The tyrosine kinase receptor Tyro3 enhances lifespan and neuropeptide Y (Npy) neuron survival in the mouse anorexia (anx) mutation

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ABSTRACT

Severe appetite and weight loss define the eating disorder anorexia nervosa, and can also accompany the progression of some neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS). Although acute loss of hypothalamic neurons that produce appetite-stimulating neuropeptide Y (Npy) and agouti-related peptide (Agrp) in adult mice or in mice homozygous for the anorexia (anx) mutation causes aphagia, our understanding of the factors that help maintain appetite regulatory circuitry is limited. Here we identify a mutation (C19T) that converts an arginine to a tyrosine in the TYRO3 protein tyrosine kinase 3 (Tyro3) gene, which resides within the anx critical interval, as contributing to the severity of anx phenotypes. Our observation that, like Tyro3+/−/− mice, anx/anx mice exhibit abnormal secondary platelet aggregation suggested that the C19T Tyro3 variant might have functional consequences. Tyro3 is expressed in the hypothalamus and other brain regions affected by the anx mutation, and its mRNA localization appeared abnormal in anx/anx brains by postnatal day 19 (P19). The presence of wild-type Tyro3 transgenes, but not an R7W-Tyro3 transgene, doubled the weight and lifespans of anx/anx mice and near-normal numbers of hypothalamic Npy-expressing neurons were present in Tyro3-transgenic anx/anx mice at P19. Although no differences in R7W-Tyro3 signal sequence function or protein localization were discernible in vitro, distribution of R7W-Tyro3 protein differed from that of Tyro3 protein in the cerebellum of transgenic wild-type mice. Thus, R7W-Tyro3 protein localization deficits are only detectable in vivo. Further analyses revealed that the C19T Tyro3 mutation is present in a few other mouse strains, and hence is not the causative anx mutation, but rather an anx modifier. Our work shows that Tyro3 has prosurvival roles in the appetite regulatory circuitry and could also provide useful insights towards the development of interventions targeting detrimental weight loss.

INTRODUCTION

Detrimental appetite and weight loss are the defining features of the restrictive subtype of the eating disorder anorexia nervosa (ANR) (Kaye et al., 1998, 2000; Walsh and Devlin, 1998), and also often accompany a range of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) (Ahmed et al., 2016), Alzheimer’s disease (AD) (Ringman et al., 2015; Burke et al., 2016; Sergi et al., 2013) and Huntington’s disease (HD) (Petersén et al., 2005; Gabery et al., 2010). Eating disorders have the highest mortality rate of any mental illness (Sullivan, 1995). Moreover, appetite and weight loss are linked to poor mood and quality of life in ALS patients and can be major issues for many during late disease progression. In spite of their impact, the neurobiological systems that link hypothalamus to peripheral energy levels and regulate food intake (reviewed in Stemson, 2013; Morton et al., 2006). Within the hypothalamus, orexigenic neurons co-express and release neuropeptide Y (Npy) (Allen et al., 1983; Chronwall et al., 1985; de Quind et al. and Emson, 1986; Hahn et al., 1998) and agouti-related protein (AgRP) (Hahn et al., 1998; Shutter et al., 1997) to promote feeding by inhibiting anorexic neurons expressing pro-opiomelanocortin (Pome) (Biloc et al., 1978; Watson et al., 1978) and cocaine- and amphetamine-regulated transcript (Cart, also known as Cartpt) (Elia et al., 1998; Kristensen et al., 1998). Serotonin can also act directly on 5HT2c receptors present in hypothalamic orexigenic neurons and 5HT1b receptors in anorexic neurons (Garfield and Heisler, 2009; Heisler et al., 2002, 2006; Lam et al., 2008; Xu et al., 2008). In the adult mouse, acute ablation of arcuate nucleus AgRP neurons leads to starvation (Luquet et al., 2005; Xu et al., 2005; Xu et al., 2005; Wu et al., 2008). AgRP neurons also project long-range axons to other brain regions, including the parabrachial nucleus (PBN), which can augment or suppress AgRP neuron actions. Further analyses in the mouse have shown that the severe aphagia caused by acute adult AgRP neuron loss can be reversed by eliminating glutamatergic excitatory drive or increasing GABAergic inhibition from the PBN or by inhibiting serotonergic activation of the nucleus of the solitary tract, which innervates and activates the PBN (Wu et al., 2012). Thus, in brief, loss of hypothalamic NPY/AGRP neurons, increased glutamatergic signaling from the PBN or serotonergic hyperinnervation of the...
nucleus tractus solitarius (NTS) could individually or together promote pathological appetite and weight loss.

Understanding how appetite regulatory circuits are involved or have been compromised in individual ANR cases, let alone in ANR populations, is largely unknown. Because prior to their diagnosis ANR patients have already been undernourished for an extended period of time, it is difficult to distinguish between the neurobiological causes and effects of ANR. Furthermore, imaging deeper brain regions such as the hypothalamus can be more challenging (Val-Laillet et al., 2015). Nonetheless, data concerning appetite regulating peptide levels in ANR patients have been obtained by assessing cerebrospinal fluid and plasma levels, and persistent changes in appetite regulating neuropeptides, such as Npy (Connan et al., 2007; Lob et al., 2003; Bronsky et al., 2011; Stöving et al., 2009; Gendall et al., 1999; Kaye et al., 1990) and in the serotonin (Baier et al., 2005; Galusca et al., 2008) neurotransmitter system have been found a long time after weight restoration in restrictive anorexia nervosa. With respect to neurodegenerative disorders, reduced expression of the orexigenic Npy and its receptor has been associated with Alzheimer’s disease and other neurodegenerative disorders (Spencer et al., 2016; Davies et al., 1990; Minthon et al., 1990, 1996; Martel et al., 1990; Koide et al., 1995; Nilsson et al., 2001; Alom et al., 1990; Martignoni et al., 1992). Thus, in both ANR and neurodegenerative disorders the appetite regulatory system is detectably impacted, albeit in an as-yet poorly defined manner.

