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Genetics of congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV:

Clinical, biological and molecular aspects of mutations in *TRKA(NTRK1)* gene encoding the receptor tyrosine kinase for nerve growth factor

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Summary

Congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV (HSAN-IV) is an autosomal recessive disorder characterized by recurrent episodic fevers, anhidrosis (inability to sweat), absence of reaction to noxious (or painful) stimuli, self-mutilating behavior and mental retardation. The anomalous pain and temperature sensation and anhidrosis in CIPA are due to the absence of afferent neurons activated by tissue-damaging stimuli and a loss of innervation of eccrine sweat glands, respectively. Nerve growth factor (NGF) supports the survival of nociceptive sensory and autonomic sympathetic neurons as well as cholinergic neurons of the basal forebrain. The human *TRKA(NTRK1)* gene located on chromosome 1 (1q21-q22) encodes a receptor tyrosine kinase (RTK) which is autophosphorylated in response to NGF, thus, activating various pathways of intracellular signal transduction. We earlier identified the genetic basis of CIPA by detecting mutations in *TRKA* gene of patients. Defects in NGF signal transduction at its receptor lead to failure to survive as various NGF dependent neurons are not maintained, most probably due to apoptosis during development. *TRKA* mutations are distributed in an extracellular domain involved in NGF binding, as well as in the intracellular signal-transduction domain. Missense mutations with loss of function provide considerable insight into the structure-function relationship in the RTK family. In view of the fact that defects in *TRKA* cause CIPA, the molecular pathology of CIPA provides unique opportunities to explore critical roles of the NGF-TRKA receptor system. Thus, CIPA can serve as a useful model to determine mechanisms of development and maintenance of NGF-dependent neurons in autonomic, sensory and central nervous systems, as well as the physiology of these neurons in humans.

Key words: Congenital insensitivity to pain with anhidrosis (CIPA); hereditary sensory and autonomic neuropathy type IV (HSAN-IV); *TRKA(NTRK1)* ; nerve growth factor (NGF); receptor tyrosine kinase (RTK)

Introduction

Congenital insensitivity to pain with anhidrosis (CIPA: MIM 256800) or hereditary sensory and autonomic neuropathy type IV (HSAN-IV) is an autosomal recessive disorder characterized by recurrent episodic fever, anhidrosis (inability to sweat), absence of reaction to noxious (or painful) stimuli, self-mutilating behavior and mental retardation [1,2]. The anomalous pain and temperature sensation and anhidrosis in CIPA are due to the absence of afferent neurons activated by tissue-damaging stimuli and a loss of sympathetic innervation of eccrine sweat glands, respectively [3-5]. Diminished pain perception results in decreased ability to protect oneself from external or self-inflicted-damage. Therefore children with CIPA can sustain severe and unrecognized injury. Loss of sympathetic innervation of eccrine sweat gland explains the phenomenon of anhidrosis [3, 5]. Because sweating plays an important role in maintaining normothermia under hot environmental conditions, anhidrosis disturbs homeostasis and increases susceptibility to recurrent febrile episodes.

During the past decade there have been major advances in understanding factors related to survival as well as the death of neuronal cells. Although mature neurons are among the most long-lived cell types in mammals, immature neurons die in large numbers during development [6]. Neuronal survival requires trophic support. Nerve growth factor (NGF), a neurotrophic factor, induces neurite outgrowth and promotes survival of embryonic sensory and sympathetic neurons derived from the neural crest and ascending cholinergic neurons of the basal forebrain [7]. Nociceptive sensory neurons that respond to tissue damage are NGF-dependent for development, survival and maintenance.

The *TRKA(NTRK1)* gene located on chromosome 1 (1q21-q22) encodes a receptor tyrosine kinase (RTK) which is autophosphorylated in response to NGF, thus, activating various pathways of intracellular signal transduction [8, 9]. Mice lacking the gene for NGF or *TRKA* (*trkA*) share phenotypic features with individuals affected with CIPA [10], including loss of responses to painful stimuli, although anhidrosis is not apparent in these animals. Using a candidate gene strategy, the genetic basis of CIPA was identified when loss-of-function mutations in *TRKA (NTRK1)* were noted in patients [11].

CIPA is the first human genetic disorder implicated in the signal transduction system of neurotrophic factors. Defects in NGF signal transduction at its receptor lead to failure to survive as various NGF dependent neurons are not maintained, most probably due to apoptosis during development. CIPA can serve as a useful model to determine mechanisms of development and maintenance of NGF-dependent neurons in autonomic, sensory and central

nervous system. In view of the fact that defects in *TRKA* cause CIPA, study of the pathophysiology of CIPA can provide unique opportunities to explore critical roles for the NGF-TRKA receptor system in humans, in particular the role of the NGF-TRKA systems in the development and function of nociceptive reception as well as thermoregulation via sweating.

Clinical aspects of CIPA

Swanson was the first to describe two male siblings with congenital insensitivity to pain and anhidrosis and noted that temperature sensation was also defective [1]. Pinsky and DiGeorge described the same disorder in three mentally retarded children, including two siblings [12]. All five patients manifested recurrent febrile episodes, repeated traumatic and thermal injuries, self-mutilating behavior and mental retardation. Sweating could not be elicited by thermal, painful, emotional, or chemical stimuli. In 1951, a case of “generalized anhidrosis” was reported in Japan that had many features similar to CIPA [13]. Rosenberg compiled a large review of 32 cases in 1994 [14].

Clinical features

Recurrent episodic fevers are usually the first clinical sign and can begin in infancy or early childhood depending on environmental temperature. Because the children do not sweat, they develop hyperthermia when they are in a hot environment. They can also develop hypothermia when exposed to extreme cold [1].

Initially impaired pain perception may not be apparent but parents may recall that their children did not cry during blood sampling. Biting of tongue or lip starts after the first teeth erupt and can result in a bifid or absent tongue. Biting of the fingers and ulcerated fingertips are common. Bruises, cuts, and burns do not elicit normal reactions and are often unrecognized. Accidental injuries such as falls or burns lead to multiple scars, and bone or joint fractures can be complicated by deep infections such as osteomyelitis. Amputations of fingers or limbs are common as a result of these complications. Abnormal deformity of the joints (neurogenic arthropathy) is often present and need surgical procedures. Life span is variable. Twenty percent of affected children succumbed to hyperpyrexia within the first 3 years of life [14]. Generally, toilet training is delayed and some CIPA children remain

incontinent of urine and feces [12,15].

