



(51) International Patent Classification:

A61K 38/18 (2006.01) A61P 9/00 (2006.01)
A61K 38/17 (2006.01)

(21) International Application Number:

PCT/US2016/012186

(22) International Filing Date:

5 January 2016 (05.01.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/100,093 6 January 2015 (06.01.2015) US

(71) Applicants: **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 3400 N. Charles Street, Baltimore, Maryland 21218 (US). **TEMPLE UNIVERSITY- OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION** [US/US]; Broad Street And Montgomery Avenue, Philadelphia, PA 19122 (US).

(72) Inventors: **PAOLOCCI, Nazareno**; 509 Anneslie Rd., Baltimore, Maryland 21212 (US). **FENG, Ning**; 239 Stanmore Rd., Baltimore, Maryland 21212 (US). **TOCCHETTI, Carlo G.**; 509 Anneslie Rd., Baltimore, Maryland 21212 (US). **ZHU, Guangshuo**; 2110 Western Run Dr., Baltimore, Maryland 21209 (US). **KOCH, Walter, J.**; 31 Laurel Circle, Malvern, Pennsylvania 19355 (US).

CANNAVO, Alessandro; 1232 Chestnut Street, Apt. 4001, Philadelphia, Pennsylvania 19107 (US). **RENGO, Giuseppe**; Via Vincenzo Padula n.2 (Parco Manzoni), 80123 Napoli (IT).

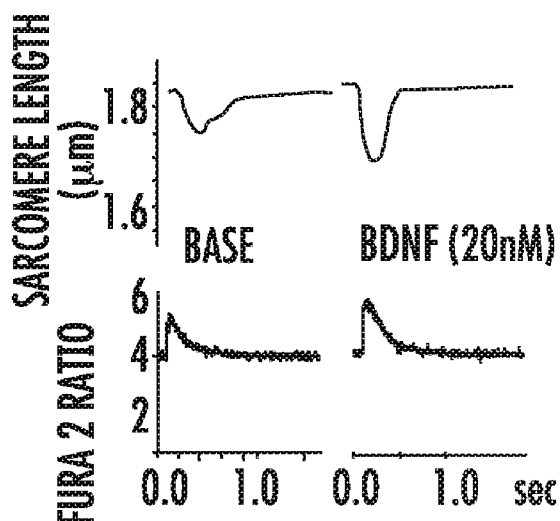
(74) Agent: **CONTRERA, Joseph**; 100 N. Charles Street, 5th Floor, Baltimore, Maryland 21201 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: TRKB AGONISTS AS NOVEL TREATMENT FOR ACUTE AND CHRONIC CARDIAC DISEASE

**FIG. 1B**

(57) Abstract: The present inventors have found that that BDNF/TrkB signaling acts constitutively to sustain in vivo myocardial performance. Thus, the present invention provides methods for improving cardiac contractility and methods for treating heart failure in a subject comprising administering to the subject, an effective amount of one or more TrkB agonists. Pharmaceutical compositions comprising one or more TrkB agonists and at least one additional biologically active agent and their uses are also provided.



Published:

— *with international search report (Art. 21(3))*

TRKB AGONISTS AS NOVEL TREATMENT FOR ACUTE AND CHRONIC CARDIAC DISEASE

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/100,093, filed on January 6, 2015, which is hereby incorporated by reference for all purposes as if fully set forth herein.

BACKGROUND OF THE INVENTION

[0002] In the brain, BDNF has a pleiotropic profile, preserving cell viability and function, preventing neuronal degeneration during stress and acting as an anti-depressant. BDNF is also expressed in various non-neuronal tissues, including smooth and skeletal muscles cells where it enhances airway smooth muscle cell contractility and acts as a metabolic enhancer, respectively.

[0003] In the mammalian heart, BDNF is essential for organ development because its genetic deletion leads to the thinning of cardiac chambers, microvascular leakage, and ultimately early death in mice. In adult mammals, BDNF governs autonomic transmission to the heart and exerts prominent angiogenic effects. Recent evidence also points to the presence of the BDNF receptor, TrkB, in the myocardium. However, the role of myocardial BDNF/TrkB signaling in cardiac physiology and myocardial response to pathological stress, independent from its well-known trophic actions on blood vessels and autonomic efferent neurons, is largely unknown. Old and new studies have examined the role of BDNF in acute and chronic cardiomyopathy due to cardiac ischemia (at least 11 could be found in the last 15 years). At a close look, whether exogenous BDNF is beneficial or detrimental to the myocardium after ischemia remains very controversial. Indeed, the number of studies showing positive effects equalizes that on which BDNF was reported to be deleterious. This divergent outcome could be due, among others, to dosing, route and timing of administration, experimental model and/or conditions of ischemia (i.e. ischemia alone or combined with other interventions/procedures such as fasting, hypothermia or co-infusion of other agent such as VEGF). However, it should be pointed out that no studies have examined yet the impact of BDNF/TrkB in a diseased model of heart failure of non-ischemic origin, i.e. heart failure induced by chronic hemodynamic stress.

SUMMARY OF THE INVENTION

[0004] The present inventors have found that cardiac-specific TrkB knockout mice (TrkB^{-/-}) display impaired cardiac contraction and relaxation, showing that BDNF/TrkB signaling acts constitutively to sustain *in vivo* myocardial performance. BDNF enhances normal cardiomyocyte Ca²⁺ cycling, contractility and relaxation via Ca²⁺/calmodulin-dependent protein kinase II (CaMKII). Conversely, failing myocytes, which have increased truncated TrkB lacking tyrosine kinase activity and chronically activated CaMKII, are insensitive to BDNF. Thus, BDNF/TrkB signaling provides a novel pathway by which the peripheral nervous system directly and tonically influences myocardial function, in parallel with β -adrenergic control. Deficits in this system are likely new contributors to acute and chronic cardiac dysfunction.

[0005] In accordance with an embodiment, the present invention provides a method for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with a composition comprising an effective amount of at least one TrkB agonist.

[0006] In accordance with another embodiment, the present invention provides a method for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with a composition comprising an effective amount of at least one a TrkB agonist and at least one additional biologically active agent.

[0007] In accordance with an embodiment, the present invention provides a method for increasing contractility in the cardiac tissue of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of at least one TrkB agonist and a pharmaceutically acceptable carrier.

[0008] In accordance with another embodiment, the present invention provides a method for increasing contractility in the cardiac tissue of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of at least one TrkB agonist, at least one additional biologically active agent, and a pharmaceutically acceptable carrier.

[0009] In accordance with an embodiment, the present invention provides a method for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of at least one TrkB agonist and a pharmaceutically acceptable carrier.

[0010] In accordance with another embodiment, the present invention provides a method for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of at least one TrkB agonist, at least one additional biologically active agent, and a pharmaceutically acceptable carrier.

[0011] In accordance with an embodiment, the present invention provides a method for identifying a test compound as a TrkB agonist comprising: a) obtaining a culture of one or more isolated wild type murine ventricular myocytes and obtaining a culture of one or more isolated TrkB^{-/-} murine ventricular myocytes; b) contacting both cultures of a) separately with vehicle, a positive control compound which is a known TrkB agonist, and one or more test compounds; and c) measuring contractile function and/or Ca²⁺ transients in the cultures of a); wherein when both the positive control compound and the one or more test compounds increase contractile function and/or Ca²⁺ transients when compared to controls in the isolated wild type murine ventricular myocytes, and the positive control compound and the one or more test compounds do not increase contractile function and/or Ca²⁺ transients when compared to controls in the isolated TrkB^{-/-} murine ventricular myocytes, then the one or more test compounds are identified as TrkB agonists.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A-1E. BDNF increases cardiomyocyte contractility and relaxation due to enhanced Ca²⁺ cycling, in a TrkB receptor-dependent manner. A) TrkB protein expression is detected in isolated adult murine cardiac myocytes by western blot (140 KD). TrkB is located on plasma membrane of cardiac myocytes by immunohistochemical study, using confocal microscopy. B) Representative traces of sarcomere shortening and Ca²⁺ transients, with or without BDNF treatment. Incubating isolated murine cardiomyocytes with BDNF (20 nM) increases myocyte fractional shortening and whole cell Ca²⁺ transients while accelerating myocyte relaxation; the latter documented by decreased relaxation time (n=19). C) Representative confocal imaging of Ca²⁺ spark events in isolated rat adult cardiomyocytes: BDNF augments Ca²⁺ spark frequency in these cells. D) Consistent with data in the mice, BDNF increases Ca²⁺ transients and also SR Ca²⁺ fractional release in isolated rat adult cardiomyocytes, without significantly affecting diastolic Ca²⁺ levels or SR Ca²⁺ load, measured by caffeine induced total SR Ca²⁺ release. E) Raw traces of BDNF's impact on L-type Ca²⁺ channel activity (LTCC) measured in isolated guinea-pig ventricular myocytes:

BDNF enhances peak LTCC activity from baseline ($p < 0.05$). F) BDNF inotropy is abolished in cardiomyocytes isolated from $\text{TrkB}^{\text{F616A}}$ mice with 1-NNMP1 pretreatment.

[0013] Figures 2A-2B. Constitutive BDNF/TrkB signaling is required for normal cardiac contraction and relaxation. A) Representative pressure-volume loops obtained in WT littermate mice and in cardiac-specific $\text{TrkB}^{-/-}$ mice. B) Cardiac contractility and relaxation are impaired in $\text{TrkB}^{-/-}$ mice as indexed by $\text{dp}/\text{dt}_{\text{max}}$, dpdt/EDV , dpdt/ip , PRSW, Ees/Ea ratio and Tau logistic, respectively.

[0014] Figures 3A-3B. β -adrenergic response is intact in $\text{TrkB}^{-/-}$ mice. A) Representative pressure-volume loops obtained before and after the infusion of the β_1 - β_2 agonist isoproterenol (ISO, 40 ng/kg/min). B) ISO increases *in vivo* contractility ($\text{dP}/\text{dt}_{\text{max}}$ and dPdt/IP) with the same magnitude in WT and $\text{TrkB}^{-/-}$ mice, despite the lower basal contractile values found in the $\text{TrkB}^{-/-}$ mice ($n=5$ each group).

[0015] Figures 4A-4B. CaMKII is the major mediator of BDNF influence on myocardial mechanics. A) BDNF induces CaMKII phosphorylation in adult murine cardiac myocytes, and increases the phosphorylation of CaMKII-dependent sites on RyR (serine 2814) and PLN (threonine 17), respectively; $\text{TrkB}^{-/-}$ mice display significantly reduced levels of constitutive phosphorylation of CaMKII as indexed by the P-CaMKII / T-CaMKII ratio. B) Representative traces of BDNF's impact on isolated mouse cardiomyocytes, in absence and presence of the CaMKII inhibitor KN93: pre-treating cells with KN93 prevents BDNF enhancement of myocyte contraction.

[0016] Figures 5A-5C. BDNF evoked enhancement of cardiac contractility is lost in failing cardiomyocytes that display increased truncated TrkB and altered CaMKII signaling/targets. A) Myocytes isolated from $G_{\alpha q}$ mice are insensitive to BDNF (20 nM): raw traces and cumulative data for sarcomere shortening and whole cell Ca^{2+} transients. B) The expression of full length TrkB (TrkB-FL) is unchanged in $G_{\alpha q}$ OE hearts; however, the truncated TrkB (Trk-T1) is markedly increased. C) In $G_{\alpha q}$ OE mice hearts, CaMKII phosphorylation is constitutively up-regulated, as shown by the increased P-CaMKII / T-CaMKII ratio. The expression of SERCA2a is markedly decreased in $G_{\alpha q}$ hearts; this change is coupled to unchanged expression of total PLN, but to reduced levels of PLN phosphorylation levels at the T17 residue (* $P < 0.05$, ** $P < 0.01$).

[0017] Figure 6 illustrates that TrkB expression is absent in cardiac myocytes isolated from $\text{TrkB}^{-/-}$ mice.

[0018] Figures 7A-7C. There is no difference in myocardial capillary density between WT and TrkB^{-/-} mice. Representative images of isolectin staining obtained from WT (A) and TrkB^{-/-} mice (B) (Scale bar = 50 μ M). C) Measurements of isolectin B4 stained capillary profiles in myocardium in WT and TrkB^{-/-} mice (n=3 each group).

[0019] Figures 8A-8B. Elevated truncated TrkB receptor and increased CaMKII signaling is present in TAC hearts. A) The expression of full length TrkB (TrkB-FL) is unchanged in TAC hearts; however, the truncated TrkB (TrkB-T1) and the TrkB-T1/FL ratio are markedly increased in hearts with pressure overload. B) In these hearts, CaMKII phosphorylation is constitutively up-regulated, along with increased T-CaMKII. The expression of SERCA2a is markedly decreased in TAC hearts; this change is coupled to unchanged expression of total PLN, but to reduced levels of PLN phosphorylation levels at the T17 residue (*P<0.05, **P<0.01).