Just as in severe ANR cases, mice homozygous for the recessive mouse anorexia (anx/anx) mutation restrict their eating and become severely emaciated (Maltais et al., 1984). Thus, genetic analyses of the anx mutation might identify factors important for the maintenance of appetite regulatory circuitry and could provide insights towards the development of interventions targeting detrimental weight loss. In anx/anx mice, hypothalamic Npy-expressing (Npy+) neurons begin to degenerate at postnatal day (P)10-12 (Nilsson et al., 2011). By P19-22 most Npy+ processes and soma have disappeared, and anx/anx mice have become severely emaciated and perish (Maltais et al., 1984). In addition, 5-HT immunoreactive fibers innervate their target areas abnormally in anx/anx mice (Huynh et al., 2011; Son et al., 1994), and their body tremors, head weaving and hyperactivity can be improved by administration of 5-HT antagonists (Maltais et al., 1984). In mice lacking both Axl and Tyro3 the migration and survival of gonadotrophin release hormone (GnRH)-expressing neurons into the hypothalamus is compromised (Pierce et al., 2008). Moreover, loss of Tyro3 accelerates retinal degeneration in Mer−/− mice (Vollrath et al., 2015). Triple Tyro3/Axl/Mer mutants exhibit cellular degeneration in the neocortex, hippocampus, cerebellum, and of rods and cones in the retina (reviewed in Ji et al., 2015). These studies illustrate the redundant reinforcing neuroprotective roles of TAM receptors, and open the door to the possibility that Tyro3 on its own might have significant neuroprotective potential.

Here, we identify a point mutation (C19T) in the signal sequence of Tyro3 (R7W-Tyro3), and detect a defect in secondary platelet aggregation in anx/anx mice that resembles one previously reported in mice lacking Tyro3. We show that Tyro3 is expressed in brain regions known to be affected by the anx mutation: the hypothalamic arcuate nucleus, the dentate gyros and serotoninergic neurons, and detect anomalies in RNA localization in anx/anx brains at P19. In the presence of wild-type Tyro3 transgenes, but not of a C19T mutated Tyro3 transgene (referred to here as R7W-Tyro3), the weight and lifespans of anx/anx mice was doubled relative to that of their non-transgenic anx/anx littermates and hypothalamic Npy+ neuron degeneration was delayed. Finally, we show that in the cerebella of transgenic mice, R7W-Tyro3 protein appeared mislocalized. Additional sequencing analyses revealed that the R7W-Tyro3 variant is present in AKR/J, FVB/NJ and NZW/lacZ mice, and thus R7W-Tyro3 is not the primary anx causative event, but rather is a strain-specific modifier of anx phenotypes. Taken together, these studies identify Tyro3 as having important neuroprotective roles in the appetite regulatory circuitry.

RESULTS

A point mutation in the signal sequence of Tyro3 is associated with the anx phenotype

The Tyro3 gene is located within the anx critical interval, which we had refined to a 3.5 Mb region between D2Mit484 and D2Mit3 by using simple sequence length polymorphisms (SSLPs) to analyze 335 progeny (670 meioses) from heterozygous intercrosses on C57BL/6J (n=111) and Mus molossinus/Ei (MOLF/EiJ) (n=224) strain backgrounds (Fig. S1). Upon sequencing the exons and flanking intronic regions of Tyro3, we identified a cytosine-to-
thymine (C19T) mutation in the signal sequence of Tyro3 (Fig. 1A). This mutation converts an arginine to a tryptophan at the seventh amino acid position, and also eliminates an NlaIV restriction site (Fig. 1B), which we used in a PCR-based assay to genotype 995 additional animals. All 176 affected progeny were homozygous for the R7W-Tyro3 mutation, whereas 819 unaffected animals were R7W-Tyro3/+ or +/+ (Fig. 1C). This sequence variant was absent in seven other inbred strains (C57BL/6J, C3H/HeJ, Balb/cJ, MolEi/J, CastEi/J, A/J, 129Sv/J) analyzed at the time. We identified two additional single nucleotide polymorphisms that led to non-synonymous changes (SNP rs47863852: Val811Leu and SNP rs13459232: Gly824Ser, ENSEMBL build GRCM38), but these were present in Tyro3 from some of the other strains sequenced here. Thus, we decided to test whether the R7W-Tyro3 variant might affect anx phenotypes.

**anx/anx mice exhibit platelet deficits similar to those reported in Tyro3 nulls**

As a first step to test whether the R7W-Tyro3 variant might have functional biological consequences, we tested whether anx/anx mice exhibit deficits in platelet aggregation, as had been observed in Tyro3°/° mice (Angelillo-Scherrer et al., 2005; Gould et al., 2005). When we examined platelet aggregation in anx/anx, anx/+ and +/+ mice, we found that, upon activation with thrombin, P-selectin levels on the platelet surface were significantly reduced in anx/anx platelets (Fig. 2A-D). In platelets lacking Tyro3, Axl or Mer, reductions in P-selectin levels were observed upon ADP stimulation. The effect of thrombin on P-selectin levels has not been reported for Tyro3°/° platelets, nor did we examine the effect of ADP-stimulation on anx/anx platelets to further delineate the parallels of these aggregation deficits. Electron microscopy revealed that anx/anx platelet morphology appears normal, and dense and alpha granules are present in equivalent numbers per platelet surface area (Fig. 2E,F). However, anx/anx platelets appear smaller than +/+ and anx/+ platelets (P<0.001) and 17.5±5.9% (mean±s.e.m.) (57-63 platelets per replicate; n=4) of anx/anx platelets contained large vacuoles, which were never observed in +/+ or anx/+ platelets (Fig. 2F). Given the presence of these platelet deficits in anx/anx mice, we pursued the possibility that R7W-Tyro3 might further contribute to anx phenotypes.

