

The Pathogenesis of Proventricular Dilatation Disease Caused by Parrot Bornaviruses: A Possible Role For Neuropeptide Y (Npy)

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Research article

Keywords: Bornavirus, psittacine, neuropeptide Y, proventriculus, RNA-seq

Posted Date: October 5th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-49954/v1>

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Abstract

Background

Psittacine Bornaviruses cause a unique disease syndrome in parrots and related birds. Known as proventricular dilatation disease (PDD), it is characterized by massive dilatation of their proventriculus as a result of excessive food accumulation within that organ. This leads to gastric obstruction and eventually, to death by starvation.

Results

In a preliminary study on the transcriptome of psittacine bornavirus-infected human astroglia it was noted that the gene encoding neuropeptide Y was significantly upregulated. In a subsequent study of cockatiels experimentally infected with the same strain of psittacine bornavirus, their brains were examined by RNA-seq to determine which genes were being actively transcribed. It was confirmed that among the genes whose expression was significantly increased relative to control, uninfected birds was that for neuropeptide Y.

Conclusions

Neuropeptide Y is known to cause overeating in birds. We hypothesize therefore that the clinical manifestations of proventricular dilatation disease are a result of the excessive production of neuropeptide Y by bornavirus-infected brain cells acting in association with damage to the neurons of the proventricular myenteric plexus.

Background

Bornaviruses of the order Mononegavirales, family *Bornaviridae*, are enveloped viruses with a non-segmented, negative-strand, 9 kb RNA genome. They are unique among RNA viruses in that they replicate within the nuclei of infected cells and employ RNA splicing machinery for their gene expression [1]. They are highly cell associated and as a result, very few infectious particles are released from infected cells.[2] Bornaviruses are probably transmitted between adjacent cells in the form of ribonucleoprotein complexes [3]).

Prior to 2008, the only known bornavirus was Borna Disease virus (BoDV-1). This causes lethal neurologic disease in horses, sheep and humans in Central Europe. Since then new mammalian bornaviruses have been isolated from horses (BoDV-2), and captive squirrels [4]. Beginning in 2008, numerous bornaviruses have also been isolated from wild birds and bornaviral taxonomy has had to be revised significantly [5]. While most of these avian bornaviruses infect psittacines, others infect canaries, finches and numerous waterfowl species [6, 7, 8]. Bornaviruses have also been isolated from snakes [9].

Mammalian bornaviruses cause a lethal meningoencephalitis in horses, sheep and humans as well as laboratory rodents. Avian bornaviruses also cause lethal disease in some species of birds but it has a very different clinical manifestation. For example, several species of Psittaciform 1 bornaviruses (PaBV) cause a unique neurologic and gastrointestinal disease. In these birds, the virus initially causes an immune-mediated encephalitis. The virus then spreads centrifugally along the cranial nerves and spinal cord to infect numerous other organs. Thus, invasion of the optic nerve can result in blindness. Invasion of the spinal cord can result in tremors, paralysis and ataxia. Invasion of the vagal nerve results in cardiac abnormalities and most notably, altered innervation of the anterior stomach – the proventriculus. This results in dilatation and eventual blockage of the proventriculus by accumulated food resulting in death by starvation. This Proventricular dilatation disease (PDD) was originally observed in large psittacines of the genus *Ara* and as a result was initially known as macaw wasting disease.

As its name suggests, PDD is characterized by massive distention of the avian proventriculus [10, 11]. This dilatation results from overfilling of the proventriculus due to its failure to empty into the ventriculus. As a result, it fills with undigested food. The proventricular wall becomes thin and atrophic to the extent that it may become translucent. Dilatation of other parts of the anterior gastrointestinal tract such as the crop, the ventriculus and the duodenum has also been reported.