[0020] Figures 9A-9B depict the impact of the TrkB agonist LM22A-4 (0.2 μ g/kg/day dose, infused via Alzet mouse intraperitoneal infusion pumps) vs. vehicle (saline) on TAC hearts. Vehicle or agonist was started 1 week after TAC. The TrkB agonist LM22A-4 preserved LV function as indexed by % fractional shortening (Fig. 9A, and time course in lower panel) and ejection fraction measured by echo in conscious (non-sedated) animals (Fig. 9B, and time course in lower panel).

[0021] Figures 10A-10B illustrate that the impact of the TrkB agonist LM22A-4 (solved in physiological solution) on ventricular myocytes isolated from control mice (of 8-12 weeks of age). As shown, LM22A-4 exerted a direct positive inotropic effect on these cells which was dose-dependent. This enhancement in contractile function (sarcomere shortening expressed as % increase from baseline value, panel 10A) was coupled to an equally marked and dose-dependent increase in whole Ca²⁺ transients (panel 10B). These data demonstrate that, in addition or independently from possible effects on vessels and nerve fibers serving the heart, LM22A-4 directly modulates myocardial function by binding on sarcolemmal TrkB receptors.

DETAILED DESCRIPTION OF THE INVENTION

[0022] During its lifetime, the heart is under the constant influence of the autonomic nervous system. Sympathetic efferent fiber activation is designed to release cardioactive neurotransmitters, crucial to adjust cardiac performance to increased workload. This on-

demand mechanism is essential during exercise and, at least initially, it maintains adequate cardiac output in presence of chronic hemodynamic stress such as hypertension. Yet, autonomic fibers also contain and release neurotrophins, but our understanding of their influence on myocardial function has been mostly confined to their ability of exerting trophic actions on autonomic efferents and vessels serving the heart.

[0023] Here, the present inventors have found that endogenous BDNF, via stimulation of sarcolemmal TrkB receptors and CaMKII-associated signaling, establishes a tonic control on basal cardiac contractility and relaxation. Thus, the present invention establishes a new role for BDNF/TrkB signaling as a direct modulator of myocardial mechanical function. Several physiological- and pathophysiological implications arise from these findings. BDNF/TrkB signaling may contribute to enhanced cardiac performance during exercise. In fact, exercise augments BDNF levels in the brain, the skeletal muscle and plasma, enhancing function and improving energy metabolism in these organs. The present invention may also provide a potential explanation for recent clinical reports showing that in heart failure patients a correlation exists between low circulating levels of BDNF and worsening of symptoms.

[0024] While it is known that growth factors such as IGF-1 and VEGF have pharmacologic actions to modulate cardiac contractility, no studies have established their relevance *in vivo* or their signaling, in this respect. The present invention shows that a tyrosine kinase-associated receptor such as TrkB, and its endogenous ligand BDNF, directly controls the cardiac E-C coupling process, independently and in parallel to G protein-coupled receptor signaling. Thus, disruption of tyrosine kinase-based signaling and consequent perturbations in cardiac mechanical work can largely contribute to loss in ventricular systolic function that accompanies the use of tyrosine kinase inhibitors during anti-cancer therapies.

[0025] Thus, in accordance with an embodiment, the present invention provides a method for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with a composition comprising an effective amount of a TrkB agonist.

[0026] In accordance with an embodiment, the present invention provides the use of a composition comprising a TrkB agonist for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with an effective amount of a composition comprising a TrkB agonist.

[0027] In accordance with another embodiment, the present invention provides a pharmaceutical composition as described herein, suitable for use as a medicament, preferably for use in the treatment of heart failure in a subject suffering therefrom.

[0028] In accordance with another embodiment, the present invention provides a method for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with a composition comprising an effective amount of a TrkB agonist and at least one additional biologically active agent.

[0029] In accordance with a further embodiment, the present invention provides the use of a composition comprising a TrkB agonist for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with an effective amount of a composition comprising a TrkB agonist and at least one additional biologically active agent.

[0030] As used herein, the term "TrkB agonist" has its usual meaning and in general, means a biologically active agent, such as any ligand, protein, peptide, small molecule or peptidomimetic which activates the TrkB kinase and TrkB signaling of the TrkB receptor. Examples of TrkB agonists useful in the present invention can include, but are not limited to, LM22A-1, (5-oxo-1-prolyl-1-histidyl-1-tryptophan methyl ester, LM22A-2 (2-[2,7-bis[(2-hydroxyethyl)amino]sulfonyl]-9H-fluoren-9-ylidene]-hydrazinecarboxamide, LM22A-3 (N-[4-[2-[5-amino-4-cyano-1-(2-hydroxyethyl)-1H-pyrazol-3-yl]-2-cyanoethenyl]phenyl]-acetamide), and LM22A-4 (N,N',N''-tris(2-hydroxyethyl)-1,3,5-benzenetricarboxamide), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), N-acetylserotonin, N-(2-(5-hydroxy-1H-indol-3-yl)ethyl)-2-oxopiperidine-3-carboximide (HIOC), amitriptyline, 7,8-dihydroxyflavone, 7,8,3'-trihydroxyflavone, 4'-dimethylamino-7,8-dihydroxyflavone, and deoxygedunin.

[0031] The term "ligand" refers to molecules, usually members of the family of BDNF like peptides that bind to the receptor via the segments involved in peptide ligand binding. Also, a ligand is a molecule which serves either as a natural ligand to which the receptor, or an analog thereof, binds, or a molecule which is a functional analog of a natural ligand. The functional analog may be a ligand with structural modifications, or may be a wholly unrelated molecule which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists, see, e.g., Goodman, et al. (eds.) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed.), Pergamon Press.

[0032] An active agent, therapeutic agent, and a biologically active agent are used interchangeably herein to refer to a chemical or biological compound that induces a desired pharmacological and/or physiological effect, wherein the effect may be prophylactic or therapeutic. The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of those active agents specifically mentioned herein, including, but not limited to, salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the terms “active agent,” “pharmacologically active agent” and “drug” are used, then, it is to be understood that the invention includes the active agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs etc. The active agent can be a biological entity, such as a virus or cell, whether naturally occurring or manipulated, such as transformed.

[0033] The biologically active agent may vary widely with the intended purpose for the composition. The term active is art-recognized and refers to any moiety that is a biologically, physiologically, or pharmacologically active substance that acts locally or systemically in a subject. Examples of biologically active agents, that may be referred to as “drugs”, are described in well-known literature references such as the Merck Index, the Physicians’ Desk Reference, and The Pharmacological Basis of Therapeutics, and they include, without limitation, medicaments; vitamins; mineral supplements; substances used for the treatment, prevention, diagnosis, cure or mitigation of a disease or illness; substances which affect the structure or function of the body; or pro-drugs, which become biologically active or more active after they have been placed in a physiological environment. Various forms of a biologically active agent may be used which are capable of being released the subject composition, for example, into adjacent tissues or fluids upon administration to a subject.

[0034] Further examples of biologically active agents include, without limitation, enzymes, receptor antagonists or agonists, hormones, growth factors, autogenous bone marrow, antibiotics, antimicrobial agents, and antibodies.

[0035] Non-limiting examples of biologically active agents include following: adrenergic blocking agents, anti-cholesterolemic and anti-lipid agents, anti-cholinergics and sympathomimetics, anti-coagulants, anti-hypertensive agents, anti-inflammatory agents such as steroids, non-steroidal anti-inflammatory agents, anti-pyretic and analgesic agents, anti-thrombotic agents, anti-anginal agents, cardioactive agents, coronary dilators, diuretics, diagnostic agents, erythropoietic agents, peripheral vasodilators, prostaglandins, stimulants, and prodrugs.

[0036] More specifically, non-limiting examples of useful biologically active agents include the following therapeutic categories: nonsteroidal anti-inflammatory drugs, salicylates; H1-blockers and H2-blockers; parasympathomimetics, cholinergic agonist parasympathomimetics, cholinesterase inhibitor parasympathomimetics, sympatholytics, α -blocker sympatholytics, sympatholytics, sympathomimetics, and adrenergic agonist sympathomimetics; cardiovascular agents, such as antianginals, antianginals, calcium-channel blocker antianginals, nitrate antianginals, antiarrhythmics, cardiac glycoside antiarrhythmics, class I antiarrhythmics, class antiarrhythmics, class antiarrhythmics, class IV antiarrhythmics, antihypertensive agents, α -blocker antihypertensives, angiotensin-converting enzyme inhibitor (ACE inhibitor) antihypertensives, β -blocker antihypertensives, calcium-channel blocker antihypertensives, central-acting adrenergic antihypertensives, diuretic antihypertensive agents, peripheral vasodilator antihypertensives, antilipemics, bile acid sequestrant antilipemics, reductase inhibitor antilipemics, inotropes, cardiac glycoside inotropes, and thrombolytic agents; electrolytic and renal agents, such as acidifying agents, alkalinizing agents, diuretics, carbonic anhydrase inhibitor diuretics, loop diuretics, osmotic diuretics, potassium-sparing diuretics, thiazide diuretics, electrolyte replacements, and uricosuric agents; enzymes, such as pancreatic enzymes and thrombolytic enzymes; hematological agents, such as antianemia agents, hematopoietic antianemia agents, coagulation agents, anticoagulants, hemostatic coagulation agents, platelet inhibitor coagulation agents, thrombolytic enzyme coagulation agents, and plasma volume expanders; corticosteroid anti-inflammatory agents, gold compound anti-inflammatory agents, immunosuppressive anti-inflammatory agents, nonsteroidal anti-inflammatory drugs, salicylate anti-inflammatory agents, skeletal muscle relaxants, neuromuscular blocker skeletal muscle relaxants, and reverse neuromuscular blocker skeletal muscle relaxants.

[0037] Various forms of the biologically active agents may be used. These include, without limitation, such forms as uncharged molecules, molecular complexes, salts, ethers, esters, amides, prodrug forms and the like, which are biologically activated when implanted, injected or otherwise placed into a subject.

[0038] In accordance with an embodiment, the present invention provides a method for increasing contractility in the cardiac tissue (i.e. the intrinsic ability of the heart to contract and a major determinant of cardiac output) of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist and a pharmaceutically acceptable carrier.

[0039] In accordance with another embodiment, the present invention provides a method for increasing contractility in the cardiac tissue of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist, at least one additional biologically active agent, and a pharmaceutically acceptable carrier.

[0040] In accordance with an embodiment, the present invention provides the use of a TrkB agonist for increasing contractility in the cardiac tissue of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist and a pharmaceutically acceptable carrier.

[0041] In accordance with another embodiment, the present invention provides the use of a TrkB agonist for increasing contractility in the cardiac tissue of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist, at least one additional biologically active agent, and a pharmaceutically acceptable carrier.

[0042] In accordance with an embodiment, the present invention provides a method for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist and a pharmaceutically acceptable carrier.

[0043] In accordance with another embodiment, the present invention provides a method for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist, at least one additional biologically active agent, and a pharmaceutically acceptable carrier.

[0044] In accordance with an embodiment, the present invention provides the use of a TrkB agonist for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist and a pharmaceutically acceptable carrier.

[0045] In accordance with a further embodiment, the present invention provides the use of a TrkB agonist for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist, at least one additional biologically active agent, and a pharmaceutically acceptable carrier.

[0046] As used herein, the term “heart failure” or “congestive heart failure” means a physiological condition resulting from the inability of the ventricular myocardium to maintain adequate blood flow to the peripheral body tissues and includes congestive heart

failure, backward and forward heart failure, right ventricular and left ventricular heart failure, and low-output heart failure. Heart failure can be caused by myocardial ischemia, myocardial infarction, excessive alcohol usage, pulmonary embolism, infection, anemia, arrhythmias, and systemic hypertension. Symptoms include tachycardia, fatigue with exertion, dyspnea, orthopnea and pulmonary edema.

[0047] “Treating” or “treatment” is an art-recognized term which includes curing as well as ameliorating at least one symptom of any condition or disease. Treating includes reducing the likelihood of a disease, disorder or condition from occurring in an animal which may be predisposed to the disease, disorder and/or condition but has not yet been diagnosed as having it; inhibiting the disease, disorder or condition, e.g., impeding its progress; and relieving the disease, disorder or condition, e.g., causing any level of regression of the disease; inhibiting the disease, disorder or condition, e.g., impeding its progress; and relieving the disease, disorder or condition, even if the underlying pathophysiology is not affected or other symptoms remain at the same level.

[0048] “Prophylactic” or “therapeutic” treatment is art-recognized and includes administration to the host of one or more of the subject compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, i.e., it protects the host against developing the unwanted condition, whereas if it is administered after manifestation of the unwanted condition, the treatment is therapeutic (i.e., it is intended to diminish, ameliorate, or stabilize the existing unwanted condition or side effects thereof).