**Tyro3 expression in areas affected by the anx mutation and in anx/anx mutant brains**

Next, we examined Tyro3 expression in wild-type and anx/anx brains. Overall levels of wild-type and mutant Tyro3 RNA were not affected, as determined by qRT-PCR and northern blot analyses performed on +/+ and anx/anx brains at P21 (Fig. S2A). Previously, Tyro3 mRNA had been detected in the subventricular zone and layers 2/3, 5 and 6 of the cerebral cortex, the CA1 region of the hippocampus, the median eminence of the hypothalamus, and granule cells of the cerebellum in the adult mouse brain (Lai et al., 1994; Schulz et al., 1995; Gely-Pernot et al., 2012). Deficits in these regions have not been reported in anx/anx mice. Using RNA in situ hybridization on P10 and P21 wild-type brains, we confirmed Tyro3 expression in these regions (Fig. 3A,E,Q; Fig. S3), but also detected Tyro3 mRNA in areas affected by the anx mutation. Tyro3 was strongly expressed in the arcuate nucleus, ventromedial nucleus and, at lower levels, in the paraventricular nucleus of the hypothalamus.
hypothalamus and in ependymal cells lining the third ventricle (Fig. 3I).

In the hippocampus, Tyro3 was expressed in CA1, but also CA3 and, in the dentate gyrus (DG). At higher magnification, Tyro3 is expressed in cells within the granule cell and polymorphic layers of the +/+ DG. In anx/anx, only a few cells in the granule cell and polymorphic layers of the DG express C19T-Tyro3. (I) In the +/+ hypothalamus, Tyro3 is expressed in the median eminence, arcuate nucleus (ac), and ventromedial hypothalamus (VMH), and at lower levels in the paraventricular nucleus of hypothalamus (PVN). (J) In the anx/anx hypothalamus, C19T-Tyro3 expression appeared more diffuse at low magnification that it is difficult to distinguish its specific subregions. At higher magnification, (K) in the +/+ arcuate nucleus, Tyro3 RNA is localized in processes emerging from neuronal soma, but (L) in the anx/anx arcuate nucleus, C19T-Tyro3 RNA localization appears disorganized. Tyro3 is expressed in neurons within the dorsal raphe nuclei of (M) +/+ and (N) anx/anx mice. At high magnification, Tyro3 RNA is seen at the edges of (O) +/+ neuronal soma and in their emerging processes, but appears disorganized in (P) anx/anx neurons within the raphe nuclei. (Q) In the +/+ cerebellum, Tyro3 is expressed in granule cells and Purkinje cells, shown at higher magnification in (R) (marked with arrows). In the cerebellar molecular layer, Tyro3-expressing processes can be seen at high magnification. Tyro3 RNA faintly outlines the soma of cells – likely cerebellar stellate and basket cells (marked with arrowheads in R). (S) In anx/anx cerebella, C19T variant Tyro3 RNA is present in granule and Purkinje cells. (T) At higher magnification, mutant Tyro3-positive cell bodies (marked with arrowheads), which are likely stellate or basket cells, can be seen in the molecular layer. Mutant Tyro3-expressing processes are not apparent. Asterisks in A,B,E,F,I,J,M,N,O and S indicate regions shown at higher magnification directly below each respective panel in C,D,G,H,K,L,O,P,R and T.

At P10, the expression pattern of Tyro3 appeared equivalent throughout the brains of anx/anx and +/+ animals (data not shown). However, at P21, anx/anx brains showed marked differences in Tyro3 expression. At low magnification, mutated (C19T) Tyro3 expression appeared overall more diffuse in specific cell populations and throughout the brain, and mislocalized in some regions (Fig. 3B,F,J,N,S). Most noticeably, in the dentate gyrus, mutant Tyro3 was highly expressed in the molecular layers of +/+ and anx/anx mice, but...
relative to +/+ littermates (Fig. 3E,G), the granule and polymorphic layers from anx/anx mice contain very few cells expressing C19T-mutated Tyro3 (Fig. 3F,H). At higher magnification, subcellular localization of the mutated mRNA appeared aberrant in P21 anx/anx brains. Generally, Tyro3 mRNA appeared excluded from the nucleus, concentrated instead to regions within the soma from which processes were extending and, at times, could be detected in processes. In anx/anx neurons, C19T variant Tyro3 RNA appeared concentrated in soma and was often seen in aggregates (Fig. 3D,H,L, P). For example, in wild-type cerebella, Tyro3 mRNA was seen in processes in the molecular layer and only faintly outlining presumptive stellate and basket cell neurons (Fig. 3Q,R). However, in anx/anx cerebella the mutant Tyro3 mRNA was detected only minimally in processes and was concentrated in the soma of presumptive basket and stellate cells (Fig. 3S,T). In summary, Tyro3 was expressed in all neuronal populations – specifically the arcuate nucleus of the hypothalamus, the dentate gyrus of the hippocampus and presumptive serotonergic neurons in the raphe nuclei – for which anomalies have been reported in anx homozygotes, and the localization of C19T variant Tyro3 RNA seemed to be affected throughout the brains of P21 anx/anx mice.