One feature we have observed in experimentally infected cockatiels (*Nymphicus hollandicus*) is that the onset of clinical disease can be anticipated following a significant increase in the bird's appetite. Animal keepers have reported voracious eating and on occasion, bird seeking to feed from a dish even before it has been placed in a cage (Turner D, Escandon P. Unpublished Observations). As a result, their food consumption increases significantly. This occurs two to three days prior to the onset of obvious proventricular dilatation. It is apparent that affected birds no longer receive satiation signals from the proventriculus. As a result, food intake increases at a time when the proventriculus is no longer capable of emptying. The proventriculus gets packed with food leading to blockage and eventual death as a result of starvation or secondary microbial infections.

While the natural route of PaBV infection is unknown, Leal et al [12] have tracked the spread of avian bornavirus from an injection site in the pectoral muscle through adjacent nerves, to the brachial plexus. It spreads from there into the thoracic spinal cord and thence to the brain. The virus subsequently spread from the brain to the ganglia in the gastrointestinal tract, the adrenal gland and the cardiac ganglia. Thus, the virus first targets the brain and central nervous system before spreading by way of nerves, most notably the vagal nerve, to other autonomic ganglia. Histologically, the primary disease lesions appear to occur within brain, the gastric plexus, the myenteric plexus in the proventricular wall and the celiac plexus. In these lesions, there appears to be a loss of neurons accompanied by a lymphocytic infiltration [13, 14].

Detailed studies on the location of brain lesions in experimentally infected cockatiels indicate that the virus is first detected in the thalamic nuclei and the hindbrain [15]. The presence of detectable virus coincides with the development of an obvious lymphocytic encephalitis. While initially restricted to the central nuclei of the brain, by about day 60 post-challenge the virus spreads throughout both gray and

white matter. This distribution of the virus and the lesions in birds are similar to those observed in naturally infected horses and sheep as well as experimentally infected mice. Thus Bornaviruses appear to have a predilection for the hippocampus, caudate nucleus, substantia nigra, the mid brain, and the hypothalamus [15].

Following the establishment of an encephalitis, the virus then spreads centrifugally [12] along the vagal nerve to the ganglia in the gastrointestinal tract, adrenal, heart, and kidneys. As a result, the neurons in the myenteric plexus are reduced in number and replaced with a lymphoplasmacytic infiltration [14, 16]. This plexus regulates gastrointestinal motility and smooth muscle contraction. While damage to the myenteric ganglion may contribute to the disease pathogenesis, the reduction in signaling does not appear to be sufficient to account for the gross dilatation of the proventriculus. Sectioning of the vagal nerve in birds, while slowing the passage of ingesta from the proventriculus into the ventriculus does not result in proventricular dilatation [17].

In an effort to further elucidate the pathogenesis of PDD in birds, preliminary transcriptome studies were performed in vitro on a human astrocytoma cell line that is readily infected by psittacine bornaviruses. As a result of these in vitro studies, our attention was drawn to a significant increase in transcription of neuropeptide Y. Neuropeptide Y is a major regulator of appetite and food intake. In order to follow up this observation, we performed transcriptome analysis on the brains of cockatiels experimentally challenged with avian bornavirus PaBV-2. They were humanely euthanized as they developed disease and their brains were assayed for RNA transcripts. The brains of four normal, uninfected cockatiels were analyzed at the same time. These confirmed the significant increase in NPY expression in infected birds. We suggest that this increase, together with myenteric ganglion damage, may explain the pathogenesis of proventricular dilatation disease.

Methods

Astrocytes

Cells of the CCF-STTG1 human astrocytoma cell line were obtained from ATCC[®]. The cells were cultured with DMEM containing 10% fetal bovine serum (FBS). The cells were infected with PaBV genotype 4 when 90% confluency was reached. The infected cell cultures were harvested for RNA purification after three passages .

Parrot Bornavirus

PaBV-2 was isolated from the brain of an experimentally infected cockatiel [18] and was grown as previously described [19]. It was grown in primary duck embryo fibroblasts cultured in Dulbecco's modified eagle medium (Gibco[®], Life Technologies Co., Thermo Fisher Scientific, Waltham, MA, USA) together with 10% FBS (Gibco[®], Life Technologies Co) at 37°C in an atmosphere of 5% CO₂. Birds were inoculated by a combined intranasal and intramuscular administration of a suspension of infected cells containing 8×10^4 focus forming units of the virus.