[0049] The term, “carrier,” refers to a diluent, adjuvant, excipient or vehicle with which the therapeutic is administered. Such physiological carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a suitable carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions also can be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

[0050] Pharmaceutically acceptable salts are art-recognized, and include relatively non-toxic, inorganic and organic acid addition salts of compositions of the present invention, including without limitation, therapeutic agents, excipients, other materials and the like. Examples of pharmaceutically acceptable salts include those derived from mineral acids, such as hydrochloric acid and sulfuric acid, and those derived from organic acids, such as ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, and the like. Examples of suitable inorganic bases for the formation of salts include the hydroxides, carbonates, and bicarbonates of ammonia, sodium, lithium, potassium, calcium, magnesium, aluminum, zinc and the like. Salts may also be formed with suitable organic bases, including those that are non-toxic and strong enough to form such salts. For purposes of illustration, the class of such organic bases may include mono-, di-, and trialkylamines, such as methylamine, dimethylamine, and triethylamine; mono-, di-, or trihydroxyalkylamines such as mono-, di-, and triethanolamine; amino acids, such as arginine and lysine; guanidine; N-methylglucosamine; N-methylglucamine; L-glutamine; N-methylpiperazine; morpholine; ethylenediamine; N-benzylphenethylamine; (trihydroxymethyl) aminoethane; and the like, see, for example, J. Pharm. Sci., 66: 1-19 (1977).

[0051] Buffers, acids and bases may be incorporated in the compositions to adjust pH. Agents to increase the diffusion distance of agents released from the composition may also be included.

[0052] The charge, lipophilicity or hydrophilicity of a composition may be modified by employing an additive. For example, surfactants may be used to enhance miscibility of poorly miscible liquids. Examples of suitable surfactants include dextran, polysorbates and sodium lauryl sulfate. In general, surfactants are used in low concentrations, generally less than about 5%.

[0053] The specific method used to formulate the novel formulations described herein is not critical to the present invention and can be selected from a physiological buffer (Feigner et al., U.S. Pat. No. 5,589,466 (1996)).

[0054] Therapeutic formulations of the product may be prepared for storage as lyophilized formulations or aqueous solutions by mixing the product having the desired degree of purity with optional pharmaceutically acceptable carriers, diluents, excipients or stabilizers typically employed in the art, i.e., buffering agents, stabilizing agents, preservatives, isotonicifiers, non-ionic detergents, antioxidants and other miscellaneous additives, see Remington's Pharmaceutical Sciences, 16th ed., Osol, ed. (1980). Such

additives are generally nontoxic to the recipients at the dosages and concentrations employed, hence, the excipients, diluents, carriers and so on are pharmaceutically acceptable.

[0055] The compositions can take the form of solutions, suspensions, emulsions, powders, sustained-release formulations, depots and the like. Examples of suitable carriers are described in "Remington's Pharmaceutical Sciences," Martin. Such compositions will contain an effective amount of the biopolymer of interest, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. As known in the art, the formulation will be constructed to suit the mode of administration.

[0056] Buffering agents help to maintain the pH in the range which approximates physiological conditions. Buffers are preferably present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use with the instant invention include both organic and inorganic acids, and salts thereof, such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture etc.), succinate buffers (e.g., succinic acid monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture etc.), oxalate buffers (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture etc.). Phosphate buffers, carbonate buffers, histidine buffers, trimethylamine salts, such as Tris, HEPES and other such known buffers can be used.

[0057] Preservatives may be added to retard microbial growth, and may be added in amounts ranging from 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, m-cresol, octadecyldimethylbenzyl ammonium chloride, benzyaconium halides (e.g., chloride, bromide and iodide), hexamethonium

chloride, alkyl parabens, such as, methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0058] Isotonicifiers are present to ensure physiological isotonicity of liquid compositions of the instant invention and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount of between about 0.1 % to about 25%, by weight, preferably 1% to 5% taking into account the relative amounts of the other ingredients.

[0059] Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols; amino acids, such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine etc.; organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, arabitol, erythritol, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, α -monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e., <10 residues); proteins, such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers, such as polyvinylpyrrolidone, saccharides, monosaccharides, such as xylose, mannose, fructose or glucose; disaccharides, such as lactose, maltose and sucrose; trisaccharides, such as raffinose; polysaccharides, such as, dextran and so on.

[0060] Additional miscellaneous excipients include bulking agents, (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine or vitamin E) and cosolvents.

[0061] Non-ionic surfactants or detergents (also known as “wetting agents”) may be added to help solubilize the therapeutic agent, as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stresses without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80 etc.), polyoxamers (184, 188 etc.), Pluronic® polyols and polyoxyethylene sorbitan monoethers (TWEEN-20®, TWEEN-80® etc.). Non-ionic surfactants may be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, preferably about 0.07 mg/ml to about 0.2 mg/ml.

[0062] The instant invention encompasses formulations, such as, liquid formulations having stability at temperatures found in a commercial refrigerator and freezer found in the office of a physician or laboratory, such as from about 20° C to about 5° C, said stability assessed, for example, by microscopic analysis, for storage purposes, such as for about 60 days, for about 120 days, for about 180 days, for about a year, for about 2 years or more. The liquid formulations of the present invention also exhibit stability, as assessed, for example, by particle analysis, at room temperatures, for at least a few hours, such as one hour, two hours or about three hours prior to use.

[0063] Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the bladder, such as citrate buffer (pH 7.4) containing sucrose, bicarbonate buffer (pH 7.4) alone, or bicarbonate buffer (pH 7.4) containing ascorbic acid, lactose, or aspartame. Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g., sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-90% (w/v) but preferably at a range of 1-10%

[0064] The formulations to be used for in vivo administration must be sterile. That can be accomplished, for example, by filtration through sterile filtration membranes. For example, the formulations of the present invention may be sterilized by filtration.

[0065] The TrkB agonists can be in the form of peptides having two or more amino acids. The term, "amino acid" includes the residues of the natural α -amino acids (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Hyl, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) in D or L form, as well as β -amino acids, synthetic and unnatural amino acids. Many types of amino acid residues are useful in the TrkB agonist polypeptides and the invention is not limited to natural, genetically-encoded amino acids. Examples of amino acids that can be utilized in the peptides described herein can be found, for example, in Fasman, 1989, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Inc., and the reference cited therein. Another source of a wide array of amino acid residues is provided by the website of RSP Amino Acids LLC.

[0066] The term, "peptide," as used herein, includes a sequence of from four to sixteen amino acid residues in which the α -carboxyl group of one amino acid is joined by an amide bond to the main chain (α - or β -) amino group of the adjacent amino acid. The peptides provided herein for use in the described and claimed methods and compositions can be cyclic.

[0067] TrkB agonists which are peptides, polypeptides, and/or proteins (including functional portions and functional variants thereof) can be obtained by methods known in the art. Suitable methods of *de novo* synthesizing polypeptides and proteins are described in references, such as Chan et al., Fmoc Solid Phase Peptide Synthesis, Oxford University Press, Oxford, United Kingdom, 2005; Peptide and Protein Drug Analysis, ed. Reid, R., Marcel Dekker, Inc., 2000; Epitope Mapping, ed. Westwood et al., Oxford University Press, Oxford, United Kingdom, 2000; and U.S. Patent No. 5,449,752. Also, polypeptides and proteins can be recombinantly produced using the nucleic acids described herein using standard recombinant methods. See, for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY 2001; and Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley & Sons, NY, 1994. Methods of isolation and purification are well-known in the art. Alternatively, TrkB agonists, such as polypeptides, and/or proteins described herein (including functional portions and functional variants thereof) can be commercially synthesized by companies, such as Synpep (Dublin, CA), Peptide Technologies Corp. (Gaithersburg, MD), and Multiple Peptide Systems (San Diego, CA).

[0068] The term, "amount effective to increase cardiac contractility or treat heart failure" is that amount effective to treat, ameliorate, or prevent or symptoms relating to decreased cardiac contractility or heart failure in a subject, or to exhibit a detectable therapeutic or preventative effect.

[0069] As used herein, the term "subject" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

[0070] The precise effective amount for a human subject will depend upon the severity of the subject's disease state, general health, age, weight, gender, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance or response to therapy. A routine experimentation can determine this amount and is within the judgment of

the medical professional. Compositions may be administered individually to a patient, or they may be administered in combination with other drugs, hormones, agents, and the like.

[0071] Accordingly, it will be necessary and routine for the practitioner to titer the dosage and modify the route of administration, as required, to obtain the optimal therapeutic effect. A typical daily dosage might range from about 0.01 mg/kg to up to about 100 mg/kg or more, preferably from about 0.1 to about 10 mg/kg/day depending on the above-mentioned factors. Typically, the clinician will administer antibody until a dosage is reached that achieves the desired effect (from 0.01 mg/kg to up to 100 mg/kg or more). The progress of this therapy is easily monitored by conventional assays.

[0072] In accordance with an embodiment, the present invention provides a method for identifying a test compound as a TrkB agonist comprising: a) obtaining a culture of one or more isolated wild type murine ventricular myocytes and obtaining a culture of one or more isolated TrkB^{-/-} murine ventricular myocytes; b) contacting both cultures of a) separately with vehicle, a positive control compound which is a known TrkB agonist, and one or more test compounds; and c) measuring contractile function and/or Ca²⁺ transients in the cultures of a); wherein when both the positive control compound and the one or more test compounds increase contractile function and/or Ca²⁺ transients when compared to controls in the isolated wild type murine ventricular myocytes, and the positive control compound and the one or more test compounds do not increase contractile function and/or Ca²⁺ transients when compared to controls in the isolated TrkB^{-/-} murine ventricular myocytes, then the one or more test compounds are identified as TrkB agonists.

[0073] As used herein, the methods for identifying TrkB agonists can be via use of known cell culture methods and myocyte cells, either primary cultures or in myocyte cell lines.

EXAMPLES

[0074] Mouse lines. The TrkB^{F616A} and TrkB^{-/-} mice have been described previously (Chen, X., et al., *Neuron* 46(1):13-21 (2005); Liu, Y., et al. *Science* 338(6112):1357-1360 (2012)). Homologous recombination was performed using a 3.2 kb sequence containing exon 15, harboring the F616A mutation for TrkB^{F616A} allele, and the same 3.2 kb sequence containing exon 15 with a FRT-Neo-FRT cassette flanked by two loxP sites for TrkB^{-/-} allele. The 129.1 mouse strain embryonic stem (ES) cells were used for homologous recombination

and ES cell clones that exhibited homologous recombination were screened by PCR, and further confirmed by Southern blotting. Correctly targeted ES clones were injected into C57BL/6 blastocysts, which were then introduced into pseudopregnant females. Heterozygous mice were generated by crossing chimeric mice with C57BL/6 mice. These mice were subsequently crossed with mice expressing FlpE recombinase in germ cells to excise the Neo cassette. Both lines were backcrossed and maintained on a C57BL/6 background. Cardiac specific $TrkB^{-/-}$ mice were generated by crossing $TrkB^{-/-}$ with MHC promoter driven Cre mice obtained from Jackson Lab. $G_{\alpha q}$ over-expressing mice ($G_{\alpha q}$ OE) were also obtained from Jackson Lab. All animal protocols were approved by The Johns Hopkins University Animal Care and Use Committee and followed established NIH guidelines.

[0075] Reagents. BDNF recombinant protein was purchased from Calbiochem. TrkB antibody was obtained from BD, CaMKII antibody and phospho-CaMKII antibody were from Thermo Scientific, anti-phospho-PLN Thr-17 (PT17), and anti-phospho-RyR-2814 were from Badrilla, (UK). KN93 and all other compounds were purchased from Sigma-Aldrich.

[0076] Confocal immunohistochemistry. Mouse adult ventricular myocytes were isolated, fixed, and stained for confocal immunohistochemistry as previously (Zhang M, *et al.* (2010) *J Am Coll Cardiol* 56(24):2021-2030). Cells were fixed with 50% methanol and 50% acetone, permeabilized with 0.1% saponin in PBS, blocked in 10% BSA in PBS, incubated overnight with primary antibodies at 4°C (rabbit anti-TrkB, Millipore Inc) and subsequently incubated with secondary antibodies for 1 hour at room temperature (Alexa Fluor 488–conjugated donkey anti-rabbit; Invitrogen). Imaging was performed using an argon-krypton laser confocal scanning microscope (UltraView; Perkin Elmer Life Science Inc.).

[0077] Cardiac Myocyte Isolation. Isolation of rat and mouse ventricular myocytes was carried out as previously described (Tocchetti CG, *et al.* (2007) *Circ Res* 100(1):96-104), and approved by the Animal Care and Use Committee of Johns Hopkins University and Loyola University. Wild type 2-4 month old mice were anesthetized with intraperitoneal pentobarbital sodium (100 mg/kg). To assess for sarcomere shortening, cells were imaged using field stimulation (0.5 Hz) in an inverted fluorescence microscope (Diaphot 200; Nikon, Inc). Sarcomere length was measured by real-time Fourier transform (IonOptix MyoCam, CCM100M). Twitch amplitude is expressed as a percentage of resting cell length. Twitch kinetics was quantified by measuring the time to peak shortening and the time from peak shortening to 50% relaxation. For whole Ca^{2+} transient measurements, myocytes were loaded

with the Ca^{2+} indicator fluo-4/AM (Molecular Probes, 20 μM for 30 min) and Ca^{2+} transients were measured under field-stimulation (0.5 Hz) in perfusion solution by confocal laser scanning microscope (LSM510, Carl Zeiss). Digital image analysis used customer-designed programs coded in Interactive Data Language (IDL).