**Rescue of anx phenotypes by Tyro3 transgenes**

Next, we performed transgenic rescue experiments. Because phylogenetic sequence comparisons between Tyro3 non-coding regions showed highest conservation within the first 9.5 kb upstream of exon 2c, we postulated that these regions might suffice to direct transgene expression in endogenous Tyro3-expressing domains, and used these regions from C57BL/6J genomic DNA to generate the T3Xpress vector (Fig. 4A). We generated three varieties of transgenic mice: five lines expressing mouse Tyro3 protein C-terminally tagged with GFP (Tyro3-GFP), a line expressing mouse R7W-Tyro3 C-terminally tagged with GFP (R7W-Tyro3-GFP) and three lines expressing untagged human TYRO3 (HuTyro3) (Fig. 4B). The R7W-Tyro3 expression constructs were identical to the normal Tyro3 cDNA constructs except for the C19T polymorphism. GFP-tagged lines can facilitate tracking protein localization, but the GFP tag might compromise protein function. Untagged human TYRO3 should have full protein function, and its RNA can be distinguished from endogenous murine Tyro3. Tyro3-GFP and R7W-Tyro3-GFP protein localization in P21 +/+ brain regions recapitulated endogenous Tyro3 RNA expression, as shown for the hypothalamus (Fig. S4) and serotonergic neurons (Fig. S5). In the arcuate nucleus and median eminence, a subset of Tyro3-GFP- and R7W-Tyro3-GFP-expressing neurons co-express Npy (Fig. S4). In the hindbrain raphe nuclei, Tyro3-GFP- and R7W-Tyro3-GFP-expressing neurons express the 5-HT marker tryptophan hydroxylase 2 (Tph2) (Zhang et al., 2004), thus identifying them as 5-HT neurons (Fig. S5). +/+ and anx/+ mice transgenic for HuTyro3 or Tyro3-GFP were indistinguishable from non-transgenic +/+ and anx/+ littermates. Tyro3 transgenic wild-type mice showed no adverse effects with regards to viability, fertility and overall health.

Notably, all anx/anx mice transgenic for Tyro3-GFP or HuTyro3 showed mild or no neurobiological anx-related behaviors such as ataxia, shaking and headweaving at P21, and survived at least until P35, as shown for all Tyro3-GFP and HuTyro3 transgenic lines (Fig. 4C; P<0.01). By contrast, the R7W-Tyro3-GFP transgene, even though expressed at levels similar to some of the rescuing Tyro3 transgenes (Fig. S2B), could not delay onset of the behavioral phenotypes by P12-15 or extend the lifespans of anx/anx mice past P21 (Fig. 4C).

At P21, all Tyro3-GFP and HuTyro3 transgenic anx/anx animals had significant body weight increases compared with non-transgenic anx/anx littermates. For example, anx/anx mice transgenic for Tyro3-GFP#1 or HuTyro3 line#1 had an average increased body weight of 83.5±8.2% or 92.8±8.5%, respectively, relative to their non-transgenic anx/anx littermates (P<0.01, one-way ANOVA; Fig. 4D,E). On average Tyro3-GFP and HuTyro3 transgenic anx/anx mice showed a 82.3±3.6% increase in body weight at P21 (Fig. 4F).

Because Npy+ neuron degeneration has been linked to appetite deregulation in anx/anx mice (Nilsson et al., 2011), we examined the effects of the Tyro3-GFP transgene on Npy+ neurons in anx/anx mice. Normally, Npy+ neurons are found in the arcuate nucleus and innervate the parvo- and magnocellular paraventricular nucleus of hypothalamus (PVN) and dorsomedial nucleus of the hypothalamus, as detected by silver-enhanced immunoperoxidase staining (Fig. 5A,D). At P19, the percentage of Npy+ soma present in the arcuate nucleus of anx/anx mice had been reduced to 20.2±1.8% relative to wild-type littermates, whereas in Tyro3-GFP#1; anx/anx mice 83.58±7.9% were still present. Moreover, at P19, Npy-immunoreactive cell bodies are consistently clustered aberrantly within and adjacent to the median eminence in anx/anx mice. In all anx/anx mice examined no punctate Npy staining suggestive of Npy localization in processes could be seen extending out of the arcuate nucleus towards the PVN or dorsomedial nucleus of the hypothalamus (Fig. 5B,E). In Tyro3-GFP#1; anx/anx mice, Npy+ soma were distributed throughout the arcuate nucleus and punctate Npy staining suggestive of Npy+ processes extended towards and into the PVN as in +/+ mice (Fig. 5C,F). However, qualitatively, Npy immunoreactivity still appeared stronger in Tyro3-GFP; anx/anx neuronal soma than in those of +/+ mice. Thus, in the presence of the Tyro3-GFP transgene, more Npy+ neurons and Npy+ processes can be detected within the arcuate nucleus in P19 anx/anx mice an observation consistent with a delay in neurodegeneration. This increase in Npy-containing processes and hence Npy signaling is likely responsible for the increased weight gain seen in anx/anx; Tyro3-GFP mice. In summary, rescue of these anx phenotypes by HuTyro3 and Tyro3-GFP transgenes, but not the R7W-Tyro3 transgene, suggest that the C19T Tyro3 mutation might contribute to the anx phenotypes.

**Effects of the R7W-Tyro3 variant**

To understand the possible consequences of the C19T mutation on Tyro3 localization and function, we examined whether the mutation impinged on signal sequence function in vitro. Point mutations within signal sequences can impact protein secretion and function in a variety of ways. The Signal P program (www.cbs.dtu.dk/services/SignalP) (Bendtsen et al., 2004), which examines amino acid sequences for characteristics of protein signal sequences (Bendtsen et al., 2004), did not predict that R7W-Tyro3 would substantially alter the signal sequence cleavage site (Fig. S6). Signal sequence mutations can also affect translocation from the cytoplasm to the endoplasmic reticulum and post-translational modifications, including N-glycosylation, that occur during protein secretion. In accordance with predictive analysis, when we assessed proteolytic processing of in vitro transcribed and translated Tyro3 and R7W-Tyro3 proteins mixed with signal peptide from canine pancreatic rough microsomes, the extent of signal sequence cleavage and glycosylation of the R7W-Tyro3 protein seemed equivalent to that of Tyro3 (Fig. S7). Thus, the C19T mutation in Tyro3 does not impact signal sequence function in vitro.
In the presence of GAS6, an established Tyro3 ligand present in serum, Tyro3 activation leads to Akt phosphorylation (Lan et al., 2000; Zhong et al., 2010). Therefore, we examined whether serum-dependent Akt phosphorylation was affected in vitro and in vivo. We detected no differences in Tyro3- or R7W-Tyro3-dependent Akt phosphorylation in the absence or presence of serum in transiently transfected COS7 cells, in stably transfected Neuro2A cells or in protein extracts from +/+ and anx/anx cerebella or hippocampi (Fig. 6 and data not shown). In the absence or presence of serum, immunoprecipitation of Tyro3-RFP and R7W-Tyro3-RFP using an anti-RFP antibody and subsequent immunoblot analysis with an anti-phosphotyrosine antibody also revealed no detectable differences in tyrosine phosphorylation (Fig. 6). Thus, signaling function does not appear to be overtly compromised by the C19T Tyro3 mutation.