Physical examination

The skin is warm and dry with thickening of the soles and the palms, and often there are scars and abrasions [15]. Distribution of hair on the scalp and body is normal. Palmoplantar hyperkeratosis with significant fissuring of the plantar skin is common. Some CIPA children develop painless deep heel ulcers that are slow to heal [16]. The tip of the tongue, lip edges, and the distal ends of fingers are often missing. Fungiform papillae are invariably observed on the tongue. Orthopedic deformities may be noted, especially at the elbow, knee, and ankle joints [3]. Microcephaly is reported occasionally [14].

Neurological examination

Insensitivity to superficial and deep painful stimuli is confirmed when painful stimuli fail to evoke either withdrawal or emotional change [1]. No tenderness or pain sensation is elicited even when apparently injured or broken joints are moved passively or actively. Temperature perception is impaired. Consistent errors are made in distinguishing between hot and cold moist substances. Extreme cold or heat fails to elicit the usual withdrawal response. Visceral pain perception is also impaired. Touch, vibration and position senses are normal, but ability to discriminate between sharp and dull stimuli may be disturbed.

Most children with CIPA are mentally retarded, but test results can be variable [14]. Children demonstrate defects in conceptual thinking, abstract reasoning, and social behavior and manifest symptoms of moderate to severe emotional disturbance [1, 12]. Behavior is characterized as labile, hyperactive, and erratic. They have a low frustration tolerance, resort to tantrums in an effort to gratify impulsive wishes, and avoid attempts to establish interpersonal relationships. Recurrent febrile convulsions are also observed and can be induced by high environmental temperature [17].

Peripheral motor and cranial nerve function are normal. Deep tendon reflexes are normal, as well as superficial abdominal and cremasteric reflexes; there are no pathological reflexes [12]. Sense of smell is assumed to be normal. Corneal reflexes are inconsistently present. Blinking and lacrimation are normal [14]. Although chronic conjunctivitis and corneal ulcerations are uncommon, they may occur as a result of some corneal hypoesthesia and develop into corneal opacities or active corneal ulcers [18]. These corneal ulcers are

characterized by very poor healing, and sometimes require surgical interventions.

Laboratory examination

Routine examinations of blood, urine, and cerebrospinal fluid are normal. Chromosome analysis in the majority of the patients is normal. Radiographic studies may show numerous fractures of the lower extremity long bones [3, 14]. The knees and ankles often show neurogenic arthropathy (Charcot joint). Bone fractures are slow healing, and may develop chronic inflammation resulting in systemic amyloidosis [19]. Some patients may show a multisystem involvement extending beyond the nervous system, suggesting immunological abnormalities.

Neurological laboratory studies such as EEG and CT scan are normal in the majority of the patients, but nonspecific dilatation of the third or the lateral cerebral ventricle has been observed in some patients [1, 20]. Electrophysiological studies reveal interesting findings. Conventional motor and orthodromic sensory conduction velocities by electrical and mechanical stimuli are usually normal. Similarly, somatosensory, visual and brainstem evoked potentials are normal. However, repeated trauma with severe neurogenic arthropathy can cause abnormalities of evoked sensory potential in the lower extremities [3]. Microneurography shows neural activity from A-beta sensory fibers connected to low-threshold mechanoreceptors, while nociceptive and skin sympathetic C fiber nerve activity is absent [21]. Moreover, intraneural electrical stimulation that produces unbearable pain in normal controls does not evoke any painful sensation. In a pure motor bundle, stretching of the muscle evokes intraneural activity from mechanoreceptor units. The sympathetic skin response is absent.

Special studies

Pharmacological tests and evaluation of the autonomic function are useful for clinical diagnosis as well as for elucidation of pathophysiology.

<u>Pain tests</u>

Painful stimuli that fail to evoke either withdrawal or emotional change include pin prick; vigorous pressure on the Achilles tendons, the testes, the stylomastoid processes, and the superior orbital rim; burning heat; immersion of the limbs in ice water; galvanic electrical

stimulation of skin; and prolonged ischemia of the limbs [1]. Visceral pain perception is impaired since no discomfort is elicited when a balloon is inflated in the lower esophagus. There is no discomfort during or after pneumoencephalograms, which often induce headaches. Strong electrical shocks, intramuscular injection of potassium chloride, and urinary catheterization fail to produce painful responses [15]. Stimulation of the tooth pulp did not produce evoked cortical sensory potentials in Swanson's surviving patient [22]. Interestingly, ultra-violet light does not produce erythema or tanning after prolonged exposure [15].

Histamine test

The axon reflex results from a stimulus applied to one branch of a nerve that induces an impulse that moves centrally to the point of division of the nerve (Fig. 1). The impulse is reflected down the other branch to the effector organ. Cutaneous pain fibers have branches reaching the arterioles and afferent (orthodromic) pain impulses pass antidromatically to the arterioles causing reflex vasodilation. The axon reflex is studied with an intradermal injection of histamine [1], which produces a wheal but no diffuse area of erythema that would correspond to a flare response. In individuals with CIPA and other HSANs, the injection and the post-injection period is not perceived as painful in contrast to control subjects who complain of burning pain or pruritis in the axon flare area.

Sweating tests

Sweat glands can be directly stimulated by intradermal injection of a cholinergic reagent and the sweat secretion visualized by the iodine-starch method [23]. In individuals with CIPA, neither pilocarpine nor methacholine (mecholyl) stimulate local sweating [12]. With warming, slight moisture of the intertriginous areas of the skin of the neck, axillae, and groin can occur, but no definite sweating is noted. The intertriginous moisture is probably caused by delayed evaporation of insensible water [1]. Emotional sweating responses are not observed by studying the electrical resistance of the palmar surfaces. Pinprick, deep pain, and startling sound fail to produce a change in skin resistance. Electrical stimulation and intradermal injection of norepinephrine also fail to evoke local sweating [1].

Autonomic nervous system

Studies of sympathetic vasomotor control are intriguing [1]. Valsalva maneuver produces a drop in blood pressure followed by a normal overshoot. There is no postural hypotension, as

tilting does not cause a blood pressure decrease. However, infusion of norepinephrine produces a rise in systolic pressure with erythematous blotching of the face and forehead, suggesting supersensitivity to exogenous vasopressor substance [12]. The cold pressor test, submersion of the forearm in ice-cold water, causes no increase in blood pressure. In fact, a slight fall in blood pressure during a cold-pressor test has been reported, suggesting a paradoxical response [1]. Carotid sinus massage, ocular pressure, and external auditory meatus mechanical stimulation do not alter heart rate [15].