[0078] Whole cell Ca^{2+} transient, SR Ca^{2+} load measurements and analysis. SR Ca^{2+} load measurements were carried out as previously described (Huke S & Bers DM (2007) *J Mol Cell Cardiol* 42(3):590-599). Isolated rat ventricular myocytes were plated onto superfusion chambers, with the glass bottoms treated with natural mouse laminin (Invitrogen, Carlsbad, CA) to increase cell adhesion. The standard tyrode's solution used in all experiments contained (in mM): NaCl 140, KCl 4, MgCl_2 1, glucose 10, HEPES 5 and CaCl_2 1, pH 7.4. Myocytes were loaded with 6 μM fluo-4/AM for 25 minutes and subsequently superfused for at least 30 minutes to allow for deesterification of the dye. All experiments were done at room temperature (23–25 °C) using field stimulation. Fluo-4 was excited at 480 ± 5 nm and emission measured using a 535 ± 20 bandpass-filter. Ca^{2+} -transients were recorded with Clampex 8.0 and data analyzed with Clampfit. SR Ca^{2+} load was assessed by rapid application of 10 mM caffeine. Cells were stimulated at 0.5 Hz and after steady-state was achieved exposed to 20 nM BDNF (Calbiochem) for 5 minutes.

[0079] Spontaneous Ca^{2+} sparks and measurement of spark frequency. Spontaneous Ca^{2+} sparks were assessed using confocal imaging as described previously (Picht E., et al., *Am J Physiol Cell Physiol* 293(3):C1073-1081). Isolated rat cardiac myocytes were loaded with the Ca^{2+} indicator fluo-4 acetoxymethyl ester (fluo-4/AM) (Molecular Probes, 20 $\mu\text{mol/L}$ for 30 minutes). Confocal images were acquired using a confocal laser-scanning microscope (LSM510, Carl Zeiss) with a Zeiss Plan-Neofluor $\times 40$ oil immersion objective (NA=1.3). Fluo-4/AM was excited by an argon laser (488 nm), and fluorescence was measured at >505 nm. Images were taken in the line-scan mode, with the scan line parallel to the long axis of the myocytes. Each image consisted of 512 line scans obtained at 1.92-ms intervals, each comprising 512 pixels at 0.10- μm separation. Digital image analysis used customer-designed programs coded in interactive data language and a modified spark detection algorithm

[0080] Patch clamp experiments. Whole cell voltage-clamp was performed to measure $\text{I}_{\text{Ca,L}}$ elicited by hyperpolarizing pulse from a holding potential of -40 mV using an Axopatch 200A patch-clamp amplifier (Axon Instruments) at room temperature (22 °C) as described previously (Aiba T, et al. (2009) *Circulation* 119(9):1220-1230). Voltage command protocols were generated by the custom-written software. Capacitance compensation was

optimized and series resistance was compensated by 40-80%. Membrane currents were filtered at 5 kHz and digitized with 12-bit resolution through a DigiData-1200 interface (Axon Instruments). The patch pipettes had $\approx 3.0 \text{ M}\Omega$ tip resistances when filled with pipette solution containing (in mM): 80 Cs-glutamate, 40 CsCl, 10 HEPES, 5 EGTA and 5 Mg-ATP adjusted to pH 7.2 with CsOH, and the bath solution was Tyrode's solution with equimolar replacement of KCl by CsCl. Cell capacitance was estimated by integrating the area under an uncompensated depolarizing step of 10 mV from a holding potential of -80 mV.

[0081] Myocardial capillary density measurements. Hearts were immersion fixed in 10% neutral buffered formalin, paraffin embedded, sectioned into 8- μm slices, and then stained with isolectin B4 (GS-IB4, Alexa Fluor 488 conjugated, Life Technologies) to assess capillary density (Koitabashi N, *et al.* (2011) *J Clin. Invest.* 121(6):2301-2312). Paraffin-embedded sections were microwaved in sodium citrate buffer pH 6 and then incubated with isolectin B4 (1:100) at room temperature for 2 hours. Five cross-sectional myocardial images (0.0675 mm²/field) were captured per animal using a Nikon E600 fluorescence microscope at 400X magnification; images were then analyzed by iVision software (iVision, version 4.01; BioVision Technologies). All histological quantifications were performed in a blinded manner.

[0082] *In vivo* hemodynamics. *In vivo* LV function was assessed by PV catheter as described previously (Takimoto E, *et al.* (2005) *Nat Med* 11(2):214-222). Briefly, mice were anesthetized with 1%-2% isoflurane, 750–100 mg/kg urethane i.p., 5–10 mg/kg etomidate i.p., and 1–2 mg/kg morphine i.p.; animals were subjected to tracheostomy; and were ventilated with 6–7 $\mu\text{l/g}$ tidal volume and 130 breaths/min. Volume expansion (12.5% human albumin, 50–100 μl over 5 min) was provided through a 30-gauge cannula via the right external jugular vein. The LV apex was exposed through an incision between the seventh and eighth ribs, and a 1.4-Fr PV catheter (SPR 839; Millar Instruments Inc.) was advanced through the apex to lie along the longitudinal axis. Absolute volume was calibrated, and PV data were measured at steady state and during transient reduction of venous return by occluding the inferior vena cava with a 6-0 silk snare suture. Data were digitized at 2 kHz, stored to disk, and analyzed with custom software. From the 10–15 successive cardiac cycles during the inferior vena cava occlusion, the end-systolic PV relation slope was derived.

[0083] Statistical analysis. Results are expressed as means $\pm$ SEM. Significance was estimated by one-way repeated measures ANOVA and/or Student's t-test for paired observations as appropriate. $P \leq 0.05$ was considered significant.

EXAMPLE 1

[0084] BDNF directly increases myocyte function, enhancing cardiac Ca^{2+} cycling. We determined the impact of exogenous application of BDNF on isolated rodent cardiomyocytes. These cells contain surface TrkB receptors (Fig. 1A), consistent with previous reports. Incubating mouse cardiac myocytes with BDNF (20 nM) resulted in increased cell contractility ($43 \pm 7.5\%$) and enhanced relaxation ($11 \pm 3\%$) ($n=19$, $P < 0.01$ for both, Fig. 1B). This enhancement of function was accompanied by larger whole cell Ca^{2+} transients ($10 \pm 4\%$, $P < 0.01$) (Fig. 1B). Ca^{2+} spark frequency was also increased after BDNF stimulation (1.3 ± 0.3 to 3.4 ± 0.7 sparks/100 $\mu\text{m}^2/\text{s}$, $P < 0.05$, $n=6$) (Fig. 1C). We next asked whether this stimulatory action stems from an increase in sarcoplasmic reticulum (SR) Ca^{2+} fractional release and/or activity of L-type Ca^{2+} channels. BDNF markedly augmented SR Ca^{2+} fractional release (from 54 ± 6 to $68 \pm 6\%$, $P < 0.05$, $n=11$), without affecting total SR Ca^{2+} load as assessed by caffeine transients (Fig. 1D). In isolated guinea-pig myocytes, BDNF increased peak L-type Ca^{2+} current density by $\approx 25\%$ compared to baseline (-3.5 ± 0.2 vs. -2.8 ± 0.4 mV; $p < 0.05$, $n=6$, Fig. 1E). Thus, BDNF enhances myocyte Ca^{2+} cycling without altering diastolic Ca^{2+} levels or Ca^{2+} SR load (Fig. 1D).

EXAMPLE 2

[0085] TrkB is required for BDNF-induced enhancement of myocyte function. We then tested whether the TrkB receptor is required for the inotropic/lusitropic action of BDNF. To do so, we used isolated myocytes from TrkB^{F616A} mice (kindly provided by Dr. David D. Ginty). In these mice a phenylalanine-to-alanine substitution within the kinase subdomain of the TrkB receptor renders it sensitive to specific inhibition by membrane-permeable, small-molecule PP1 derivatives, including 1NMPP1. BDNF had no impact on TrkB^{F616A} myocytes pretreated with 1-NMMP1 (100 nM) (Fig. 1F), and 1-NMMP1 did not alter basal myocyte function: fractional shortening was 3.19 ± 0.43 (baseline) vs. 2.92 ± 0.2 (after 1NMPP1) ($n=10$, $P=0.52$).

EXAMPLE 3

[0086] Constitutive TrkB stimulation is required for optimal cardiac contraction and relaxation. To determine if BDNF/TrkB stimulation is constitutively active *in vivo* and the relevance of this signaling in maintaining normal heart function, cardiac-specific TrkB knockout mice (TrkB^{-/-}) were generated by crossing conditional TrkB^{-/-} mice (from Dr. David D. Ginty) with cardiomyocyte specific (MHC)-Cre mice (Fig. 6). BDNF is a modulator of angiogenesis, thus changes in vascularity may contribute to alter cardiac performance in TrkB^{-/-} mice. Therefore, we first measured capillary density in TrkB^{-/-} mice and their control littermates. The capillary density measured by counting isolectin B4 stained capillary profiles in myocardium did not differ between WT and TrkB^{-/-} mice (Fig. 7).

[0087] Next, we examined the impact of BDNF/TrkB signaling on cardiac contraction and relaxation *in vivo*. Pressure-volume relationships were used to distinguish intrinsic changes in myocardial contractility/relaxation from confounding effects due to potential changes in vascular loading conditions (i.e., preload and afterload). TrkB^{-/-} mice displayed reduced myocardial performance, as determined by dP/dt_{max} (a load-dependent index) and load-independent parameters of myocardial contractility such as ventricular elastance (Ees), dPdt/EDV, dPdt ip and pre-recrutable stroke work (PRSW) (Fig 2 and Table 1). All these indices were consistent with reduced systolic function in TrkB^{-/-} mice, though this was not severe enough for triggering chamber dilation or reducing ejection fraction. Cardiac relaxation was also impaired, with prolongation of the relaxation time constant (Tau) from 4.0±0.2 to 5.3±0.3 msec (P=0.0019) (Fig. 2B and Table 1). Total systemic vascular resistance (SVR) and ventricular afterload (indexed by arterial elastance, Ea) were similar in both genotypes, so systemic load was not impacted. The ventricular/arterial coupling ratio (Ees/Ea) declined due solely to the fall in contractility, which would indicate a fall in efficiency of blood transfer in TrkB^{-/-} mice. Thus, BDNF/TrkB signaling is an important independent contributor to basal cardiac contraction and relaxation *in vivo*. We further examined whether TrkB^{-/-} mice had altered responses to β-adrenergic receptor stimulation. Isoproterenol (from 10 to 40 ng/kg/min) infusion produced similar augmentation in both genotypes (n=5 each group, Fig. 3 and Table 1). These findings indicate that a lack of BDNF/TrkB modulation does not impair β-AR-cAMP/PKA signaling.

[0088] TABLE 1: Hemodynamic values in WT and TrkB^{-/-} via pressure-volume relationships.

Basal Hemodynamics	WT (n=6)	TrkB ^{-/-} (n=6)	P value
HR (min ⁻¹)	611.7±13.5	574±25.1	0.22
SBP (mmHg)	100.1±3	96±5.2	0.53
SV (μl)	23.9±1.7	21.6±1.9	0.39
CO (ml/min)	14.6±1	12.4±1.1	0.17
EF (%)	56.0±2.3	56.7±2.0	0.59
dPdt_{max} (mmHg/sec)	10944.7±496.3	7647.2±498.9	0.0009
Ees (mmHg/ml)	15.2±3.2	6.6±1.0	0.041
dPdt/EDV	417.2±56.4	209.2±25	0.0073
dPdt_{ip} (sec ⁻¹)	185.3±8.3	142.4±9.8	0.0075
PRSW (mmHg)	108.3±2.4	85.8±7.6	0.018
Tau_{log} (msec)	4.0±0.2	5.3±0.3	0.0019
Ea (mmHg/ml)	4.0±0.4	4.3±0.4	0.56
SVR (mmHg.min.ml ⁻¹)	6.5±0.7	7.5±0.6	0.26
Ees/Ea	4.1±1.1	1.5±0.6	0.033
Isoproterenol (ISO, 40 ng/kg/min)	WT (n=5)	TrkB^{-/-} (n=5)	
dPdt_{max} (mmHg/sec)	13973.7±558.8	10583.7±655.0	0.005
% increase over baseline	22.3±3.0	38.8±11.1	0.19

EXAMPLE 4

[0089] CaMKII mediates BDNF-induced enhancement of myocyte function. In the brain, BDNF/TrkB is mainly coupled to CaMKII. We found that BDNF also increased the activated and phosphorylated state of CaMKII in isolated murine ventricular myocytes. This change was paralleled by augmented phosphorylation of CaMKII-dependent sites on the ryanodine receptor 2 (RyR2)(S2814), and phospholamban (PLN) (T17) (Fig. 4A). TrkB^{-/-} mice displayed decreased levels of P-CaMKII and the reduction of P-CaMKII/T-CaMKII ratio (Fig. 4A). Moreover, pharmacological inhibition of CaMKII activity by KN93 completely abolished BDNF Ca²⁺ transients and contractility (Fig. 4B). Thus, CaMKII is the main mediator of BDNF/TrkB-evoked cardiac stimulatory actions that operates in parallel to β-adrenergic signaling to regulate myocardial contraction and relaxation.