Experimental infections

Birds

Eight cockatiels (*Nymphicus hollandicus*), ranging from 79 to 145 g (mean 101 g), were used in this study. These birds originated from a breeding colony maintained by the Schubot Avian Health Center at Texas A&M University. Four birds had been used as infected controls in an approved drug therapy trial and according to protocol were scheduled for euthanasia. The euthanasia was performed between 60 and 120 days post-challenge depending upon the time of onset of clinical disease. Four other birds were normal, uninfected controls. An animal use protocol detailing the drug therapy trial was reviewed and approved by the Texas A&M University Office of Research Compliance, complying with guidelines included in the National Research Council of the National Academies' publication Guide for the Care and Use of Laboratory Animals, 8th edition.

RNA-seq

Total RNA was collected from bornavirus-infected and control human astrocytes using an RNeasy Mini Kit (Qiagen). Purified total RNA was analyzed for quality and quantity using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific). The RNA samples submitted to the Genomics and Bioinformatic Service Laboratory at Texas A&M University for the preparation and sequencing of the library.

Quantitative polymerase chain reaction (qPCR)

The brains from the infected and control birds were frozen immediately on necropsy. Total RNA of infected human astrocytes and experimentally infected cockatiels brain was isolated using Trizol (Qiagen) according to the manufacturer's protocol. RNA was eluted using 50 ml of RNase-free water and then stored at -80°C. Quantitative polymerase chain reaction (qPCR) was performed using iTaq Universal SYBR Green One-step Kit (Bio-Rad, Hercules, CA). The following primers were used: human-GAPDH forward, AATGGACAACCTGGTCGTGGAC; human-GAPDH reverse CCCTCCAGGGGATCTGTTTG; human-NPY forward, TGTGTCTACCGTTCCTTACC; human-NPY reverse, GATTGTGGATACTTGTACTGCCA; cockatiel-GAPDH forward, TGACGTGCAGCAGGAACACT; cockatiel-GAPDH reverse, GTGACCAGGCGCCAATAC

cockatiel-NPY forward, AGGACATGGCCAGATACTACT; cockatiel-NPY reverse, GTCTCTGGGCTTGATCTCTTTC.

Statistical Analysis

RNA-seq data was analyzed using CLC Genomics Workbench software. Ingenuity pathway analysis (IPA) was used to define the top upregulated/downregulated genes and any significantly affected gene pathways. Measurements were presented as mean \pm standard errors of the mean (SEM). Significance was tested only for experiments using biological replicates, using two-way repeated measures Analysis of Variance (ANOVA). In all figures, * = $p < 0.05$.

Results

In Vitro studies.

Based on the transcriptome analysis of parrot bornavirus-infected human astrocytes, numerous changes were detected in gene expression patterns between normal uninfected astroglia and control cells (Supplemental data S1). Few obvious or relevant patterns could be discerned in the upregulated genes. Many of the upregulated genes encoded synapse structure or inflammatory proteins but only one stood out as being of potential relevance to the pathogenesis of PDD. This was the gene for neuropeptide Y that showed a 20.6-fold increase in RNA content. However, once controlled for relative concentration, the true increase in RNA expression of the NPY gene was 4.137 Log 2. This was effectively more than a 40-fold increase in NPY mRNA (Table 1, Figure 1). Likewise of the down-regulated genes (Supplemental data S1), none appeared to be immediately relevant to the disease pathogenesis.

In Vivo studies

Based on the preliminary findings of the in vitro astrocyte studies, in vivo studies were initiated by comparing the transcriptomes of four normal cockatiel brains and the brains of four Bornavirus-infected cockatiels euthanized when they developed clinical PDD. Quantitative PCR analysis of the NPY genes was performed on each of the brains. However as in the in vitro astrocyte studies it was clear that the gene for NPY was significantly upregulated in all infected birds. (Figures 2 and 3).