Ophthalmologic examination often reveals mild bilateral ptosis [15]. The pupils are mildly miotic and equal. Ciliospinal (skin-pupillary) reflex is absent. A diagnosis of "congenital bilateral Horner's syndrome" was made in one patient, because of the combination of bilateral mild ptosis, bilateral anhidrosis, midposition pupils which failed to dilate in darkness but were briskly reactive to light, convergence and accommodation [15]. The same authors described pharmacological studies on the ocular autonomic nervous system. Epinephrine instilled into the conjunctival sac produced dilatation. Cocaine produced no response. Methacholine (Mecholyl) produced a miosis within one half hour in a dark room, but this response could not be seen in a lighted room. The pupillary response to adrenaline and cocaine is consistent with sympathetic denervation and, possibly, some degree of secondary end-organ hyposensitivity [15]. Subcutaneous administration of methacholine or neostigmine, in a dose capable of producing lacrimation in normal children, fails to do so in the CIPA children, despite their ability to lacrimate spontaneously [12].

Histopathological studies

Nerve biopsies

Electron microscopy studies of the cutaneous branch of the radial nerve reveal complete absence of small myelinated and unmyelinated fibers without degenerative or regenerative changes [3]. Reduction in the number of unmyelinated and small myelinated fibers of the sural nerve was found in most of the specimens from this disorder studied by electron microscopy and these results were confirmed by morphometric analysis [24-26]. The histogram of the control nerve is typically bimodal; the histogram of the CIPA individual's nerve is unimodal. Very few Schwann cells, apparently related to unmyelinated axons, are seen [3].

Skin biopsy

The pathology of the skin is normal in most cases of CIPA. Nonspecific changes are occasionally in the epidermis that are considered secondary to repeated trauma [3]. Dermal nerves are normal in number and in appearance by silver stain light microscopy. Sweat glands on skin biopsy appear normal [1, 12] and histologic studies also demonstrate normal sweat and sebaceous glands and normal hair follicles [15] as well as normal nerve receptors such as Pacinian and Meissner's corpuscles [20].

Electron microscopic examination, however, reveals lack of innervation of the eccrine sweat glands with loss of unmyelinated sudomotor fibers, which could explain the anhidrosis [3, 5]. Sweat glands are not atrophic but intensively PAS-positive, in contrast to controls where most glands are PAS-negative or weakly positive [25]. The lumens of sweat glands appear smaller than in controls. Nerve bundles in the dermis seem to be slightly reduced in number and size, compared with those of controls. Normal myelinated nerve fibers are present in the subcutaneous tissue and similar but smaller nerve fibers are seen in the dermis, but no nerve endings are demonstrated in the epidermis [17]. Furthermore, no cell processes or unmyelinated axons are seen near or around the sweat glands; eccrine sweat glands are surrounded by loose collagen fibers and granular material outside the basal lamina of epithelial and myoepithelial cells.

Recently, immunohistochemistry has demonstrated absent innervation of skin as well as of sweat glands. The lack of C and A-delta fibers in the skin is consistent with the loss of unmyelinated and small myelinated fibers in the sural nerve biopsy [21] and provides a morphological basis for insensitivity to pain and anhidrosis. The same study reports an almost complete absence of dermal fibers to blood vessels and erector pilomotor muscles [21].

Autopsy

Autopsy was performed in only one case [4]. The younger of two boys that Swanson had reported died suddenly at the age of 12 years after a 24-hr febrile illness during which his body temperature exceeded 43° C. Hemorrhages were noted in many tissues and attributed to the high fever. Normal autonomic ganglion cells were found in the intestinal tract and the skin had abundant sweat glands.

No obvious gross abnormalities were recognized in the brain or brainstem. In spite of the mild dilatation of the lateral ventricles previously demonstrated by pneumoencephalography [1], the ventricular system was normal post-mortem. On microscopic examination of the

brainstem, the spinal tract of the trigeminal nerve was about one third of normal. The majority of fibers in this tract were of large diameter, and there was a marked decrease in number of fine, lightly myelinated axons. Sections of the posterior ventral lateral nucleus of the thalamus and the somatosensory cortex were normal. No abnormalities were identified in the sympathetic ganglia or in the intermediolateral cell column of the spinal cord. The autonomic ganglia also appeared normal, but quantitative counts were not performed [1].

The spinal dura mater was thickened with a unique and unexplained abnormality consisting of two slit-like cavities, one dorsal and one ventral. Lissauer's tract (dorsolateral fasciculus) could not be identified in myelin or axon-stained sections at any level of the spinal cord. Cross sections of dorsal roots demonstrated a nearly uniform large fiber population with only a few scattered small fibers, in contrast to the normal picture of intermingled large and small myelinated fibers. In dorsal ganglia, an almost uniform population of large neurons replaced the normal pattern of large and small ganglion cells. The cells of the substantia gelatinosa and the anterior and posterior commissures were normal in number and morphology. The spinothalamic tracts could not be specifically identified, but the lateral and ventral columns of the spinal cord appeared normal.

In summary, the abnormalities were absence of small neurons in the dorsal ganglia, lack of small fibers in the dorsal roots, absence of Lissauer's tract, and reduction in size of the spinal tract of the trigeminal nerve with paucity of small fibers. These findings represent almost complete absence of the first order afferent system generally considered responsible for pain and temperature sensation [1].

Diagnosis

The diagnosis of CIPA is initially based upon clinical features of impaired senses of pain and temperature, the absence of sweating, the presence of disseminated bruises and scars, bone fractures and joint deformities which may lead to amputation, and mental retardation [14]. Supporting the diagnosis is the frequent history of bouts of fever in early life. Skin tests demonstrating abnormalities in sweating and the lack of the axon reflex provide further evidence for diagnosis. Until recently, skin and nerve biopsies were used to confirm diagnosis, but now molecular DNA diagnosis using peripheral blood samples is possible.

If a *TRKA* mutation responsible for CIPA is identified in a family, it is now technically possible to do prenatal genetic testing. Genetic counseling should be provided and consideration given to ethical issues when performing the procedure.

Treatment

No specific therapy is available at present but supportive therapy is important. Clinically, CIPA is a serious illness that may be fatal in the first few years of life, if hyperpyrexia is not properly corrected. In older children, osteomyelitis and bone and/or joint deformities demand surgical procedures and may even require amputations. Special training programs to prevent self-mutilation and accidental injuries are necessary but may be hampered by the low level of intelligence of these patients [14].