When we examined whether Tyro3-GFP and R7W-Tyro3-GFP protein localization differed in transiently transfected Neuro2A, COS7 and HEK293T cells or in stably transfected Neuro2A cells, we found that in all instances their subcellular localization appeared indistinguishable (Fig. S8 and data not shown). Using
Fig. 5. Rescue of Npy+ neuron abnormalities of anx/anx mice at P19. Npy+ neuronal abnormalities are ameliorated in Tyro3-GFP; anx/anx transgenic mice as detected by immunoperoxidase enhanced with silver staining with an anti-Npy antibody, which stains Npy+ neurons and processes black. (A,D) In P19 +/+ mice, Npy+ neurons are located in the arcuate nucleus (arc) and send processes to the paraventricular nucleus. A monolayer of Npy+ cells also lines the inside of the median eminence (ME). (B,E) In P19 mice, only a few Npy+ neurons are present in the arcuate nucleus, and Npy+ processes extending towards the PVN cannot be readily detected. In addition, some Npy+ cells are located abnormally in the median eminence (ME). (C,F) In Tyro3-GFP transgenic mice, Npy+ neurons are mostly distributed throughout the arcuate nucleus and Npy+ puncta can be detected extending towards the PVN. D-F show higher magnification views of A-C, respectively. Dotted line in A-C demarcates the upper limit of the Npy+ soma included in the counts. (G) For anx/anx; Tyro3-GFP mice the percentage of Npy+ soma within the arcuate nucleus at the level of the median eminence relative to wild-type littermates are shown. Mice carrying Tyro3-GFP transgene (line #1) were analyzed. Cell counts were obtained from three sections with highest number of Npy+ neurons per animal and n=3 animals per genotype. Pairwise comparisons were performed using the Wilcoxon signed rank test; P<0.05.

**DISCUSSION**

Our study reveals Tyro3 as the first validated, linked gene that contributes to anx phenotypes and can delay hypothalamic neurodegeneration in anx/anx mice. As aforementioned, previous microarray analysis of the degenerating arcuate nucleus from P21 anx/anx mice (Lindfors et al., 2011) revealed twofold differences in the levels of Ndufa1 and Rpap1. We found that cDNAs of both Ndufa1 and Rpap1 are 100% identical to those of AKR/J mice. We did find a shared intronic SINE insertion within the Ndufa1 gene and a 222 bp deletion located 1296 bp upstream of the Rpap1 transcriptional start site in both anx/anx and AKR/J mice and these differences could conceivably interfere with Ndufa1 and Rpap1 transcription, respectively. Furthermore, whereas mitochondrial dysfunction can lead to neurodegeneration, abnormal mitochondrial respiration and localization can also be a consequence of neurodegeneration (Martin, 2012). Thus, although it remains a possibility that decreases in Rpap1 and/or Ndufa1 levels might influence anx phenotypes, in contrast to Tyro3, at present any direct experimental support for the involvement of either is lacking.

Our findings reveal roles for Tyro3 in maintaining Npy+ neurons within the appetite regulatory circuitry. Unlike anx/anx mice, no abnormalities in size or appetite have been reported for Tyro3−/− or TAM−/− mice. A series of studies in which AgRP neurons were ablated at different times and over varying timeframes might help explain why anx/anx mice show acute hypothalamic deficits and why, if such deficits are present in Tyro3−/− or TAM−/− mice or other mouse mutants, they might have been missed. AgRP/Npy+ neuron ablation that occurs acutely at birth or gradually over a period

immunofluorescence with an anti-Tyro3 antibody on cultured cortical and hippocampal neurons from anx/anx and +/+ littermates, endogenous R7W-Tyro3 and Tyro3 localization also appeared indistinguishable (Fig. S9). However, when we used silver-enhanced immunohistochemistry with an anti-GFP antibody to survey Tyro3-GFP and R7W-Tyro3-GFP protein distribution in the brains of P10 transgenic +/+ mice, we detected distinctly different localization patterns in cerebella (Fig. 7). At P10, Tyro3-GFP; +/+ and R7W-Tyro3-GFP; +/+ Purkinje cells appear normal, as determined by calbindin immunofluorescence. Tyro3-GFP protein was distributed in tiny puncta throughout the molecular layer of transgenic +/+ cerebella (Fig. 7B,B'). However, R7W-Tyro3-GFP protein was predominantly found in Purkinje cell soma and in the major shafts extending from the soma in large, sparse puncta (Fig. 7D,D'). The stereotypic organization and large dendritic arbors of Purkinje cells facilitated detection of this mislocalization, which was otherwise not obvious in other brain regions. Thus, the R7W-Tyro3 variant causes protein mislocalization in the postnatal cerebellum.

**R7W-Tyro3 is a strain-specific modifier of the anx phenotype**

The anorexia mutation arose spontaneously on a mixed Swiss Webster/Mus poschiavinus background (Maltais et al., 1984) and has subsequently been maintained on a C57Fe/a background. Recently whole genome sequence has become available for 17 inbred mouse strains in addition to C57BL/6J (Yalcin et al., 2012; Keane et al., 2011; Wong et al., 2012). We found that the R7W-Tyro3 variant is also present in FVB/NJ, AKR/J and NZW/lacI strains, which are related to the Swiss Webster strain. As none of these strains exhibit abnormalities in size or appetite, they might have been missed. AgRP/Npy+ neuron ablation that occurs acutely at birth or gradually over a period
of a few months leads to only mild decreases in weight (Bewick et al., 2005; Xu et al., 2005). By contrast, acute AgRP/Npy+ neuron ablation in adult mice (Gropp et al., 2005; Luquet et al., 2005) or in anx/anx mice starting at P10-P12, a time by which arcuate nucleus neuron projections have formed functional connections with other hypothalamic regions (Bouret et al., 2004), inhibits feeding behavior and causes starvation. Thus, appetite regulatory circuitry can adjust and compensate for gradual adult or acute neonatal loss, and gradual Npy+ neuron loss in Tyro3−/− or TAM−/− mice would have gone unnoticed. Considering that other genes within the anx critical interval do not play known roles in Tyro3 signaling, it seems likely that Tyro3 boosts neuronal survival in general rather than by interacting specifically with the as-yet-unknown primary anx causative gene.