Discussion

The upregulation of NPY expression in bornavirus-infected parrots is not a unique finding. Thus, NPY was also found to be significantly upregulated in the brains of BoDV-1-infected Lewis rats by Bette et al [4]. They found that an increase occurred in neuronal NPY expression during the acute phase of the disease. The upregulation was detected by northern blotting in the cerebral cortex, the hippocampus and the thalamus. They also found that dexamethasone treatment profoundly reduced the cerebral inflammation in these rats but had no detectable effect on cerebrocortical NPY expression. Thus, its upregulation was not causally related to the inflammation [20]. Double staining for both NPY and Bornaviral antigens indicated that the vast majority of NPY-producing neurons were not infected with the virus. Plata-Salaman et al have also examined neuropeptide expression in Bornavirus infected neonatal rats [20]. Among other changes, they observed an increase in NPY mRNA levels in the cerebral cortex hippocampus and hypothalamus at 28 days post-infection. Bornaviruses are among those viruses that can induce behavioral changes resulting in obesity [21].

Neuropeptide Y (NPY) is a 36 amino acid peptide belonging to a family of gut-brain peptides [22, 23]. It is involved in several homeostatic processes, especially those involved in feed intake and appetite. It is synthesized within the brain, especially the cortex, hippocampus, hindbrain and in the arcuate nucleus within the hypothalamus [22]. NPY receptors are however not restricted to the brain but are widely distributed in both the central and enteric nervous systems [24].

A subset of neurons in the arcuate nucleus appear to be critical in regulating appetite. [25] They produce a mediator called agouti-related peptide (AgRP) that promotes food consumption. NPY is required to stimulate their activity in a persistent manner. Deletion of NPY but not GABA suppressed their functions. Thus, AgRP and NPY-containing neurons sense peripheral signals of energy shortage such as declining glucose levels or increased ghrelin levels. As a result, the AgRP/NPY neurons influence (inhibit) several neuronal subpopulations in the paraventricular hypothalamus as a result of the release of AgRP and NPY and as a result stimulate appetite and eating behavior [23]. Our studies failed to demonstrate any significant change in the transcription levels of AgRP in vivo (S1).

One obvious question is, was the increased eating behavior observed in PDD birds secondary to hunger induced by proventricular blockage and decreased nutrient intake or was it a primary result of the virus infection. Hunger may have played a role in the in vivo studies. However, the increase in NPY transcription observed in astrocytes in vitro suggested clearly that this was a primary result of the virus infection.

When injected into the brain, NPY acts as a potent appetite stimulant and induces a robust feeding response, that lasts for 6–8 hours. This effect has been observed in both mammals and birds. It is ineffective however when infused peripherally. This effect has been confirmed by the use of NPY blocking agents such as antibodies or NPY antisense oligodeoxynucleotides that suppress food intake on infusion. NPY delays satiety and as a result increases meal size, and the time spent eating. It also appears to promote motivation to eat in a manner similar to temporarily fasting. Two lines of obese rats, the Zucker rat and the Koletsky rat both have persistently elevated levels of NPY in the arcuate nucleus of their hypothalamus as a result of dysregulation and consequently, suffer from hyperphagia leading to obesity [23, 26].

Central administration of NPY has been studied in broiler chicks in order to determine whether its appetite stimulating effect can be used to promote growth [27, 28]. These studies demonstrated that NPY will indeed induce a transient increase in appetite. Following a single intracerebral injection, the effect was maximal at 30 minutes and had begun to decline by 120 minutes. However, it has a half-life of only 20 minutes, so the effect wears off quickly [28, 29]. Neuropeptide Y also affects satiation signals and causes relaxation of the circular muscles of the proventriculus permitting it to relax and expand to a greater extent than normal.