Nerve growth factor and receptor tyrosine kinase, TRKA

Neurotrophins and neurotrophic theory

The development and maintenance of cellular communication networks within the central and peripheral nervous system are regulated by neurotrophic factors, referred to as neurotrophins. Neurotrophins bind and activate specific cell surface receptors, generating differentiation and survival signals in various neurons. Each of the neurotrophins described to date - nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) - exerts similar functional effects, but on different neuronal populations [27, 28].

The neurotrophic hypothesis proposes that immature neurons compete for target-derived trophic factors that are of limited supply; only those neurons that are successful in establishing correct synaptic connections would obtain trophic factor support to allow for survival. Neurotrophic factors function as survival signals and suppress a cellular suicide program (apoptosis); deprivation of those factors activates cell death [6].

NGF was the first growth factor to be identified and characterized [7] and it is the prototypic neurotrophin. NGF supports the survival of sympathetic ganglion neurons and nociceptive sensory neurons in dorsal root ganglia, derived from the neural crest, and ascending cholinergic neurons of the basal forebrain [7]. For over 50 years since its initial discovery, NGF has occupied a central position in developmental neurobiology [29-31]. There are two unique features of NGF's action on neurons as opposed to those of growth factors on other cell types [29, 32]. First, NGF regulates functions of differentiated neurons,

i.e., growth as opposed to proliferation. Second, NGF is synthesized at a considerable distance from the cell body by peripheral tissues or other neurons (referred to as targets) that are contacted by axons of the NGF-sensitive neurons. The secreted NGF is thought to have local effects on axons of innervating neurons as well as more general influences on gene expression after it reaches the cell body via the process of retrograde transport. Most investigators have favored the view that target-derived NGF matches the number and properties of innervating neurons to the needs of the target tissue [30-32].

Structure and biochemistry of NGF

All neurotrophins have similar biochemical characteristics. They are secretary proteins that are synthesized as precursor proteins. The mature (or processed) part of all neurotrophins show a high degree of sequence homology, and approximately 50% of the amino acids are common to all neurotrophins, although the precursor sequences diverge significantly.

The mature active form of NGF in the mouse submandibular gland, the source for most studies, consists of a tightly associated dimer of two identical 118-amino acid polypeptide chains [33, 34]. Human mature NGF consists of 120–amino acids [35]; it contains two more residues than the mouse protein because the carboxy-terminal dipeptide is not excised [33].

Discovery of receptors for NGF

Early studies revealed that NGF receptors on various neuronal populations and on tumor cell lines were heterogeneous with regard to binding affinity and binding kinetics; the distinct receptor populations were referred as low and high affinity or as kinetically fast and slow [28]

<u>p75 neurotrophin receptor</u>

The first receptor to be cloned was the low-affinity receptor, p75 neurotrophin receptor [36-38]. This receptor binds all neurotrophins with similar affinity but its functional role and signaling capacity has remained elusive. The p75 neurotrophin receptor is the founding member of the TNF receptor superfamily [39]. NGF binding to p75 neurotrophin receptor results in the hydrolysis of sphingomyelin to ceramide [40]. The similarity in the intracellular domain of the p75 neurotrophin receptor with other members, such as the Fas antigen and the p55 TNF receptor, suggests that the p75 neurotrophin receptor might also function as a cell

death molecule.

TRKA , receptor tyrosine kinase for NGF

The TRK family of receptor tyrosine kinases was first identified in 1986 when the *trk* gene (*TRKA*) was found to be part of an oncogene isolated from a human colon carcinoma [41, 42]. The physiological role of TRKA protein was unveiled when it was shown to be the signaling receptor for NGF [8,9]. Other members of the TRK family include TRKB, the signaling receptor for BDNF and NT-4; and TRKC, the primary receptor for NT-3 [27]. NT-3 also binds to TRKA and TRKB, but with significantly lower affinity.

TRKA is phosphorylated in response to NGF and is essential for intracellular signal transduction [43]. The binding of NGF to TRKA stimulates homodimer formation and activation of tyrosine kinase activity (Fig. 2). A single transmembrane domain divides the TRKA protein into extracellular and intracellular domains [27, 44]. The extracellular domain is important for specific NGF binding and includes a signal peptide, three tandem leucine-rich motifs flanked by two cysteine clusters, and two immunoglobulin-like domains (or motifs). The intracellular domain, including a juxtamembrane region, a tyrosine kinase domain, and a very short carboxy-terminal tail, is phosphorylated in response to NGF and is critical for intracellular signaling. Phosphorylated tyrosine residues in the TRKA cytoplasmic domain serve as anchors for binding downstream signaling molecules [27, 28]. *In vitro* assessment has identified TRKA tyrosine residues - 490, 670, 674, 675 and 785 as autophosphorylation sites [45].

***Gene Knockout Mice of* NGF, p75 *Neurotrophin Receptor and* TRKA**

Mice lacking *NGF* or its receptors have remarkable neurological deficits.

NGF knockout mice

To study the effects of NGF depletion on both central and peripheral neurons, the *NGF* gene was disrupted, using homozygous recombination in embryonic stem cells [46]. Mice heterozygous for *NGF* gene disruption grow and breed normally. Mice that are homozygous for the disrupted allele display severe perinatal cell loss in the dorsal root and sympathetic ganglia, yet develop basal forebrain cholinergic neurons that survive for the life span of the mice. Animals homozygous for *NGF* disruption failed to respond to noxious mechanical stimuli. Histological analysis revealed profound cell loss in both sensory and sympathetic ganglia but examination of the central nervous system revealed that basal forebrain

cholinergic neurons differentiated and continued to express phenotypic markers for the life span of the null mutant mice. These observations confirm the critical dependence of peripheral sensory and sympathetic neurons on NGF but demonstrated that differentiation and initial survival of central NGF-responsive neurons can occur in the absence of NGF [46].

p75 neurotrophin receptor gene knockout mice

Mice with a target mutation of the *p75* neurotrophin receptor gene exhibit a substantial loss of neurons within sensory ganglia and a deficit of nociceptive function [47]. Mice homozygous for the mutation were viable and fertile. The mutation did not decrease the size of sympathetic ganglia or the density of sympathetic innervation of the iris or salivary gland. Subsequent studies, however, have revealed that there are substantial sympathetic abnormalities in these mice [48]. Pineal glands lacked innervation and sweat gland innervation was absent or reduced in particular footpads. The phenotype of homozygous mice is somewhat reminiscent of a partial NGF deprivation [47-49].