**Tyro3 and restrictive anorexia nervosa**

To date no unequivocal, well-replicated genes or loci associated with ANR have been identified in humans (reviewed in Yilmaz et al., 2015). With respect to neurotrophic factors and tyrosine kinase receptor signaling, candidate gene approaches have obtained mixed results for linkage of the rs6265 (V66M) polymorphism in brain-derived neurotrophic factor (BDNF) with ANR (Dardennes et al., 2007; Ribasés et al., 2005, 2004; Ando et al., 2012; Brandys et al., 2013; Rybakowski et al., 2007). In the case of appetite-regulating neuropeptides, so far missense mutations in POMC have been reported in one individual with ANR, but no associations with common variants have been detected in ANR populations (Yilmaz et al., 2014). Furthermore, several genome-wide linkage studies found association of the serotonin receptor 1D and opioid receptor delta 1 with ANR (Bergen et al., 2003; Brown et al., 2007). Considering that body mass index remains low in recovered individuals with ANR, and ANR predominantly affects females, a genome-wide association meta-analysis cross-referencing these traits uncovered possible associations with, amongst other genes, CTBP2 and NBEAL1 (Hinney et al., 2016). Both CTBP2 and NBEAL1 show altered hypothalamic expression in response to fasting and diet-induced obesity and are postulated to act upon or at synapses. Finally, epoxide hydrolase 2 (EPHX2) emerged as a candidate for ANR associated with increased depression and anxiety in a high-throughput sequencing study (Scott-Van Zeeland et al., 2014). The paucity of known genetic factors likely reflects the heterogeneity among ANR cases and the genetic complexity of ANR specifically and psychiatric disorders in general. Because onset of ANR is accompanied by a range of psychiatric manifestations, including anxiety, body dysmorphic disorder, and often heightened sensitivity to negative stimuli, which are not of hypothalamic origin, and because these often persist to some degree even upon recovery, it seems unlikely that...
might offset this and perhaps improve chances of recovery.

\(\text{SOD1}\) familial cases, is often caused by mutations in Cu/Zn superoxide dismutase (\(\text{SOD1}\)).

\[\text{Whether hypothalamic degeneration accompanies the ingestive disorders, such as amyotrophic lateral sclerosis (ALS), which, in some instances, some of the other mouse strains that carry the R7W-Tyro3 variant have shown enhanced vulnerability to neurodegenerative disorders. For example, mice, which lack the SMN1 RNA binding protein and act as a model for human spinal muscular atrophy (SMA), survived twice as long on a pure C57BL/6N background than on the R7W-Tyro3-carrying FVB/N background (Ackermann et al., 2013). Moreover, mice lacking the amyotrophic lateral sclerosis 2 (\(\text{ALS2}\)) gene exhibited a shorter life span than wild-type littermates when they were on a FVB/N than on a C57BL6/J background (Hadano et al., 2010). Thus, the R7W-Tyro3 variant might be one of the factors that contribute to increased disease susceptibility in these instances.}\]

\[\text{Possible consequences of the R7W-Tyro3 variant}\]

Although bioinformatic analysis did not predict an appreciable impact on Tyro3 processing and we did not observe dramatic changes in the glycosylation state of the extracellular domain or localization of the mutant protein in cell culture, the R7W-Tyro3 variant appears to have consequences \textit{in vivo}. Often, the biochemical effects of signal sequence mutations that are associated with human disorders correlate well with the disease state, as shown for prepro-parathyroid hormone (PTH) (Arnold et al., 1990). However, sometimes, even though a human disease is apparent, signal sequence function seems normal in \textit{in vitro} assays. For example, a subtle mutation in the signal sequence of the cell surface calcium-sensing receptor (CASR) resulted in less protein at the plasma membrane, but the \textit{in vitro} co-translational processing of the CASR mutant protein was unaffected (Pidasheva et al., 2005). Nonetheless the individual carrying this mutation, along with others carrying more dramatic mutations in the signal sequence of CASR, suffered from hypocalcemic hypercalcemia. Another example is a mutation in the signal sequence of thyroxin-binding globulin (TBG), which is associated with a partial deficiency of TBG-Allentown. Even though the Signal P program predicted no change in cleavage site, TBG was lowered in the blood of individuals with this deficiency (Fingerhut et al., 2004). Thus, C19T mutation in \(\text{Tyro3}\) might compromise signal sequence function subtly in general or significantly in specialized, long-lived cell types, such as neurons.

Another possibility for the apparent lack of impact of the R7W-Tyro3 variant on \textit{in vitro} signal sequence function is that it impacts RNA-sequence-based functions. Although, conventionally, the main roles of signal sequences have been based on their peptide sequences, the RNA sequence of the signal sequence coding region of some secretory proteins can activate nuclear mRNA export (Palazzo et al., 2007). Consistent with the possibility of an RNA-based mechanism, comparison of all sequenced \(\text{Tyro3}\) orthologues revealed that the cytosine mutated in the C19T \(\text{Tyro3}\) mutation is invariant and located within a larger conserved 21 nucleotide sequence. Current programs designed to identify RNA binding protein sites (RBPDB: http://rbpdb.ccbri.utoronto.ca/index.php and RegRNA: http://regrna.mbc.nctu.edu.tw/html/prediction.html) did not score disruption or creation of a binding site for a known RNA binding protein (RBP) by the C19T \(\text{Tyro3}\) mutation. Moreover, the RNA binding profiles for some neuronally important RNA binding

\[\text{Tyro3 as a strain-specific modifier}\]