The distribution of avian bornavirus in the brain of psittacine birds is essentially identical to the reported distribution of obesity-inducing strains of mammalian bornavirus in the brains of infected rats [30]. Thus, the obese phenotype had both viral antigen expression as well as a mononuclear cell infiltration in the septum, hippocampus, amygdala and ventromedian tuberal hypothalamus. This suggests that some disruption in neuroendocrine signaling is involved in the dysregulation of food appetite in both rats and birds.

It is well recognized that PaBV infected parrots may die as a result of cardiac abnormalities. Leal et al have demonstrated the presence of the virus in the cardiac ganglion. However, NPY can also affect the functioning of the heart by its actions on the vagal nerve [29, 31]. The NPY causes prolonged attenuation

of cardiac vagal action. As a result, it may cause an accelerated heartbeat. Another reported manifestation of PDD is feather picking, NPY can cause itch and skin irritation so the two may be connected.[32]

Concluding hypothesis

There are three possible, non-exclusive mechanisms of PDD [33], Excessive proventricular relaxation, proventricular outlet obstruction, and excessive feed ingestion. The reports of lesions in the myenteric plexus suggest that excessive relaxation/ failure of contraction will reduce the ability of the proventriculus to empty. As pointed out above, bilateral vagal ablation in chickens and turkeys will not in itself cause dilatation but it will markedly slow the emptying of this organ [17]. The results reported herein also point to the role of excessive intake and a failure of satiation signals leading to proventricular overfilling. In effect, the bird will continue to feed even when the proventriculus is full. Receptive relaxation will initially occur [34]. However, when the level of NPY is sufficiently high and the myenteric plexus is sufficiently damaged, then at some point, the proventriculus will no longer be able to fully recover its tone. Ventricular emptying will become impaired and when sufficiently severe, this will eventually result in food accumulation, eventual impaction and blockage.

Conclusions

We hypothesize that bornavirus infection of experimentally infected cockatiels results in disturbances in neuroendocrine functions within the thalamus and significant elevations of neuropeptide Y (Fig. 4). This chronic overproduction of NPY resulted in the observed increases appetite and feeding behavior observed in these birds. In association with the virus-induced damage to the proventricular myenteric plexus that results in proventricular relaxation this over-eating leads to proventricular dilatation. If emptying cannot keep up with intake, then the proventriculus will fill with undigested food resulting in impaction and blockage.

Abbreviations

BoDV Borna disease virus-1

FBS Fetal bovine serum

IPA Ingenuity Pathway Analysis

NPY Neuropeptide Y

PaBV Psittacine bornavirus

PDD. Proventricular dilatation disease

qPCR Quantitative polymerase chain reaction

Declarations

Ethics approval and consent to participate

An animal use protocol detailing the experimental protocol for the treatment study and euthanasia guidelines was reviewed and approved by the Texas A&M University Office of Research Compliance, complying with guidelines included in the National Research Council of the National Academies' publication *Guide for the Care and Use of Laboratory Animals*, 8th edition.

Consent for publication

Not Applicable

Competing interests

None of the authors have competing interests

Funding

The Project was funded by the Richard M. Schubot Endowment at Texas A&M University

Authors' contributions

IT and YT designed the study. JC and GJ performed the experiments. IT, YT and JC analyzed the data. All authors read and approved the final manuscript.

Acknowledgements

We wish to acknowledge the assistance Dr Paulina Escandon who provided the infected birds at the completion of her study.

Raw Data

All information is included in the paper and supplementary files.

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Tables

Table 1. The most upregulated and downregulated genes detected in Parrot bornavirus-infected human astrocytes in vitro.