TRKA (trkA) knockout mice

Mice carrying a deletion in the tyrosine-kinase domain of the *trkA* gene have been generated by homologous recombination in embryonic stem cell [10, 27]. Mice lacking *trkA* have severe sensory and sympathetic neuropathies and most die within one month of birth. Animals homozygous for this mutation appear anatomically normal at birth and feed normally. However, by postnatal day 10 (P10), the *trkA* (-/-) mice are significantly smaller in size as compared to their (+/+) and (+/-) siblings and display a wide array of sensory defects. For instance, these mice do not react to noxious olfactory stimuli, such as ammonium hydroxide, and fail to react to deep pinpricks in either their whisker pads or their rear paws, indicating defects in their trigeminal and peripheral sensory neurons, respectively. In addition, these animals exhibit deficiencies in thermoception.

By P20, surviving animals have miotic pupils and slight ptosis; when their eyes are illuminated, the iris constricts without efficient redilation, indicating that the parasympathetic component of the oculomotor nerve is intact but the sympathetic innervation from the superior cervical ganglion (SCG) is defective. In contrast, they respond normally to sound (handclap) and can discriminate 1% acetic acid from water, indicating that facial (VII), acoustic (VIII) and glossopharyngeal (IX) nerves function correctly. Basic motor functions also appear to be normal. But anhidrosis (inability to sweat) is not apparent [10]

After 3 to 4 weeks, mutant mice develop a distinct pathology. Their fur becomes mottled

and numerous scabs appear over the entire body; their paws are covered by ulcerations and digits are missing, probably due to self-mutilation; their eyes exhibit corneal opacities, indicating there may be a sensory defect affecting the blink response.

Neuroanatomical examination of the peripheral nervous system of these *trkA* (-/-) mice reveals extensive (>70%) neuronal cell loss in trigeminal, dorsal root, and sympathetic ganglia. In the dorsal root ganglia, the vast majority of the missing neurons correspond to those of small size, a population believed to be NGF dependent. At birth, the SCG of *trkA* (-/-) mice displays a significant loss of neurons, as well as large numbers of pyknotic nuclei, suggesting an active process of cell degeneration. At P10, the SCG is severely shrunken, and only a few principal sympathetic neurons can be found [10]. Disruption of the *trkA* gene does not appear to cause the loss of cholinergic neurons in the caudatoputamen and in the basal forebrain complex, two brain structures that have been previously shown to be targets for NGF [7,10]. However, adult *trkA* (-/-) mice exhibit a severe decrease in the cholinergic fibers that project from the medial septum to the hippocampus and from the nucleus basalis to the cerebral cortex. Whether TRKA receptors are required for the outgrowth of these cholinergic fibers or for maintenance of their cholinergic phenotype remains to be determined.

Comparison of CIPA with *trkA* Knockout Mice

Phenotypic features of CIPA and *trkA* knockout mice are compared in Table 1.

Anatomical changes in the DRG neurons and spinal cord in *trkA* knockout mice are similar to those noted in CIPA [10]. These include absence of small neurons in the DRG, lack of small fibers in the dorsal roots, absence of Lissauer's tract and reduction of the spinal tract of the trigeminal nerve [4]. Miotic pupils and slight ptosis associated with loss of neurons in the SCG are the features in these mice, suggesting a defect in sympathetic innervation. These findings are also reported in some patients with CIPA [15]. The autonomic ganglia and brain appeared normal in a patient with CIPA [4]. Why CIPA patients with the same apparent gene disruption do not show a deficiency in sympathetic ganglia may be due to species differences or, alternatively, sympathetic innervation in humans might be maintained or compensated for by a combination of other neurotrophin and receptor systems.

TRKA is the responsible gene for CIPA

Based on the intriguing data in animal models, we tested the hypothesis that NGF signaling might be involved in patients with CIPA. At first, we studied the *p75* neurotrophin receptor

gene in a patient with CIPA because only its knockout mice were reported at that time [47]. Then, we examined the *NGF* gene itself, but we could not detect a putative mutation in either gene. Next, we analyzed the *TRKA* gene in three unrelated CIPA patients who had consanguineous parents. Three mutations in the *TRKA* – a deletion, a splicing and a missense mutation - were detected in these three families [11], which identified the genetic basis of CIPA as mutations in the *TRKA* gene. We then developed a comprehensive strategy to screen for the *TRKA* gene mutations [16, 50] and we characterized intragenic polymorphic sites and described the haplotypic association of mutant alleles [51]. We also studied effects of *TRKA* missense mutations on autophosphorylation of the RTK for NGF.

Human *TRKA* maps to chromosome 1q21-q22 [52] and is divided into 17 exons and 16 introns [42,50, 53]. The entire sequence was estimated to span at least 23 kb, coding for a protein of 790 or 796 amino acid residues (Fig. 3). Six amino acid residues encoded by exon 9 are in the extracellular domain of the neuronal-specific TRKA receptor [54]. The two isoforms differ from each other in the presence of 6 amino acid residues (VSFSPV) located in the extracellular domain near the transmembrane region. These additional sequences do not affect NGF binding. Moreover, these isoforms appear to have similar biological properties. However, the 796 amino acid long TRKA molecule is primarily expressed in neuronal cells, whereas the 790 amino acid long isoform has been found in cells of nonneuronal origin [54].

Mutations and polymorphisms

We identified the genetic basis for CIPA when we detected loss-of-function mutations in the *TRKA* gene derived from affected patients [11]. Recently, we developed a strategy to screen for *TRKA* mutations, on the basis of the structure and organization of the gene [16, 50] and we characterized intragenic polymorphic sites [51].

We have analyzed 32 CIPA families and detected a total of 25 mutations, including six frameshift, four nonsense, 11 missense and four splice mutations (Fig. 3). Three of these missense mutations are probably polymorphisms, as described later. There is a common founder mutation in the Japanese. In addition to these mutations, we have recently reported eight novel mutations [55]. Other investigators have also detected and reported *TRKA* mutations in CIPA patients [56-59]. Subsequently, a total of 37 different mutations including nine frameshift, seven nonsense, seven splice, and 14 missense mutations have been detected in CIPA families from various countries [60]. The mutations are located both in the extracellular and intracellular domains. Most are private mutations, but relatively common

mutations have been reported for particular ethnic groups such as Japanese and Israeli-Bedouins [51, 58]. At least eight missense mutations have been confirmed to cause defects in NGF-stimulated autophosphorylation of the TRKA protein by *in vitro* expression studies [61, 62]. The effect of other missense mutations remains to be determined.