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proteins (RBPs), such as FMRP1 (Miyashiro et al., 2003; Darnell et al., 2001, 2005), Nova (Ule et al., 2003), TDP-43 (Sephton et al., 2011), RhFox1 (Lee et al., 2016) and SRRM4/nSR100 (Irmiya et al., 2014), have begun to be determined, but none have recovered wild-type Tyro3 mRNA. Endogenous Tyro3 protein is localized in neuronal processes (Fig. S9) (Prieto et al., 2007). Many regulatory elements known to govern localization of neuronal mRNAs, such as BDNF (An et al., 2008) and Arc (Rao et al., 2006), reside in 3′ UTRs. However, the transgenic Tyro3 expression constructs contain only Tyro3 coding sequences and 154 nt of the 5′ UTR, and the C19T Tyro3 mutation resides near the translational start site. Taken together, these observations might be more in line with a disruption of translational regulation. Given that we could only observe protein and possible RNA mislocalization in vivo and not in culture, neuron-specific RNA-binding and/or translational regulatory factors — possibly ones dependent on neuronal activity — might be involved, and these might either be absent in cell culture or not have been activated under normal culture conditions. However, although an intriguing speculation, it is currently unknown whether an RNA-based mechanism — and perhaps a novel one — might play a role in R7W-Tyro3 mislocalization.

Regardless of mechanism, the mislocalization of R7W-Tyro3-GFP protein in the cerebella of wild-type mice does suggest that the R7W variant causes protein mislocalization in vivo and that, given that these mice appear normal, this effect is not due to overtly compromised neuronal health. The molecular mechanism by which the R7W-Tyro3 variant disrupts Tyro3 function(s) remains unknown, and given that its effect is only apparent in vivo in the mouse and most striking in anx/anx mice, identification and validation of such a mechanism is more challenging.

CONCLUSION
In conclusion, here we have identified a mutation in the signal sequence of the Tyro3 tyrosine kinase receptor (R7W-Tyro3) as enhancing anx phenotypes, and shown that normal Tyro3 acts protectively to sustain the appetite regulatory circuitry in anx/anx mice. Whereas predictive programs and in vitro analyses could not predict or detect appreciable differences, the biological consequences of both the R7W-Tyro3 variant and normal Tyro3 are apparent in vivo. Furthermore, our findings suggest the existence of subtle, quite possibly often missed, mechanisms that might regulate protein and/or RNA subcellular localization. To date platelet function has not been systematically examined in ANR. Our observations suggest that, in the future, systematic analyses of platelet function in eating disorders and possibly other psychiatric and neurodegenerative disorders might prove useful and help categorize these into specific biological subtypes. Overall, our observations highlight the importance of analyses in the mouse or other model systems as a testbed for the search for human disease modifiers and for genetic variants with measurable biological effects. Finally, enhancing Tyro3 expression or signaling could provide a strategy for countering deficits in the appetite regulatory circuitry that might contribute — in a primary or secondary manner — to the ingestive dysfunctions seen in ANR and some neurodegenerative disorders.

MATERIALS AND METHODS
Mice
anx+/+ mice maintained on a B6C3Fe a/a hybrid background were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and crossed onto C57Bl/6J and Molf/EJ backgrounds. To identify mice carrying the C19T-Tyro3 mutation, polymerase chain reaction (PCR)-based assay using primers 5′-GATGGCGCTGAAAGCGGACATG and 5′-CGCGGC-CGGAGGTCTGGCAG, and subsequent digestion with NlaIV was used. All animal experiments were performed in strict adherence to guidelines for experimentation with laboratory animals set by the Canadian Animal Care Center (CACC).

Platelet aggregation assays
Platelet aggregation assays were performed as in Angelillo-Scherrer et al. (2005). In brief, 1×10⁶ platelets per mouse were incubated with 1 U thrombin (Sigma) for 10 min at room temperature (RT), labeled with FITC-conjugated anti-P-selectin antibody (1:1000; BD Biosciences-Pharmingen Cat. no. 561849 and analyzed by flow cytometry using a FACs instrument and Cell Quest software. Platelet aggregation was analyzed for six or more mice of each genotype anx/anx and three anx+/+ and +/+ animals was analyzed. Data was analyzed using the Mann–Whitney Test.

Platelet electron microscopy
Isolated platelets were fixed with 4% paraformaldehyde and 2% gluteraldehyde in 0.1 M sodium cacodylate buffer pH 7.3. Sectioned samples were analyzed by Tecnai 20 transmission electron microscopy followed by data analysis using Soft Imaging Systems. For morphologic analyses, 56-63 platelets from each of four anx/anx and three anx+/+ and +/+ animals were analyzed. Data was analyzed using the Mann–Whitney Test.

Quantitative RT-PCR
RNA from P19 cortices from anx/anx, anx/+ and +/+ mice was isolated using Trizol reagent (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized from total RNA using oligo-dtf primers and the Superscript III First Strand Synthesis System (Invitrogen) according to manufacturer’s instructions (2 μg of RNA used in 20 μl reverse-transcription reaction). Primers specific for Tyro3 (5′-AATGCCGGAT-TTACAATCTACCTC-3′ and 5′-GTTCCATTCGACAGACGTGAA-GCTT-3′) and GAPDH (5′-TGTGAACGGATTGCGCGAT-3′ and 5′-CATGATGACCATGTAGTGGAG-3′) were used. Reactions were run with SYBR green PCR master mix (Invitrogen) in an AB 7500 fast real-time PCR system and relative gene expression levels were calculated using Sequence Detection System 2.2 Software (Applied Biosystems). Expression values were normalized relative to the sample Gapdh mRNA expression. RNA from five anx+/+ and +/+ and three anx/anx animals was analyzed. Data were analyzed using nonparametric t-tests and Mann–Whitney test.