EXAMPLE 5

[0090] BDNF-induced enhancement of myocyte function is lost in failing hearts. We finally tested whether BDNF-evoked cardiac enhancement is preserved in failing myocytes, using cardiomyocytes isolated from $G_{\alpha q}$ over-expressing ($G_{\alpha q}$ OE) mice that display progressive cardiac dilation and reduced ejection fraction. Enhanced $G_{\alpha q}$ signaling is a common pathway mediating maladaptive cardiac hypertrophy and adverse remodeling. In response to pathological stress, $G_{\alpha q}$ signaling is activated by α -adrenergic agonist, angiotensin II, or endothelin, etc., promoting cardiac growth, apoptosis, fibrosis, and ultimately resulting in cardiac dysfunction. Therefore, $G_{\alpha q}$ OE mice mimic this pathological response and are often used as a heart failure model. In the present study, $G_{\alpha q}$ OE myocytes did not respond to BDNF (20 nM) (Fig. 5A); therefore, we sought to determine the mechanisms underlying this insensitivity. First, we examined the expression of TrkB in $G_{\alpha q}$ OE hearts. Intriguingly, the expression of full-length TrkB (TrkB-FL) was unchanged, but the truncated form of TrkB (TrkB-T1), which lacks tyrosine kinase activity, was markedly increased (Fig. 5B). TrkB-T1 is one of the TrkB splicing variants, and it often acts as a dominant negative form that suppresses BDNF/TrkB signaling; increased TrkB-T1 levels have been implicated in neurological disorders. Next, we further examined the CaMKII signaling cascade in $G_{\alpha q}$ OE hearts. We found that these hearts display elevated P-CaMKII and total T-CaMKII at baseline (Fig. 5C), consistent with previous reports. The expression of total PLN was unchanged; however decreased level of SERCA2a was evident (Fig. 5C). Moreover, the phosphorylation at threonine 17 of PLN was markedly reduced (Fig. 5C), despite chronic activation of CaMKII. Thus, chronically activated CaMKII and/or altered CaMKII downstream targets can also account for loss in BDNF/TrkB stimulatory action in $G_{\alpha q}$ OE myocytes. The above findings were recapitulated in mice with pressure overload-induced heart failure via transverse aortic constriction (TAC). TAC hearts displayed higher expression of the TrkB-T1 and chronically activated CaMKII signaling pathway (Fig. 8), demonstrating that altered TrkB receptor and chronically activated CaMKII signaling pathway are not peculiar to a transgenic mouse model, but also pertain to other heart failure models generated via chronic hemodynamic stress.

EXAMPLE 6

[0091] Impact of TrkB agonist in TAC mouse model of heart failure. Vehicle or agonist was started 1 week after TAC. The TrkB agonist LM22A-4 (0.2µg/kg/day dose) or saline (vehicle) was delivered for 4 weeks in TAC mice (via Alzet mouse intraperitoneal infusion pumps), starting one 1 week after TAC. The TrkB agonist LM22A-4 preserved LV function as indexed by % fractional shortening (Fig. 9A, and time course in lower panel) and ejection fraction measured by echo in conscious (non-sedated) animals (Fig. 9B, and time course in lower panel).

EXAMPLE 7

[0092] The impact of the TrkB agonist LM22A-4 on normal myocytes. LM22A-4 (solved in physiological solution) was administered to ventricular myocytes isolated from control mice (of 8-12 weeks of age). As shown, LM22A-4 exerted a direct positive inotropic effect on these cells which was dose-dependent. This enhancement in contractile function (sarcomere shortening expressed as % increase from baseline value, panel Fig. 10A) was coupled to an equally marked and dose-dependent increase in whole Ca^{2+} transients (panel Fig. 10B). These data demonstrate that, TrkB agonists, including LM22A-4 can directly modulate myocardial function by binding on sarcolemmal TrkB receptors, independently from any other influence it may have on the vasculature (extrinsic or intrinsic to the myocardium) and nerve fibers directed to the heart.

[0093] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0094] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value

falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0095] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Claims:

1. Use of a TrkB agonist for increasing contractility in a cardiac cell or population of cells comprising contacting the cell or population of cells with a composition comprising an effective amount of a TrkB agonist.

2. The use of claim 1, wherein the TrkB agonist is an analog, derivative, or peptidomimetic of Brain Derived Neurotrophic Factor (BDNF).

3. The use of either of claims 1 or 2, wherein the cell or population of cells is contacted with a composition comprising an effective amount of a TrkB agonist and at least one additional biologically active agent.

4. The use of either of claims 1 or 2, wherein the TRkB agonist is selected from the group consisting of LM22A-1, LM22A-2, LM22A-3, LM22A-4, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), N-acetylserotonin, N-(2-(5-hydroxy-1H-indol-3-yl)ethyl)-2-oxopiperidine-3-carboximide (HIOC), amitriptyline, 7,8-dihydroxyflavone, 7,8,3'-trihydroxyflavone, 4'-dimethylamino-7,8-dihydroxyflavone, and deoxygedunin.

5. Use of a TrkB agonist for increasing contractility in the cardiac tissue of a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist and a pharmaceutically acceptable carrier.

6. The use of claim 5, wherein the TrkB agonist is an analog, derivative, or peptidomimetic of Brain Derived Neurotrophic Factor (BDNF).

7. The use of either of claims 5 or 6, wherein the pharmaceutical composition comprises at least one additional biologically active agent.

8. The use of either of claims 5 or 6, wherein the TRkB agonist is selected from the group consisting of LM22A-1, LM22A-2, LM22A-3, LM22A-4, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), N-acetylserotonin, N-(2-(5-hydroxy-1H-indol-3-yl)ethyl)-2-oxopiperidine-3-carboximide (HIOC), amitriptyline, 7,8-dihydroxyflavone, 7,8,3'-trihydroxyflavone, 4'-dimethylamino-7,8-dihydroxyflavone, and deoxygedunin.

9. Use of a TrkB agonist for treating congestive heart failure in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a TrkB agonist and a pharmaceutically acceptable carrier.

10. The use of claim 9, wherein the TrkB agonist is an analog, derivative, or peptidomimetic of Brain Derived Neurotrophic Factor (BDNF).

11. The use of either of claims 9 or 10, wherein the pharmaceutical composition comprises at least one additional biologically active agent.

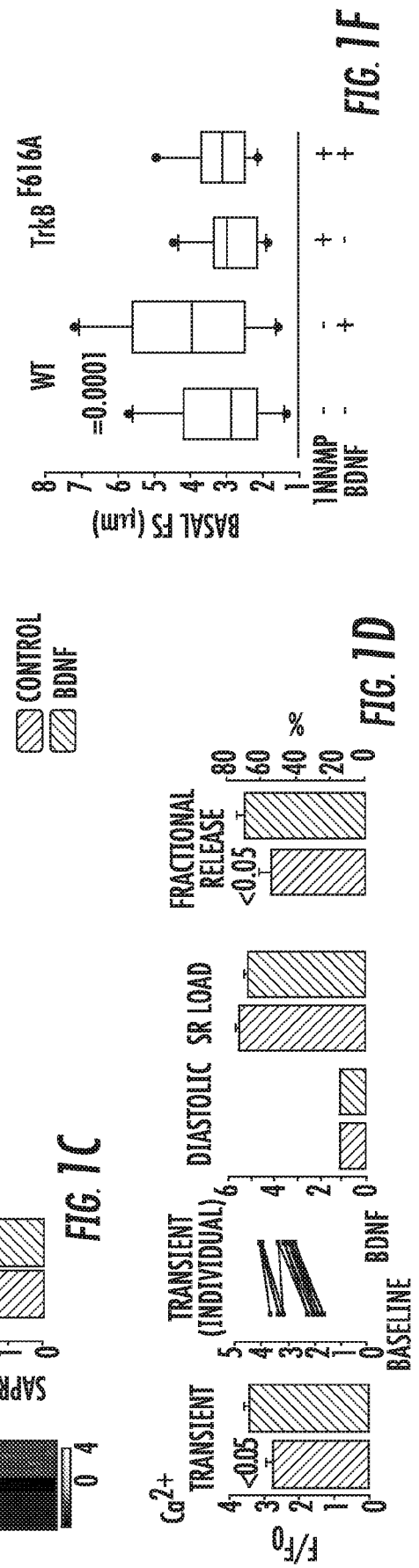
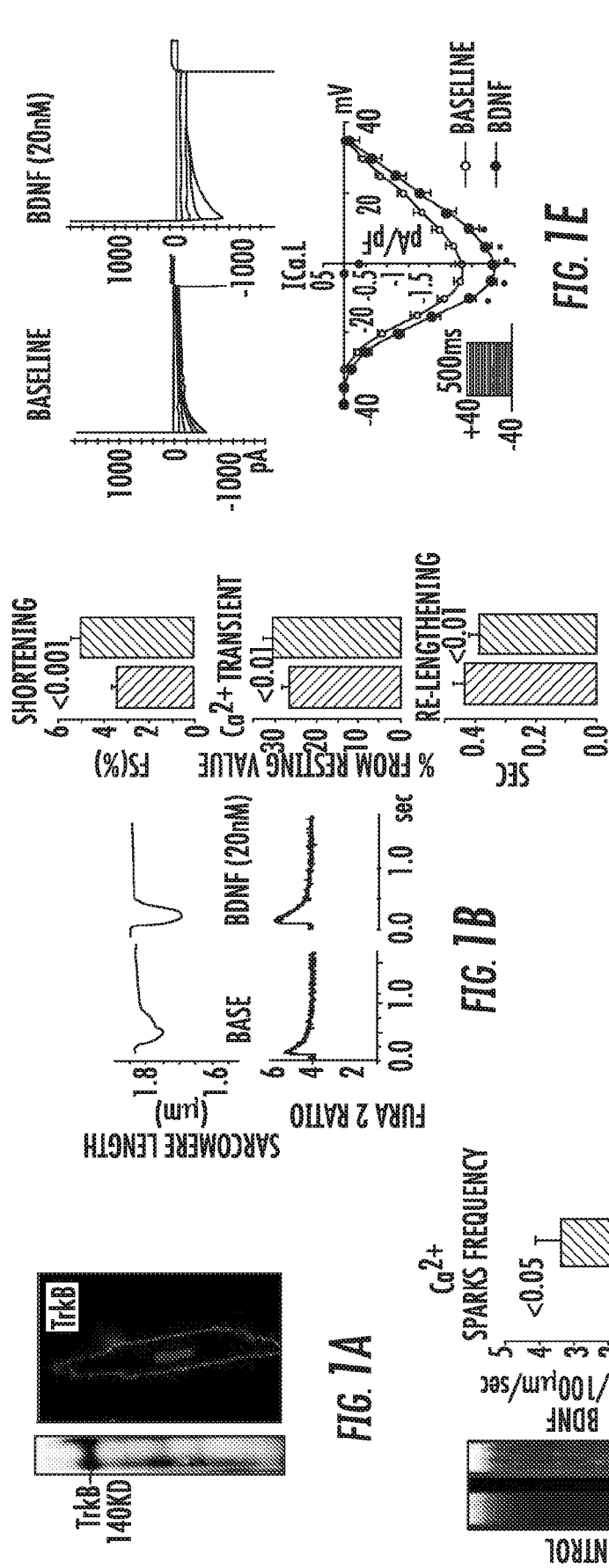
12. The use of either of claims 9 or 10, wherein the TRkB agonist is selected from the group consisting of LM22A-1, LM22A-2, LM22A-3, LM22A-4, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), N-acetylserotonin, N-(2-(5-hydroxy-1H-indol-3-yl)ethyl)-2-oxopiperidine-3-carboximide (HIOC), amitriptyline, 7,8-dihydroxyflavone, 7,8,3'-trihydroxyflavone, 4'-dimethylamino-7,8-dihydroxyflavone, and deoxygedunin.