A number of polymorphisms have been reported [60]. Six non-synonymous amino acid substitutions have been described. Three, R85S, H598Y and G607V, were detected as double and triple mutations [16]. But these three are probably polymorphisms in a particular ethnic background according to an *in vitro* expression study [62] and various mutation and polymorphism searches [56, 58, 60]. The effect of other non-synonymous amino acid substitutions (G18E, G608V and R774Q) remains to be determined.

Application of intragenic polymorphic sites for detection of unexpected mutations

The IVS7-33T>A mutation is located upstream of the consensus splice acceptor site and was detected in five chromosomes from five Japanese patients [51]. Initial screening for *TRKA* mutations failed to identify this mutation. Analyses of intragenic polymorphic sites indicated that this mutation is linked to a very rare haplotype. Re-examination of DNA from patients revealed that the intronic mutation is involved in a putative branch-site critical for intron excision. Subsequently, the exon trap analysis showed that this causes an aberrant splicing *in vitro.* Thus, analysis of intragenic polymorphic sites can aid in identifying an unexpected mutation that can often escape detection.

Functional studies of* TRKA *mutants

Loss of TRKA function is self-evident in the frameshift, splice-site or nonsense mutations. In contrast, putative missense mutations require an expression study for confirmation as a cause of CIPA. Such studies provide an opportunity to consider the structure-function relationship of the TRKA protein as well as findings in other receptor tyrosine kinase families. We reported 11 putative missense mutations in CIPA families from various ethnic groups. We have introduced the corresponding mutations into the *TRKA* cDNA and examined NGF-stimulated autophosphorylation (Fig. 4). Two mutants (L93P and L213P) in the extracellular domain were aberrantly processed and showed diminished autophosphorylation [62]. Five mutants (G516R, G571R, R643W, R648C and G708S) in the tyrosine kinase domain were processed as wild-type TRKA but showed significantly diminished

autophosphorylation [62]. The G571R mutant was also studied by another group [61]. Another mutant (R774P) in the tyrosine kinase domain also showed significantly diminished autophosphorylation [57]. To date, eight of 14 putative missense mutations were confirmed to show diminished NGF-stimulated autophosphorylation [60]. Mutated residues (except for residue Arg-774) in the tyrosine kinase domain are conserved in various RTKs and probably contribute to critical functions of these proteins. Thus, naturally occurring *TRKA* missense mutations with loss of function provide considerable insight into the structure-function relationship in the RTK family.

Genotype/phenotype relationship

The genotype/phenotype correlation in the patients was not observed in a study of limited number of subjects [16, 51, 55]. The accumulated data favor the notion that there seems to be no genetic heterogeneity in CIPA. However, one cannot completely rule out the possibility that mutation(s) in other gene(s) are responsible for clinical phenotypes similar to CIPA [58].

TRKA mutations are detected in CIPA patients from various countries and most of them are private mutations [60]. But a relatively common mutation (R548fs) was noted in the Japanese [51]. Mutation analysis of *TRKA* indicated that more than 50% of 46 Japanese CIPA chromosomes share the same frameshift mutation. Haplotype analysis of the *TRKA* gene, based on intragenic polymorphic sites, showed that this mutation shows linkage disequilibrium with a rare haplotype in normal chromosomes. These findings strongly suggest that this mutation is a common founder mutation in the Japanese population. Another relatively common mutation (P615fs) was found in 16 of 19 unrelated CIPA families from Israeli-Bedouin people [58].

Conclusion and perspective

The molecular pathology of CIPA provides unique opportunities to explore the critical role of the NGF-TRKA system in development and function of autonomic sympathetic nervous systems as well as sensory nervous systems that transmit noxious stimuli. Both *NGF* and *TRKA (trkA)* knockout mice resulted in dramatic phenotypes, but because the mice do not survive beyond one month of life behavioral studies are not feasible, whereas patients with CIPA often survive into adulthood if they receive careful treatment. Thus, CIPA can serve as

a useful human model to determine mechanisms of development and maintenance of NGF-dependent neurons in autonomic, sensory and central nervous systems, as well as the physiology of these neurons. Behavioral studies of CIPA patients may reveal critical roles of sympathetic and afferent neurons in the human physiology.

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FIGURES

Fig. 1 Neurotransmitter mediators of the axon reflex.