RNA in situ hybridization on sections
RNA in situ hybridization on sections was performed on brains perfused with 4% paraformaldehyde in diethyl-pyrocarbonate-treated 1×PBS and embedded in optimal cutting temperature medium (Sakura Finetek, Torrance, California, USA). Coronal sections of 14 μm were cut using the Leica VT1000 cryostat. RNA in situ hybridization was performed on tissue sections as described in Storn and Kingsley (1996). Sense and antisense probes were generated from a 588 bp HindIII fragment, containing the partial coding sequence of the Tyro3 intracellular kinase domain, cloned into pBluescript KSII. The sense probe was generated with Xhol digestion and T3 RNA polymerase transcription, the antisense probe upon EcoRI digestion and in vitro transcription with T7 RNA polymerase (Roche, Basel, Switzerland).

Generation of transgenic animals
Tyro3 and R7W-Tyro3 cDNA was generated from RNA from +/+ and anx/anx brains using Superscript First-Strand Synthesis RT System (Invitrogen), amplified by real-time PCR, and inserted into pDONR201 with BP Clonase (Invitrogen). To generate an untagged human TYRO3 construct, human TYRO3 cDNA (generously provided by Dr Kensaku Mizuno, Tohoku University, Sendai, Japan) was inserted into filled-in Sall sites of pDONR201. Using LR Clonase, these versions of Tyro3 were cloned into the T3Xpress vector. The T3Xpress vector consists of 9.5 kb BamHI-NorI fragment 5′ of and containing the transcriptional start site for signal sequence-containing exon 2c (Biesecker et al., 1995) and 154 nt of the 5′UTR, attR recombination sites, an in-frame C-terminal GFP, and Sva40 IVS polyA. Resulting expression constructs were digested with Nsal, purified with GeneClean (Qiobiogene, Irvine, California, USA), and used to generate
transgenic mice by standard pronuclear microinjection into FVB/N oocytes. Positive transgenic mice were identified by Southern blot analysis and by a PCR-based assay using the following primers: GWTyro3-F1:GGGGACAGTTTTGACAAAAACAAGCCGTGCCGCGGTAGGCGTCAGGGGAGCATGGGGGTG and GWTyro3-R1:GGGGACACCTTGTACCAGAAGACTGGTTAAGCTGTGAGGCGTAGCCCTTG.

**Immunostaining**

In all cases, a minimum of three mice of a given genotype and stage were analyzed. Adult brain cryosections were blocked in 10% normal donkey serum (NDS) in PBS with 0.2% Triton X-100 (PBST) for 1 h at RT. Antibodies were diluted in 2% NDS PBST and incubated overnight at 4°C. Samples were washed and incubated with Cy3 or Alexa Fluor 488 secondary antibodies (1:500; Invitrogen) for 1 h at RT. Samples were mounted with Vectashield plus DAPI (Vector Labs).

For immunofluorescence, primary neuronal culture samples were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 PBS for 5 min at RT. All samples were blocked in histoblock [3% BSA, 1% BM blocking solution (0.01 M maleic acid, 15 mM NaCl, pH 7.5), 20 mM MgCl2 and 0.3% Tween 20 in PBS] for 1 h at RT, incubated overnight in primary antibodies. Secondary antibodies were applied and samples processed as described above.

The following antibodies were used: anti-Npy (1:1000; Peninsula Labs, T4068.0500), anti-Tyro3 (1:1000; Abcam, ab79778) and mouse anti-Map2 (1:2000; Sigma, M4403), rabbit anti-calbindin (1:1000; Chemicon, AB1778), anti-SHT (1:800; Immunostar, 20080).

For silver nitrate enhanced immunoperoxidase staining, 20 µm sections were cut through hypothalami of P19 +/-, anx/anx and Tyro3-GFP; anx/anx mice for anti-Npy staining, and through cerebellum of P19 and P10 Tyro3-GFP; +/- and R7W-Tyro3-GFP; +/- mice for anti-GFP staining. Immunoperoxidase staining was performed with the Vectastain kit (Vector Laboratories) and enhancement with silver nitrate was performed as described in Kitt et al. (1987). Slides were scanned using a Zeiss Axio Scan.Z1 microscope.

For analyses of Npy-expressing neurons, hypothalami of P19 +/- mice were sectioned completely and Npy+ soma from nine sections containing the arcuate nucleus. Median eminence and highest number of Npy+ neurons for each of three animals per genotype were counted. In wild-type mice Npy+ soma were completely and Npy+ soma from nine sections containing the arcuate nucleus, hypothalami of P19 mice were sectioned through hypothalami of P19 +/-, anx/anx and Tyro3-GFP; anx/anx mice for anti-Npy staining, and through cerebellum of P19 and P10 Tyro3-GFP; +/- and R7W-Tyro3-GFP; +/- mice for anti-GFP staining. Immunoperoxidase staining was performed with the Vectastain kit (Vector Laboratories) and enhancement with silver nitrate was performed as described in Kitt et al. (1987). Slides were scanned using a Zeiss Axio Scan.Z1 microscope.

Neuronal cultures

Primary hippocampal and cortical neurons were harvested from embryonic day (E)17 mice and cultured for 10-14 days as described in (Yudowski et al., 2006) and (Boucard et al., 2005). For transection, dissociated neurons were transfected prior to plating with the Amaxa Nucleofector kit (VPG-1001, Lonza) using approximately 5 X 104 cells and 10 µg plasmid DNA per transfection.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.Y.K. performed genetic mapping, RNA in situ analyses, cloning and transgenic rescue experiments and contributed to manuscript. J.Y. performed platelet analyses, biochemical analyses, neuronal culture and qRT-PCR analyses and contributed to manuscript. R.K.M. and R.N. performed neuronal culture and immunofluorescence analyses, H.B.T. performed expression analyses. R.S. performed platelet aggregation assays with J.Y. J.W.S. designed platelet aggregation experiments S.P.C. performed cloning, sequencing, and immunohistochemical analyses, guided D.Y.K., J.Y., R.K.M., R.N., and H.B.T., designed overall study and wrote manuscript.

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