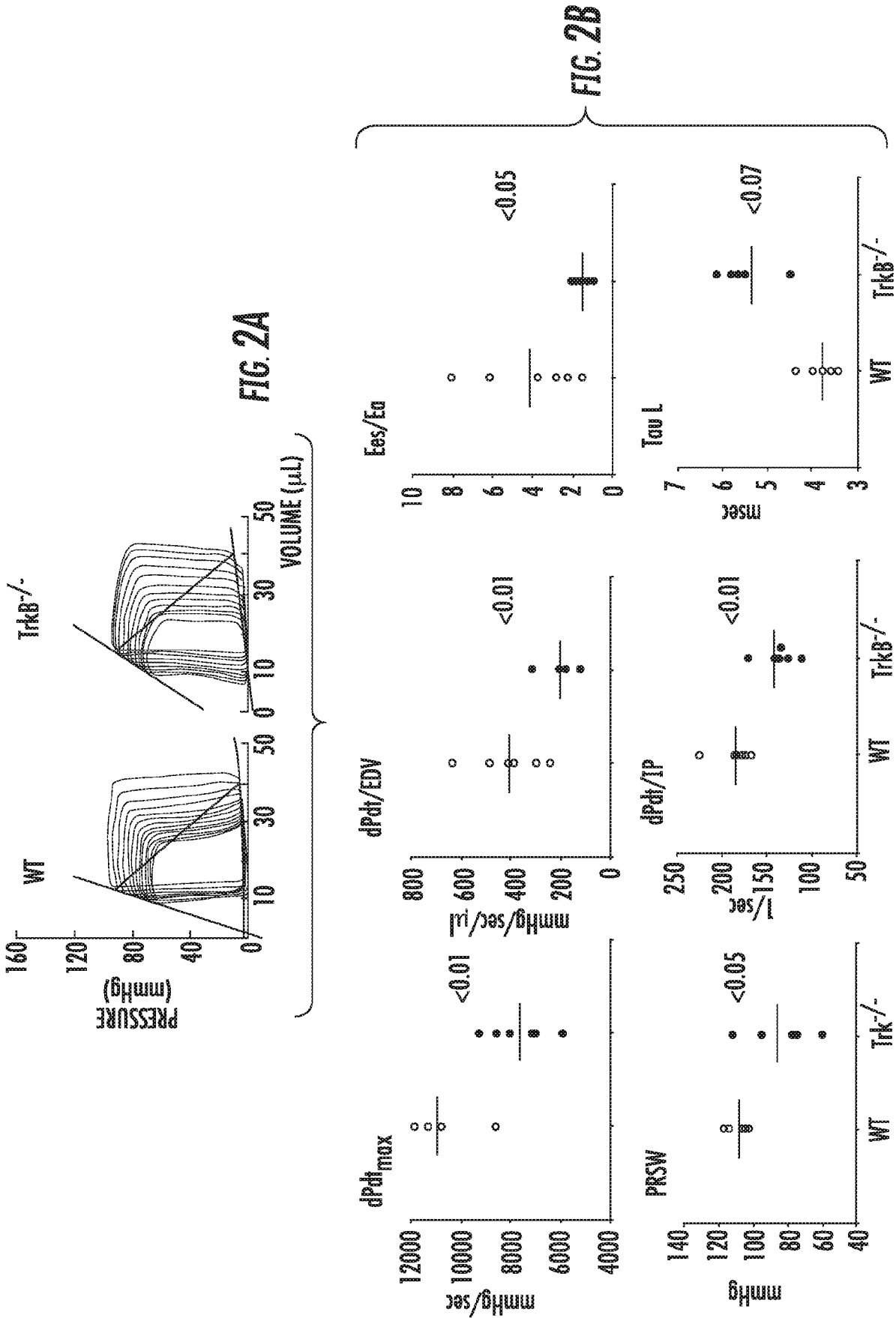
13. The use of any of claims 1 or 2, 5 or 6, or 9 or 10, wherein the at least one additional biologically active agent is selected from the group consisting of angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, digoxin and derivatives thereof, beta blockers, diuretics, aldosterone antagonists, and inotropic drugs.

14. A method for identifying a test compound as a TrkB agonist comprising:

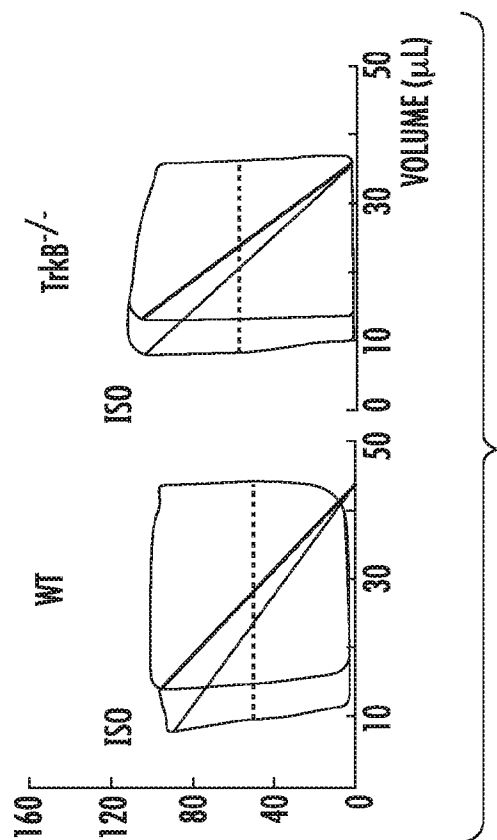
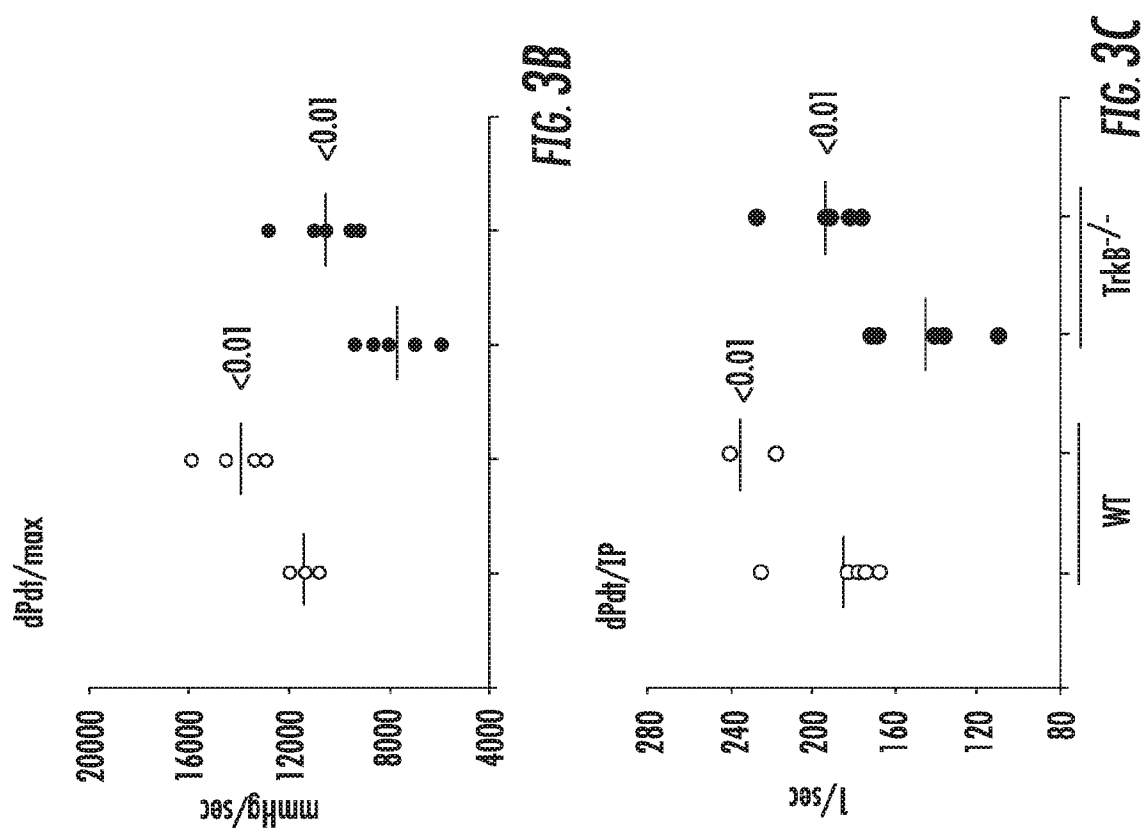
- a) obtaining a culture of one or more isolated wild type murine ventricular myocytes and obtaining a culture of one or more isolated TrkB^{-/-} murine ventricular myocytes;
 - b) contacting both cultures of a) separately with vehicle, a positive control compound which is a known TrkB agonist, and one or more test compounds; and
 - c) measuring contractile function and/or Ca²⁺ transients in the cultures of a);
- wherein when both the positive control compound and the one or more test compounds increase contractile function and/or Ca²⁺ transients when compared to controls in the isolated wild type murine ventricular myocytes, and the positive control compound and the one or more test compounds do not increase contractile function and/or Ca²⁺ transients when compared to controls in the isolated TrkB^{-/-} murine ventricular

myocytes, then the one or more test compounds are identified as TrkB agonists.

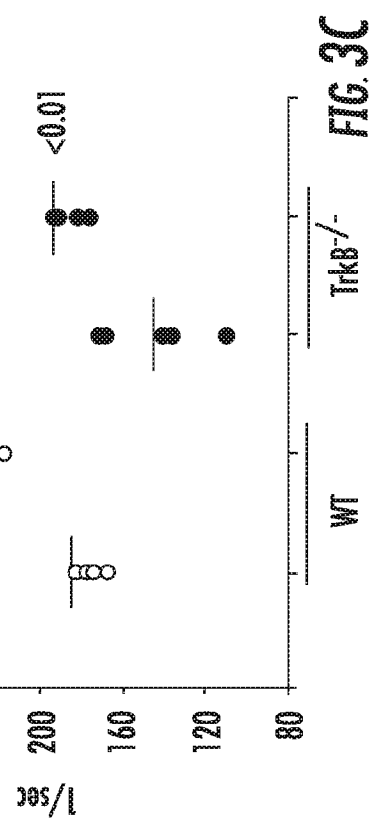


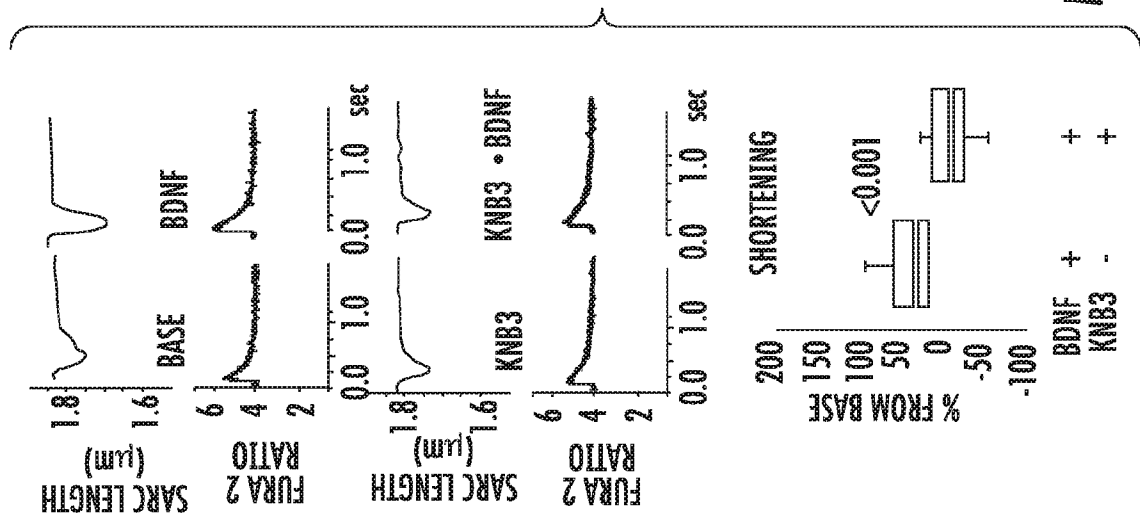
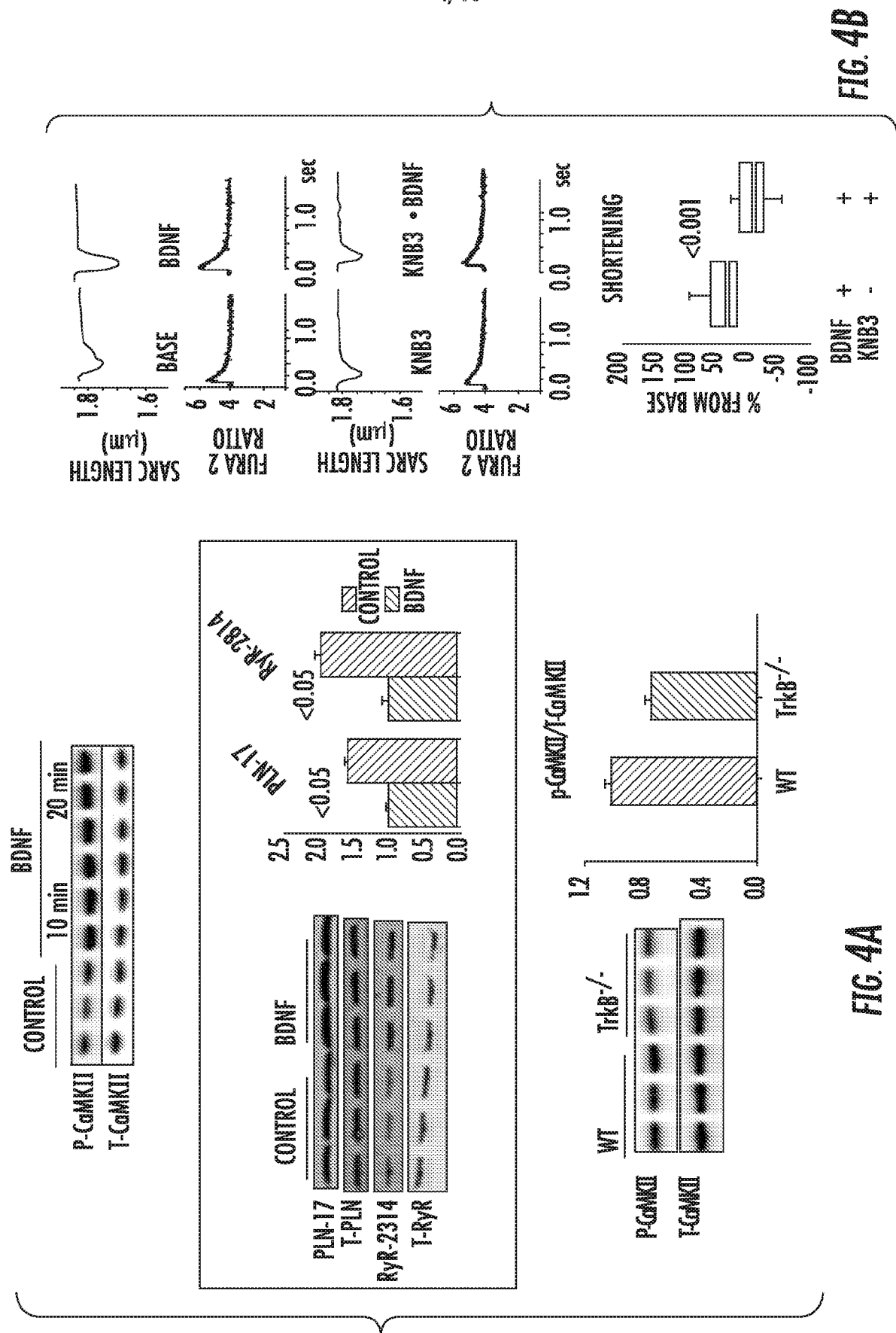