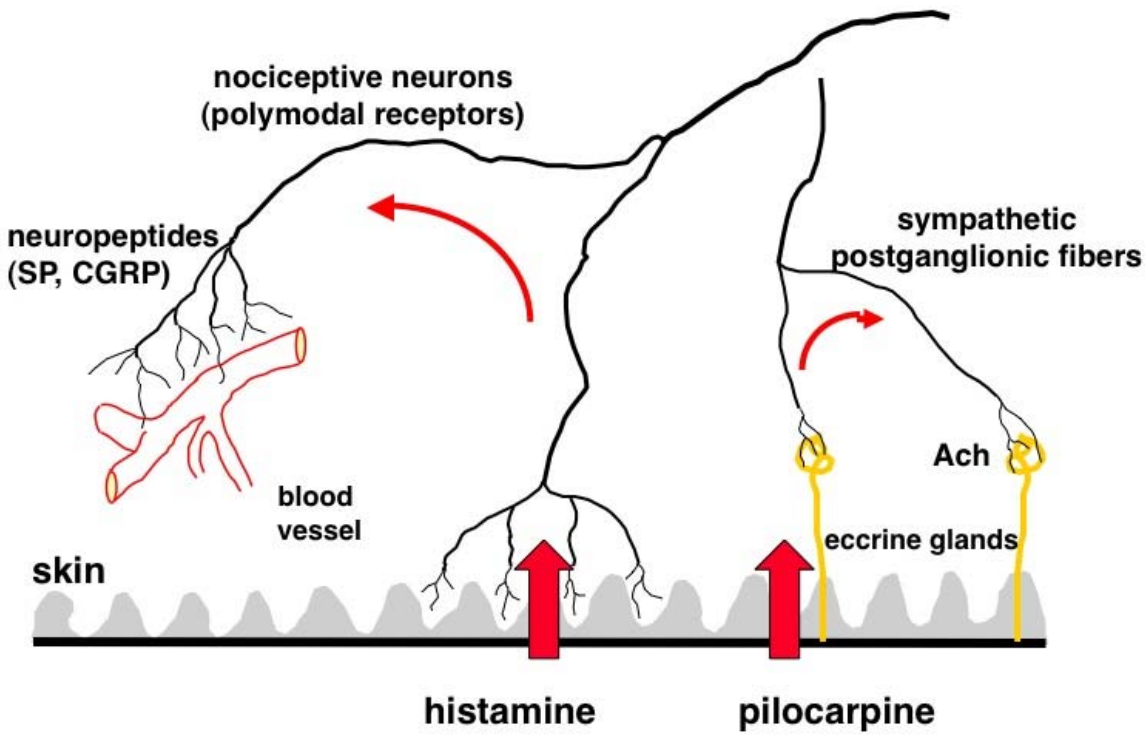

Fig. 1 Neurotransmitter mediators of the axon reflex.

Chemical mediators can sensitize and activate nociceptive neurons (or polymodal receptors) and stimulate the axon reflex. Activation of these receptors and neurons leads to the release of substance P and CGRP. Histamine, a chemical mediator and pilocarpine, a cholinergic agent probably can cause the axon reflex flare and axon reflex sweating as shown on left and right sides, respectively. Substance P produces plasma extravasation and CGRP produces dilatation of peripheral blood vessels.

(*SP* substance P, *CGRP* calcitonin gene related peptide, *Ach* acetylcholine).

Fig. 2 The binding of nerve growth factor (NGF) to the NGF/TRKA receptor.

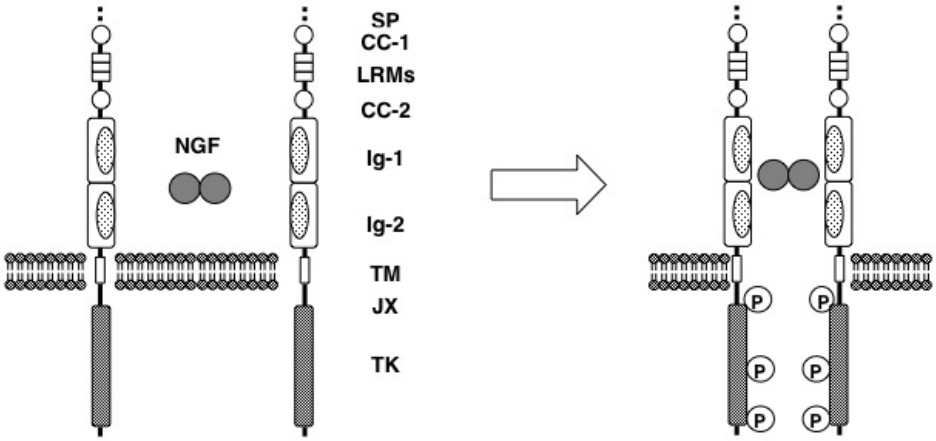

Fig. 2 The binding of nerve growth factor (NGF) to the NGF/TRKA receptor.
The binding of the tightly associated NGF dimer to TRKA protein stimulates homodimer formation [43]. TRKA is phosphorylated in response to NGF, which is essential for intracellular signal transduction. The domain structure of TRKA protein with 790 or 796 amino acids are shown [44]: *SP* signal peptide; *CC-1* and *CC-2* the first and second cysteine clusters, respectively; *LRMs* leucine-rich motifs; *Ig-1* and *Ig-2* the first and second immunoglobulin-like motifs, respectively; *TM* transmembrane; *JX* juxtamembrane; *TK* tyrosine kinase. A single transmembrane domain divides the TRKA protein into an extracellular and an intracellular domain. The extracellular domain is important for specific NGF binding. The intracellular domain includes a tyrosine kinase domain. Phosphorylated tyrosine residues –490, 670, 674, 675 and 785 in the TRKA cytoplasmic domain serve as anchor for binding downstream signaling molecules [45].

Fig. 3 Location of human *TRKA* mutations associated with congenital insensitivity to pain with anhidrosis (CIPA).

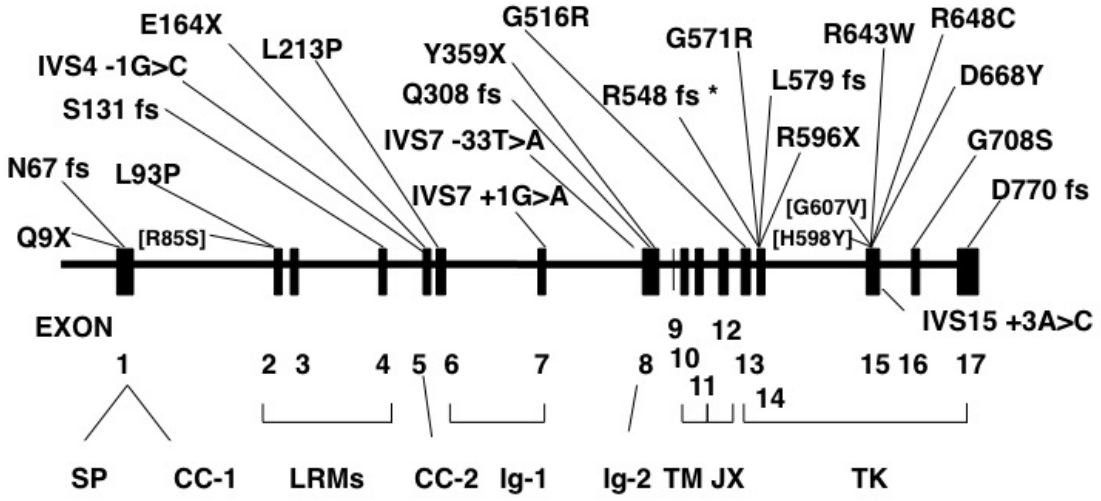

Fig. 3 Location of human *TRKA* mutations associated with congenital insensitivity to pain with anhidrosis (CIPA).

The human *TRKA* gene, located on chromosome 1 (1q21-q22), is divided into 17 exons and 16 introns [50]. The entire sequence was estimated to span at least 23 kb, coding for a protein of 790 or 796 amino acid residues. Abbreviations on the bottom row indicate the domain structures encoded by the corresponding exon(s) [44, 50]. Multiple mutations have been described.

