with heparin (5,000 U/kg i.p.) and after 10 min were anesthetized with Avertin (150 mg/kg i.p.). Hearts were quickly removed from the chest cavity and put into ice-cold calcium-free solution in ultrapure H_2O (specific resistance >21 MOhm) containing 133.5 mM NaCl, 4 mM KCl, 1.2 mM NaH_2P0_4, 1.2 mM $MgS0_4$, 10 mM N-2-hydro-xyethylpiperazine-N'-2-ethanesulfonic acid, and 11 mM glucose, adjusted to pH 7.4 with NaOH. The heart, cannulated through the aorta with a round tip 21G needle was mounted in a Langendorff perfusion system and perfused at a pressure of 80 mmHg for 5 min with Ca^{2+}-free solution containing 1 mg/ml BSA (Sigma-Aldrich) and subsequently for 8–15 min with the same solution containing 25 mM Ca^{2+} together with collagenase type II (200–350 U/mg; Worthington

Biochemical Corporation). Collagenase perfusion was determined by the coronary flow variation and stopped when it increased consistently. Ventricles were then cut into small pieces and gently triturated for 1–3 min with a fire-polished Pasteur pipette. The resulting cell suspension was then passed through a 250-mesh filter and centrifuged at 150 rpm for 5 min. After resuspension in fresh control solution with 100 mM Ca^{2+}, cells were washed again and resuspended in control solution containing 200 mM Ca^{2+} at 22–23°C (Wolska and Solaro, 1996).

Measurement of intracellular Ca^{2+} transients

Calcium tolerant adult cardiomyocytes were loaded with Indo1-AM for 1 h, washed with control solution, and left at room temperature for at least 45 min to allow deesterification of the dye. Loading solution was prepared from a control solution containing 0.2 mM Ca^{2+} and 1 mM Indo1-AM (1 mM stock in dimethyl sulfoxide; Life Technologies) dissolved first in Pluronic surfactant (PowerLoad 100×; Life Technology) with 1 mg/ml BSA. Cardiomyocytes were field stimulated (0.5/s) with platinum electrodes (FHC Inc.) and recordings were made at 27°C in 1 mM Ca^{2+} solution. BDNF was applied at 50 ng/ml using a picospritzer at 30 hPa and a glass pipette with 1-M Ohm tip resistance. Excited at 340 nM, indo1-AM emission was split into two channels and passed through 405/10-nm (calcium-bound emission) and 488/10-nm (calcium-free emission) fluorescence filters. Light collected by photodiodes (Till Photonics) was digitally converted at 10 KHz (pClamp 10 and Digidata 1322a) and computed and the information was stored.

Calcium current recordings

Ca^{2+}-tolerant cardiomyocytes cells were placed in a recording chamber and perfused with a Na^+-free solution containing 137 mM tetraethylammonium chloride, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 10 mM glucose (pH 7.4 with TEA-OH) at a flux of 1 ml/min at 27°C. The pipette solution contained 111 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM glucose, 14 mM EGTA, 10 mM Hepes, and 5 mM MgATP (pH 7.3 with CsOH). Pulled borosilicate glass pipettes at ∼3–4 MOhm filled with pipette solution were used to obtain gigaseal on cardiomyocytes and subsequent whole-cell voltage clamp. Currents (I_{Ca-L}) were elicited by applying depolarizing voltage steps from a holding potential of −60 mV (–50 mV to +70 mV, 10-mV increments) and recorded (filtered at 2 kHz through a 4-pole low-pass Bessel filter and digitized at 5 kHz) with a Multiclamp 700B (Axon) using pClamp software (Axon). The maximum absolute value of the current obtained (in pA) was divided by the cell capacitance (in pF).

Heart histology

Hearts from anesthetized mice were quickly removed from the chest cavity, placed in ice-cold calcium- and magnesium-free PBS, cannulated through the aorta with a 21G needle, and perfused at a constant flow of 1 ml/min for 2 min with calcium- and magnesium-free PBS containing 20 mM KCl to induce cardiac relaxation. After that, the heart was perfused with 4% PFA in PBS for 10 min, cut in half horizontally, and fixed overnight at 4°C before paraffin embedding, sectioning at 7 µm, and staining with hematoxylin and eosin or Masson trichromic.