3/10



dPdt/IP





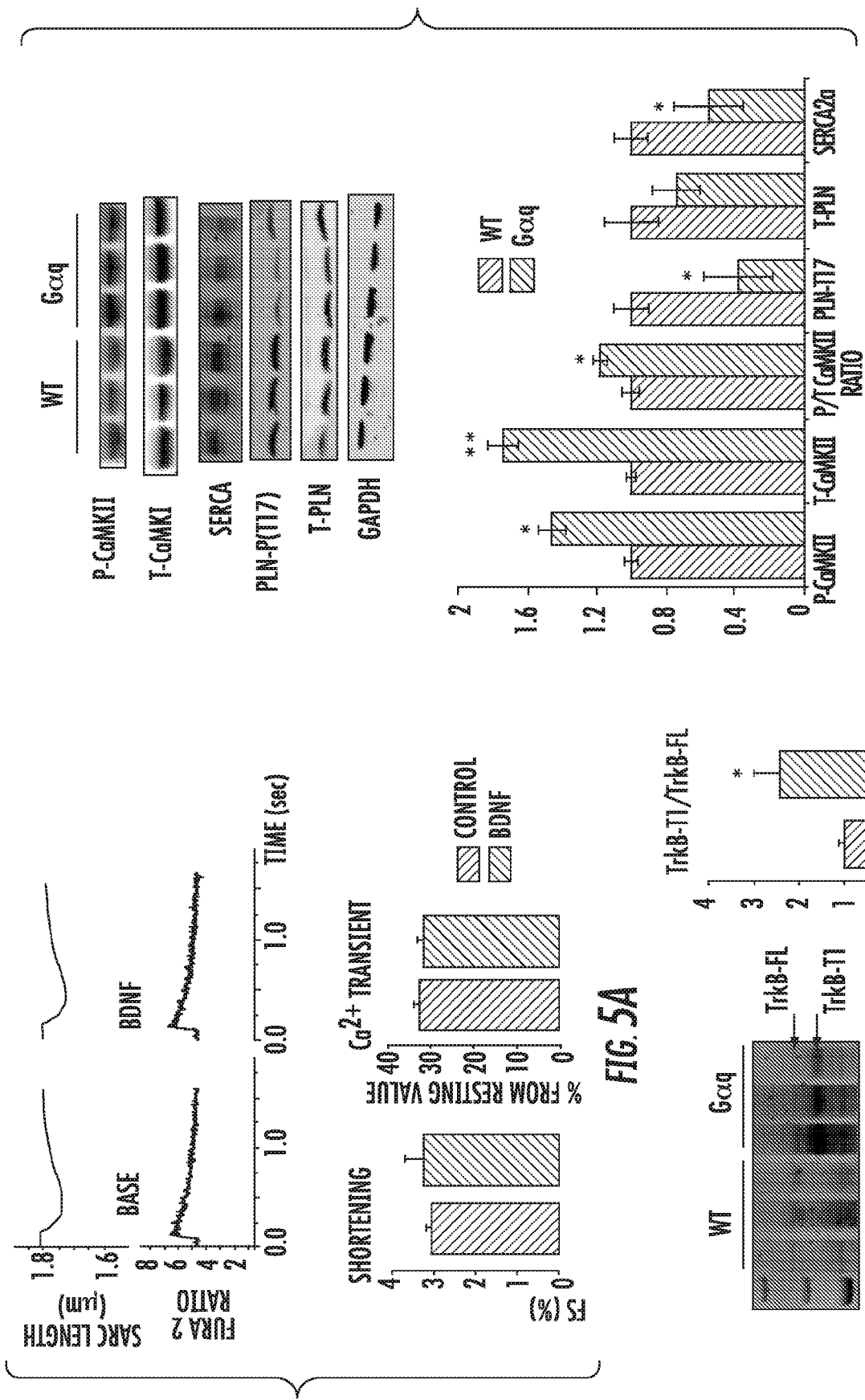
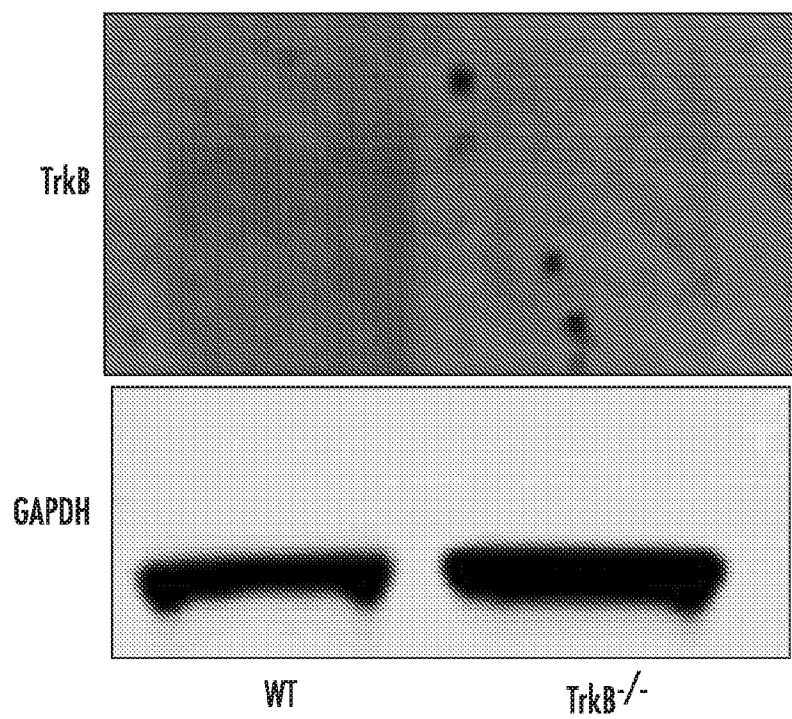