An asterisk denotes the common Japanese founder mutation (G548fs). In addition to these mutations, we have recently reported eight novel mutations [55]. Three mutations in brackets are probably polymorphisms in a particular ethnic background, as described in text.

Fig. 4　Expression study of mutant *TRKA* cDNAs and autophosphorylation of their products.

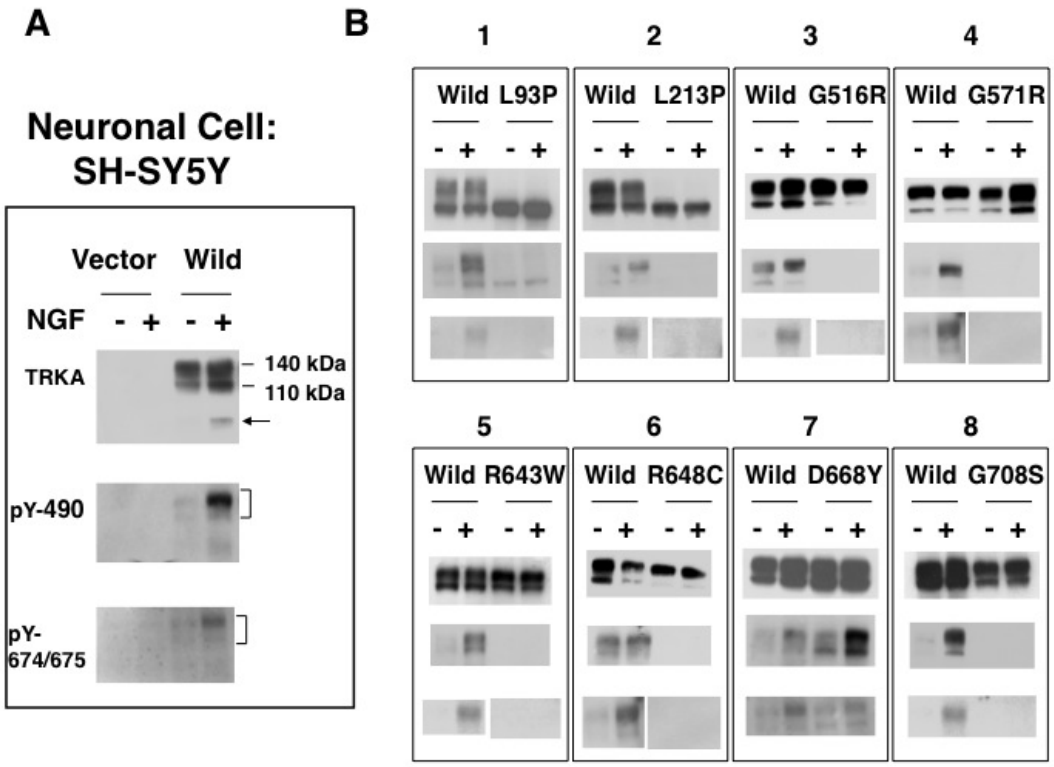

Fig. 4　Expression study of mutant *TRKA* cDNAs and autophosphorylaton of their products.

A. Expression of the wild-type *TRKA* cDNA and processing and autophosphorylation of its product in a neuronal cell line: SH-SY5Y.

The full-length cDNA for human TRKA receptor was subcloned into a mammalian expression vector (pCAGGS). The *TRKA* expression plasmid was transfected into cells. Each cell transfected with the plasmid pCAGGS vector, without the *TRKA* insert, was used as a mock-treated control. Cells were incubated in the presence (+) or absence (-) of nerve growth factor (NGF) and were harvested for detection of the total or phosphorylated TRKA proteins by immunoblotting. An antibody recognizes the C-terminal TRKA protein (TRKA: upper panel). Two phospho-specific antibodies recognize the phosphorylated Tyr-490 residue (pY-490: middle panel) and Tyr-674/675 residues (pY-674/675: lower panel) of TRKA protein. The TRKA precursor protein was detected and processed to a 140 kDa- and a 110 kDa-forms by glycosylation. An 80 kDa-form (arrow) is probably the unglycosylated

polypeptidic backbone of these proteins. The 140 kDa-form was strongly phosphorylated in response to NGF.

B. *TRKA* cDNAs harboring putative eight missense mutations.

TRKA cDNAs were prepared, using in vitro site-directed mutagenesis [62]. Each specific mutagenesis oligonucleotide was complementary to the wild-type *TRKA* cDNA, except for a region of mismatch. The mutant and the wild-type *TRKA* cDNAs were transfected into SH-SY5Y cells. Autophosphorylation of the TRKA protein was detected after stimulation with NGF. In the neuronal cell, wild-type TRKA precursor protein was processed into the 110 kDa and mature 140 kDa forms and the latter only phosphorylated in an NGF-dependent manner. Two mutants (L93P and L213P) in the extracellular domain were aberrantly processed and showed diminished autophosphorylation (Lanes 1, 2). Five mutants (G516R, G571R, R643W, R648C and G708S) in the tyrosine kinase domain were processed as wild-type TRKA but showed significantly diminished autophosphorylation (Lanes 3, 4, 5, 6, 8). One putative mutant D668Y might be a rare polymorphism or might impair the function of TRKA without compromising autophosphorylation (Lane 7). In contrast, the other three (R85S, H598Y and G607V) we detected as double or triple mutations are probably polymorphisms in a particular ethnic background (data not shown). These data are reproduced from our previous report [62].

Table 1 Phenotypes of congenital insensitivity to pain with anhidrosis (CIPA) and *TRKA(trkA)* knockout mice

Phenotype	CIPA	mice
Perinatal:		
- Abnormal birth	-	-
- Failure to thrive	-	+
- Early death	- *	+ (die within a month)
Sensory system:		
- Abnormal pain/temperature sensation	+	+
- Injury or ulcer of digits	+	+
- Self-mutilation	+	+
- Abnormal touch/position sensation	-	-
- Abnormal peripheral small nerve fibers	+	+
- Abnormal dorsal root ganglia	+	+
Sympathetic system:		
- Abnormal sweating	+	?
- Abnormal piloerection	+	?
- Miosis/ptosos	+	+
- Abnormal innervation of sweat glands	+	?
- Abnormal sympathetic ganglia	?	+
Central nervous system:		
- Mental retardation	+	?
- Abnormal basal forebrain	?	+

Symbols, + and - indicate the presence and absence of finding, respectively.
? indicates non-confirmed finding
* Early death can occur in CIPA due to hyperpyrexia.