Mouse embryonic cardiomyocytes

Mouse embryonic cardiomyocytes were prepared as previously described (Graciotti et al., 2011). In brief, embryonic 17.5-d hearts were dissected, washed with ice-cold DMEM, minced, and incubated in 0.25% trypsin (Gibco) at 37°C for 13 min. After centrifugation (1,000 rpm for 5 min), the pellet was resuspended and incubated again in 0.25% trypsin at 37°C for 13 min, centrifuged, resuspended, and incubated in DMEM with 0.2% collagenase (collagenase type II) at 37°C for 30 min. Cells were then plated in a 10-cm dish in DMEM with 10% FBS media. After 1 h, the floating cells were collected and plated in DMEM/10% FBS media at a density of 160,000 cells/cm^2 in 6-well plates pretreated with 40 µg/ml laminin for 1 h. Embryonic cardiomyocytes were cultured for at least 7 d to allow differentiation before adding fresh media for a conditioning period of 48 h. Supernatants were applied fresh (without freezing/thawing) to C2C12 cells stably expressing TrkB.Kin. Recombinant BDNF (Alamone Laboratories) and a recombinant TrkB Fc Chimera (R&D Systems) were used as controls.

Echocardiography

Echocardiography was performed on 6-mo-old mice using a VisualSonic Vevo 770 equipped with a 30-MHz probe as previously reported (Gao et al., 2011).

β-Galactosidase staining

For β-galactosidase staining, adult hearts retro-perfused with 4% PFA were embedded in OCT mounting medium before freezing and sectioning at 20 µm. Sections were washed three times for 5 min with 100 mM sodium phosphate buffer, 20 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40, and then stained for 2 h at 30°C with PBS without Mg^{2+}/Ca^{2+}, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal, rinsed in dH_2O, and counterstained with neutral red for 40 s. After three rinses in 100% ethanol, coverslips were mounted in xylene.

Online supplemental material

Fig. S1 shows that BDNF does not alter the spontaneous cardiac beating frequency. Fig. S2 shows that BDNF increases systolic and decreases diastolic pressure but has no effect on coronary flow. Fig. S3 provides evidence that the TrkBF616A mouse model is not suitable for this type of analysis because 1-NMPP1 exerts a potent inotropic and lusitropic effect in Langendorff-perfused hearts that occludes BDNF effect on contractility. Fig. S4 shows that TrkB.T1 deletion does not cause obvious early postnatal cardiac developmental defects. Fig. S5 demonstrates that BDNF is the main neurotrophin expressed in neonatal and adult cardiomyocytes although NT3 but not NGF causes an increase in the contraction force of WT but not TrkB.T1-deficient hearts. Table S1 provides the functional parameters recorded in Langendorff-perfused hearts from WT and $T1^{-/-}$ animals. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201502100/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201502100.dv.

Acknowledgments

We thank Eileen Southon, Sudhir Yanpallewar, Erkan Kiris, and Mary Ellen Palko for critical reading of the manuscript and Lisa Riffle and Joseph Kalen for echocardiography imaging.

This work was supported by the National Institutes of Health Intramural Research Program, Center for Cancer Research, and National Cancer Institute.

The authors declare no competing financial interests.

Submitted: 26 February 2015
Accepted: 21 July 2015

References

Agah, R., P.A. Frenkel, B.A. French, L.H. Michael, P.A. Overbeek, and M.D. Schneider. 1997. Gene recombination in postmitotic cells. Targeted

expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J. Clin. Invest.* 100:169–179. http://dx.doi.org/10.1172/JCI119509

Alva, J.A., A.C. Zovein, A. Monvoisin, T. Murphy, A. Salazar, N.L. Harvey, P. Carmeliet, and M.L. Iruela-Arispe. 2006. VE-Cadherin-Cre-recombinase transgenic mouse: A tool for lineage analysis and gene deletion in endothelial cells. *Dev. Dyn.* 235:759–767. http://dx.doi.org/10.1002/dvdy.20643

Anastasia, A., K. Deinhardt, S. Wang, L. Martin, D. Nichol, K. Irmady, J. Trinh, L. Parada, S. Rafii, B.L. Hempstead, and P. Kermani. 2014. Trkb signaling in pericytes is required for cardiac microvessel stabilization. *PLoS ONE.* 9:e87406. http://dx.doi.org/10.1371/journal.pone.0087406

Arganda-Carreras, I., C.S. Sorzano, R. Marabini, J. Carazo, C. Ortiz-de-Solorzano, and J. Kybic. 2006. Consistent and elastic registration of histological sections using vector-spline regularization. *In* Computer Vision Approaches to Medical Image Analysis. Vol. 4241. R. Beichel, and M. Sonka, editors. Springer-Verlag Berlin Heidelberg, Berlin/Heidelberg. 85–95. http://dx.doi.org/10.1007/11889762_8

Baydyuk, M., and B. Xu. 2014. BDNF signaling and survival of striatal neurons. *Front. Cell. Neurosci.* 8:254. http://dx.doi.org/10.3389/fncel.2014.00254

Benitah, J.P., J.L. Alvarez, and A.M. Gómez. 2010. L-type Ca^{2+} current in ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.* 48:26–36. http://dx.doi.org/10.1016/j.yjmcc.2009.07.026