FIG. 5C



FIG. 5B

6/10

**FIG. 6**

7/10

WT

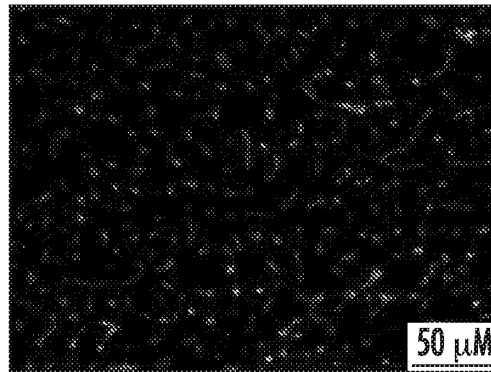


FIG. 7A

TrkB^{-/-}

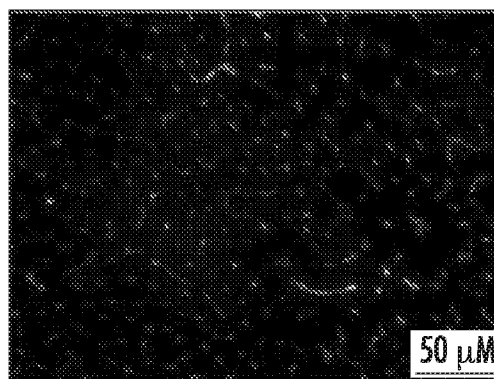


FIG. 7B

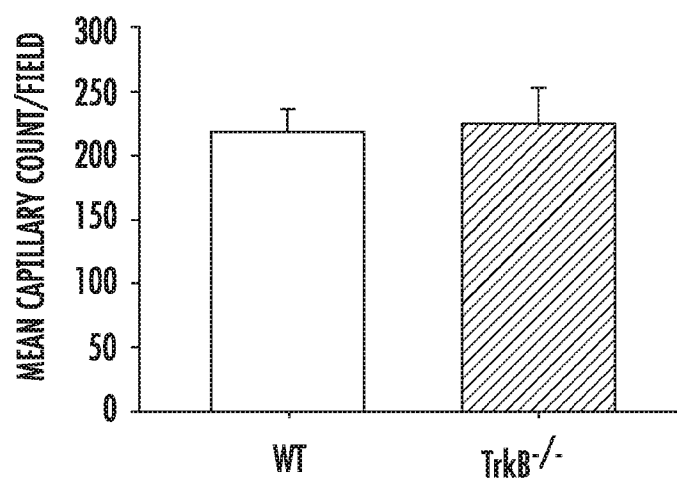


FIG. 7C

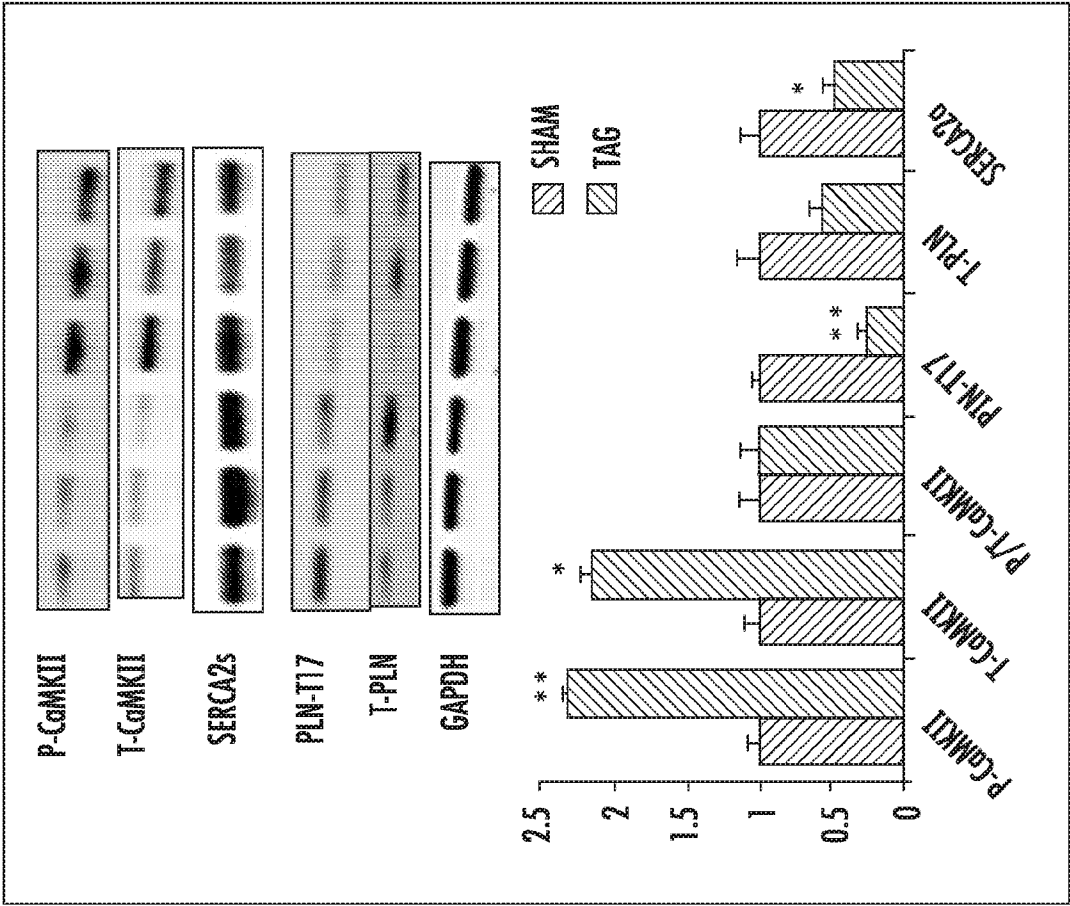


FIG. 8B

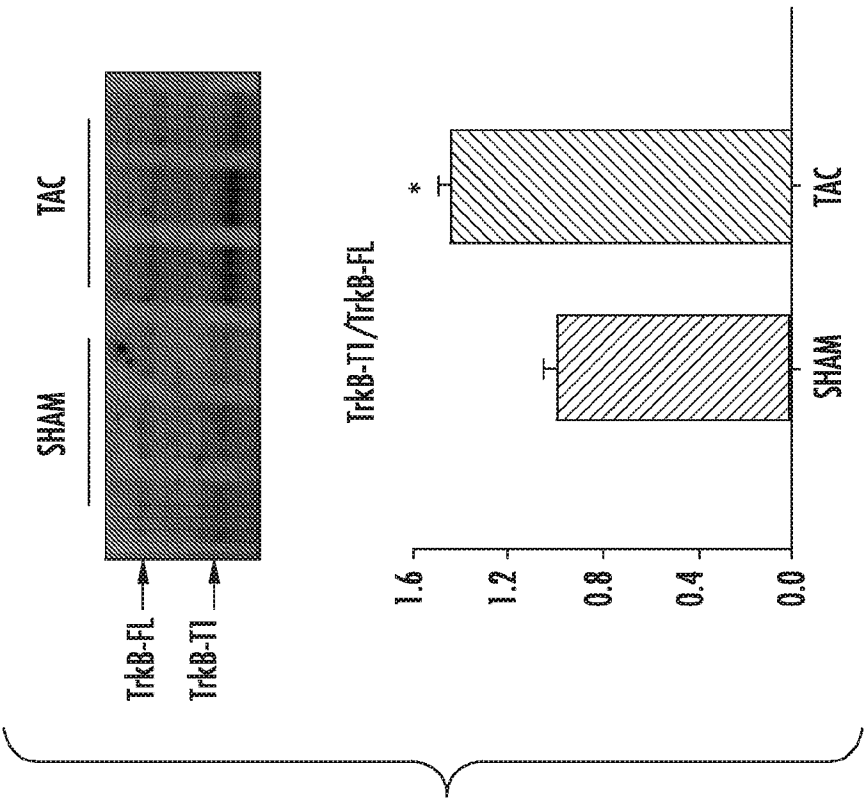


FIG. 8A

9/10

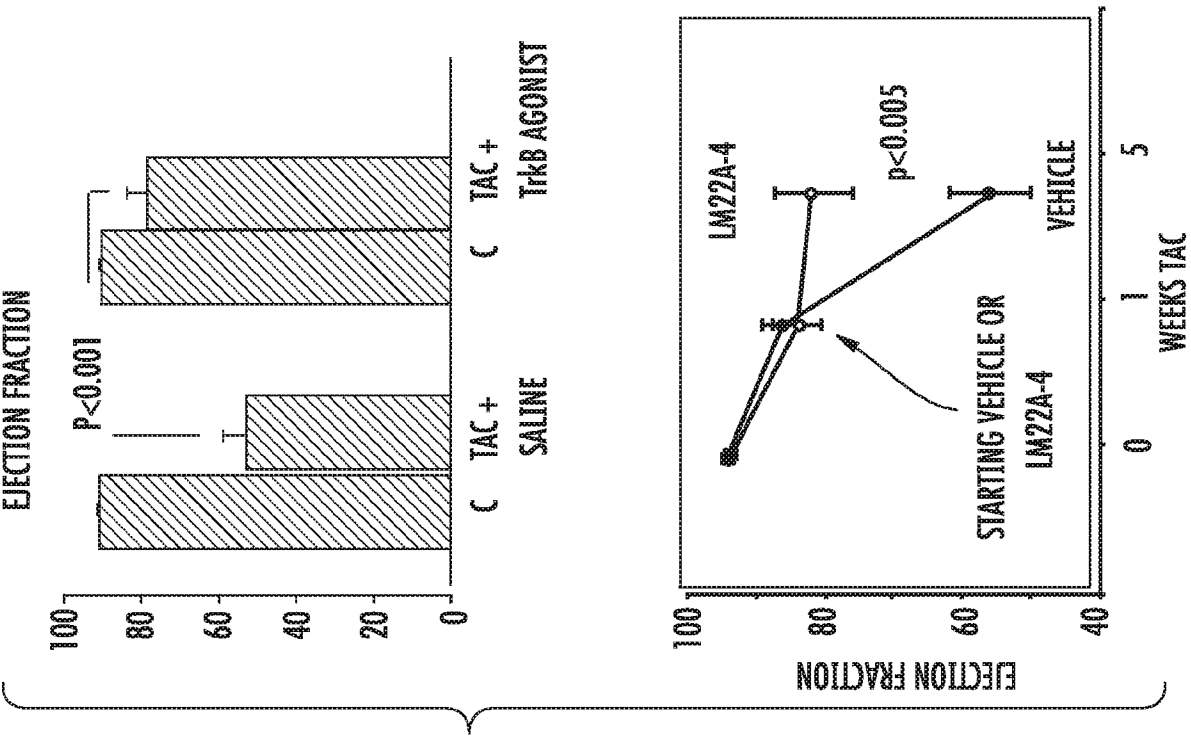


FIG. 9B

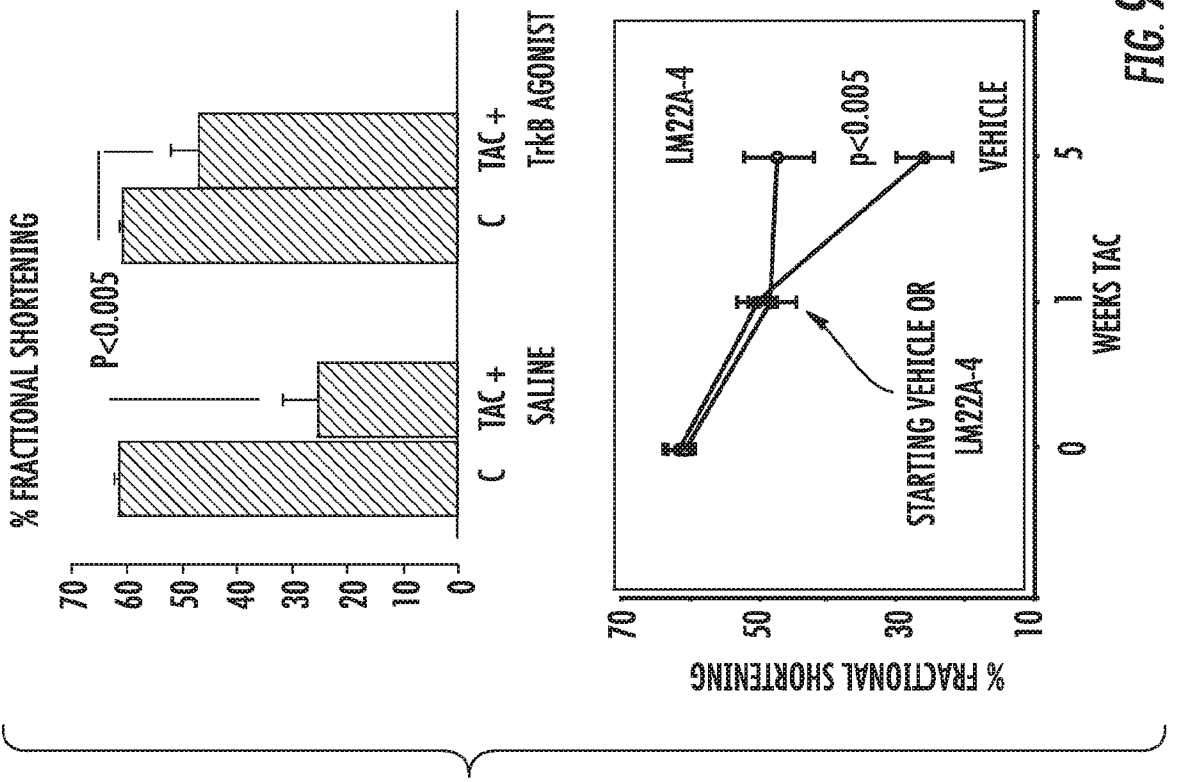
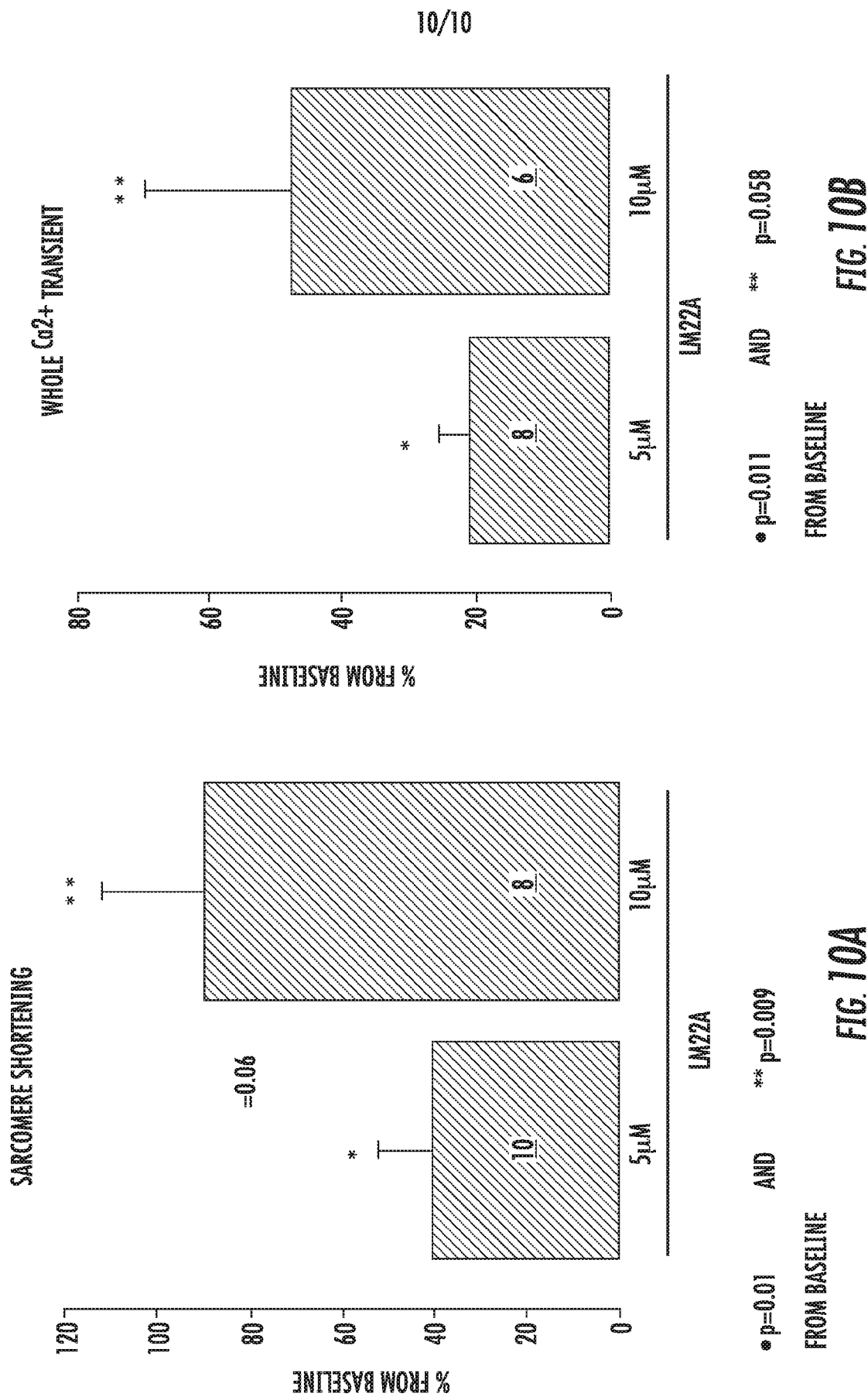


FIG. 9A



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/012186**A. CLASSIFICATION OF SUBJECT MATTER****A61K 38/18(2006.01)i, A61K 38/17(2006.01)i, A61P 9/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/18; A61K 31/353; A61K 38/17; A61P 9/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: TrkB agonist, BDNF, contractility, cardiac, congestive heart failure

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FENG et al., 'Brain-derived neurotrophic factor (BDNF) directly modulates cardiac ventricular myocyte function' The FASEB Journal, Vol.25, Meeting Abstracts, Abstract No.646.1 (2011) See the whole document, especially lines 5-7 and 19-21.	1-14
A	JANG et al., 'A selective TrkB agonist with potent neurotrophic activities by 7, 8-dihydroxyflavone' PNAS, Vol.107, No.6, pp.2687-2692 (2010) See the whole document.	1-14
A	GARY et al., 'Aerobic exercise as an adjunct therapy for improving cognitive function in heart failure' Cardiology Research and Practice, Vol.2014, Article ID 157508, Internal pages 1-8 (2014) See the whole document.	1-14
A	HASHIMOTO, 'Sigma-1 receptor chaperone and brain-derived neurotrophic factor: Emerging links between cardiovascular disease and depression' Progress in Neurobiology, Vol.100, pp.15-29 (2013) See the whole document.	1-14

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 April 2016 (15.04.2016)

Date of mailing of the international search report

15 April 2016 (15.04.2016)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea

Facsimile No. +82-42-481-8578

Authorized officer

KIM, Seung Beom

Telephone No. +82-42-481-3371



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/012186

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2014-0057837 A1 (DURING et al.) 27 February 2014 See the whole document .	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/012186

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014-0057837 A1	27/02/2014	None	