Bootman, M.D., L. Missiaen, J.B. Parys, H. De Smedt, and R. Casteels. 1995. Control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+}. *Biochem. J.* 306:445–451. http://dx.doi.org/10.1042/bj3060445

Broadley, K.J. 1979. The Langendorff heart preparation—Reappraisal of its role as a research and teaching model for coronary vasoactive drugs. *J. Pharmacol. Methods.* 2:143–156. http://dx.doi.org/10.1016/0160-5402(79)90038-X

Carim-Todd, L., K.G. Bath, G. Fulgenzi, S. Yanpallewar, D. Jing, C.A. Barrick, J. Becker, H. Buckley, S.G. Dorsey, F.S. Lee, and L. Tessarollo. 2009. Endogenous truncated TrkB.T1 receptor regulates neuronal complexity and TrkB kinase receptor function in vivo. *J. Neurosci.* 29:678–685. http://dx.doi.org/10.1523/JNEUROSCI.5060-08.2009

Chao, M.V., R. Rajagopal, and F.S. Lee. 2006. Neurotrophin signalling in health and disease. *Clin. Sci.* 110:167–173. http://dx.doi.org/10.1042/CS20050163

Chen, X., H. Ye, R. Kuruvilla, N. Ramanan, K.W. Scangos, C. Zhang, N.M. Johnson, P.M. England, K.M. Shokat, and D.D. Ginty. 2005. A chemical-genetic approach to studying neurotrophin signaling. *Neuron.* 46:13–21. http://dx.doi.org/10.1016/j.neuron.2005.03.009

Clark, C.G., E.M. Hasser, D.L. Kunze, D.M. Katz, and D.D. Kline. 2011. Endogenous brain-derived neurotrophic factor in the nucleus tractus solitarius tonically regulates synaptic and autonomic function. *J. Neurosci.* 31:12318–12329. http://dx.doi.org/10.1523/JNEUROSCI.0746-11.2011

Coppola, V., J. Kucera, M.E. Palko, J. Martinez-De Velasco, W.E. Lyons, B. Fritzsch, and L. Tessarollo. 2001. Dissection of NT3 functions in vivo by gene replacement strategy. *Development.* 128:4315–4327.

Donovan, M.J., M.I. Lin, P. Wiegn, T. Ringstedt, R. Kraemer, R. Hahn, S. Wang, C.F. Ibañez, S. Rafii, and B.L. Hempstead. 2000. Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development.* 127:4531–4540.

Dorsey, S.G., C.L. Renn, L. Carim-Todd, C.A. Barrick, L. Bambrick, B.K. Krueger, C.W. Ward, and L. Tessarollo. 2006. In vivo restoration of physiological levels of truncated TrkB.T1 receptor rescues neuronal cell death in a trisomic mouse model. *Neuron.* 51:21–28. http://dx.doi.org/10.1016/j.neuron.2006.06.009

Emanueli, C., M. Meloni, W. Hasan, and B.A. Habecker. 2014. The biology of neurotrophins: cardiovascular function. *Handbook Exp. Pharmacol.* 220:309–328. http://dx.doi.org/10.1007/978-3-642-45106-5_12

Escandón, E., D. Soppet, A. Rosenthal, J.L. Mendoza-Ramírez, E. Szönyi, L.E. Burton, C.E. Henderson, L.F. Parada, and K. Nikolics. 1994. Regulation of neurotrophin receptor expression during embryonic and postnatal development. *J. Neurosci.* 14:2054–2068.

Feng, N., S. Huke, G. Zhu, C.G. Tocchetti, S. Shi, T. Aiba, N. Kaludercic, D.B. Hoover, S.E. Beck, J.L. Mankowski, et al. 2015. Constitutive BDNF/TrkB signaling is required for normal cardiac contraction and relaxation. *Proc. Natl. Acad. Sci. USA.* 112:1880–1885. http://dx.doi.org/10.1073/pnas.1417949112

Gao, S., D. Ho, D.E. Vatner, and S.F. Vatner. 2011. Echocardiography in mice. *Curr. Protoc, Mouse Biol.* 1:71–83.

Graciotti, L., J. Becker, A.L. Granata, A.D. Procopio, L. Tessarollo, and G. Fulgenzi. 2011. Dystrophin is required for the normal function of the cardio-protective K_{ATP} channel in cardiomyocytes. *PLoS ONE.* 6:e27034. http://dx.doi.org/10.1371/journal.pone.0027034

Klein, R., D. Conway, L.F. Parada, and M. Barbacid. 1990. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell.* 61:647–656. http://dx.doi.org/10.1016/0092-8674(90)90476-U

Klein, R., R.J. Smeyne, W. Wurst, L.K. Long, B.A. Auerbach, A.L. Joyner, and M. Barbacid. 1993. Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell.* 75:113–122. http://dx.doi.org/10.1016/S0092-8674(05)80088-1

Kockskämper, J., A.V. Zima, H.L. Roderick, B. Pieske, L.A. Blatter, and M.D. Bootman. 2008. Emerging roles of inositol 1,4,5-trisphosphate signaling in cardiac myocytes. *J. Mol. Cell. Cardiol.* 45:128–147. http://dx.doi.org/10.1016/j.yjmcc.2008.05.014

Longo, F.M., and S.M. Massa. 2013. Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. *Nat. Rev. Drug Discov.* 12:507–525. http://dx.doi.org/10.1038/nrd4024

Lu, B., G. Nagappan, and Y. Lu. 2014. BDNF and synaptic plasticity, cognitive function, and dysfunction. *Handbook Exp. Pharmacol.* 220:223–250. http://dx.doi.org/10.1007/978-3-642-45106-5_9

Mende, U., A. Kagen, A. Cohen, J. Aramburu, F.J. Schoen, and E.J. Neer. 1998. Transient cardiac expression of constitutively active $G\alpha_q$ leads to hypertrophy and dilated cardiomyopathy by calcineurin-dependent and independent pathways. *Proc. Natl. Acad. Sci. USA.* 95:13893–13898. http://dx.doi.org/10.1073/pnas.95.23.13893

Missiaen, L., H. De Smedt, J.B. Parys, and R. Casteels. 1994. Co-activation of inositol trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+} is loading-dependent. *J. Biol. Chem.* 269:7238–7242.

Nagahara, A.H., and M.H. Tuszynski. 2011. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. *Nat. Rev. Drug Discov.* 10:209–219. http://dx.doi.org/10.1038/nrd3366

Ohira, K., K.J. Homma, H. Hirai, S. Nakamura, and M. Hayashi. 2006. TrkB-T1 regulates the RhoA signaling and actin cytoskeleton in glioma cells. *Biochem. Biophys. Res. Commun.* 342:867–874. http://dx.doi.org/10.1016/j.bbrc.2006.02.033

Rios, M., G. Fan, C. Fekete, J. Kelly, B. Bates, R. Kuehn, R.M. Lechan, and R. Jaenisch. 2001. Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol. Endocrinol.* 15:1748–1757. http://dx.doi.org/10.1210/mend.15.10.0706

Rose, C.R., R. Blum, B. Pichler, A. Lepier, K.W. Kafitz, and A. Konnerth. 2003. Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature.* 426:74–78. http://dx.doi.org/10.1038/nature01983

Rosenthal, A., and J.C. Lin. 2014. Modulation of neurotrophin signaling by monoclonal antibodies. *Handbook Exp. Pharmacol.* 220:497–512. http://dx.doi.org/10.1007/978-3-642-45106-5_19

Scarisbrick, I.A., E.G. Jones, and P.J. Isackson. 1993. Coexpression of mRNAs for NGF, BDNF, and NT-3 in the cardiovascular system of the pre- and postnatal rat. *J. Neurosci.* 13:875–893.

Stoilov, P., E. Castren, and S. Stamm. 2002. Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism. *Biochem. Biophys. Res. Commun.* 290:1054–1065. http://dx.doi.org/10.1006/bbrc.2001.6301

Wan, R., L.A. Weigand, R. Bateman, K. Griffioen, D. Mendelowitz, and M.P. Mattson. 2014. Evidence that BDNF regulates heart rate by a mechanism involving increased brainstem parasympathetic neuron excitability. *J. Neurochem.* 129:573–580. http://dx.doi.org/10.1111/jnc.12656

Wiese, S., S. Jablonka, B. Holtmann, N. Orel, R. Rajagopal, M.V. Chao, and M. Sendtner. 2007. Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. *Proc. Natl. Acad. Sci. USA.* 104:17210–17215. http://dx.doi.org/10.1073/pnas.0705267104

Wolska, B.M., and R.J. Solaro. 1996. Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *Am. J. Physiol.* 271:H1250–H1255.

Yang, J., C.J. Siao, G. Nagappan, T. Marinic, D. Jing, K. McGrath, Z.Y. Chen, W. Mark, L. Tessarollo, F.S. Lee, et al. 2009. Neuronal release of proBDNF. *Nat. Neurosci.* 12:113–115. http://dx.doi.org/10.1038/nn.2244

Yanpallewar, S.U., C.A. Barrick, H. Buckley, J. Becker, and L. Tessarollo. 2012. Deletion of the BDNF truncated receptor TrkB.T1 delays disease onset in a mouse model of amyotrophic lateral sclerosis. *PLoS ONE.* 7:e39946. http://dx.doi.org/10.1371/journal.pone.0039946

Zuccato, C., and E. Cattaneo. 2009. Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat. Rev. Neurol.* 5:311–322. http://dx.doi.org/10.1038/nrneurol.2009.54