Expr Log Ratio up-regulated		
Molecules	Expr. Value	Expr. Chart
CCR7	↑ 6.323	
ADAM23	↑ 5.564	
PCP4	↑ 5.278	
MID1	↑ 5.176	
SYT1	↑ 4.531	
INHBE	↑ 4.347	
LOXL4	↑ 4.141	
NPY	↑ 4.137	
FBXL7	↑ 3.939	
BARX2	↑ 3.927	

Expr Log Ratio down-regulated		
Molecules	Expr. Value	Expr. Chart
KRT23	↓ -4.771	
EPAS1	↓ -4.348	
ITGA4	↓ -4.186	
ACAN	↓ -4.151	
CRTAP	↓ -3.972	
FGF10	↓ -3.940	
ACTA2	↓ -3.847	
PCDH10	↓ -3.667	
C3	↓ -3.630	
STARD10	↓ -3.583	

Figures

NPY expression in RNA-seq

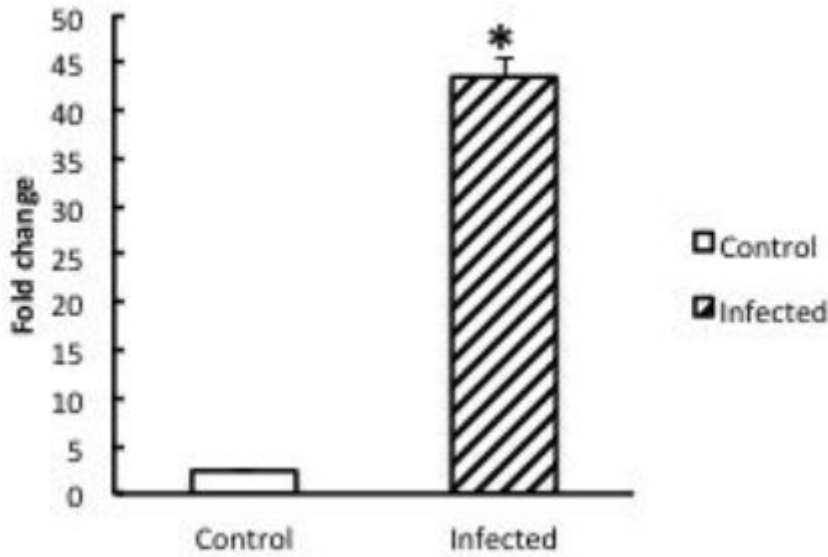


Figure 1

The relative expression of NPY RNA in Psittacine bornavirus-infected and control astrocyte culture in vitro as obtained by RNA-Seq analysis.

NPY expression using q-PCR

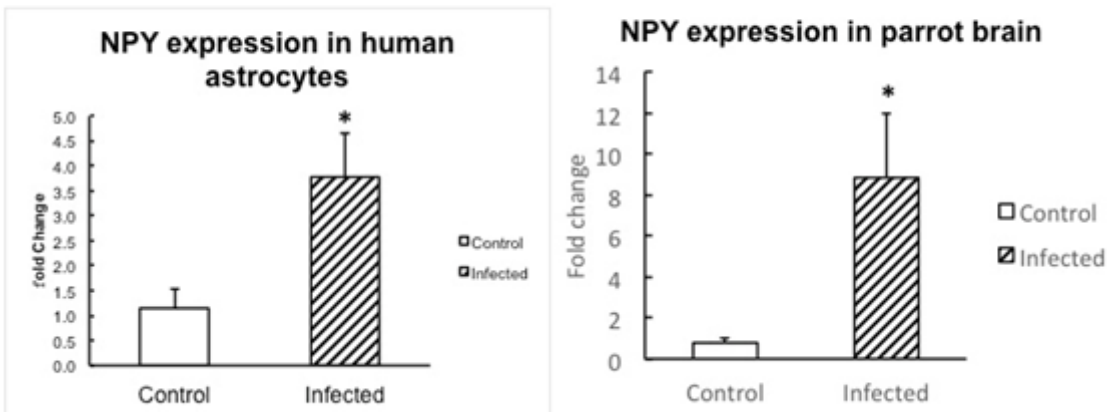


Figure 2

A. The relative expression of NPY RNA in Psittacine bornavirus-infected and control astrocyte cultures as determined by quantitative RT-PCR analysis B. The relative expression of NPY RNA in the brains of infected and non-infected cockatiels using the same quantitative RT-PCR assay.

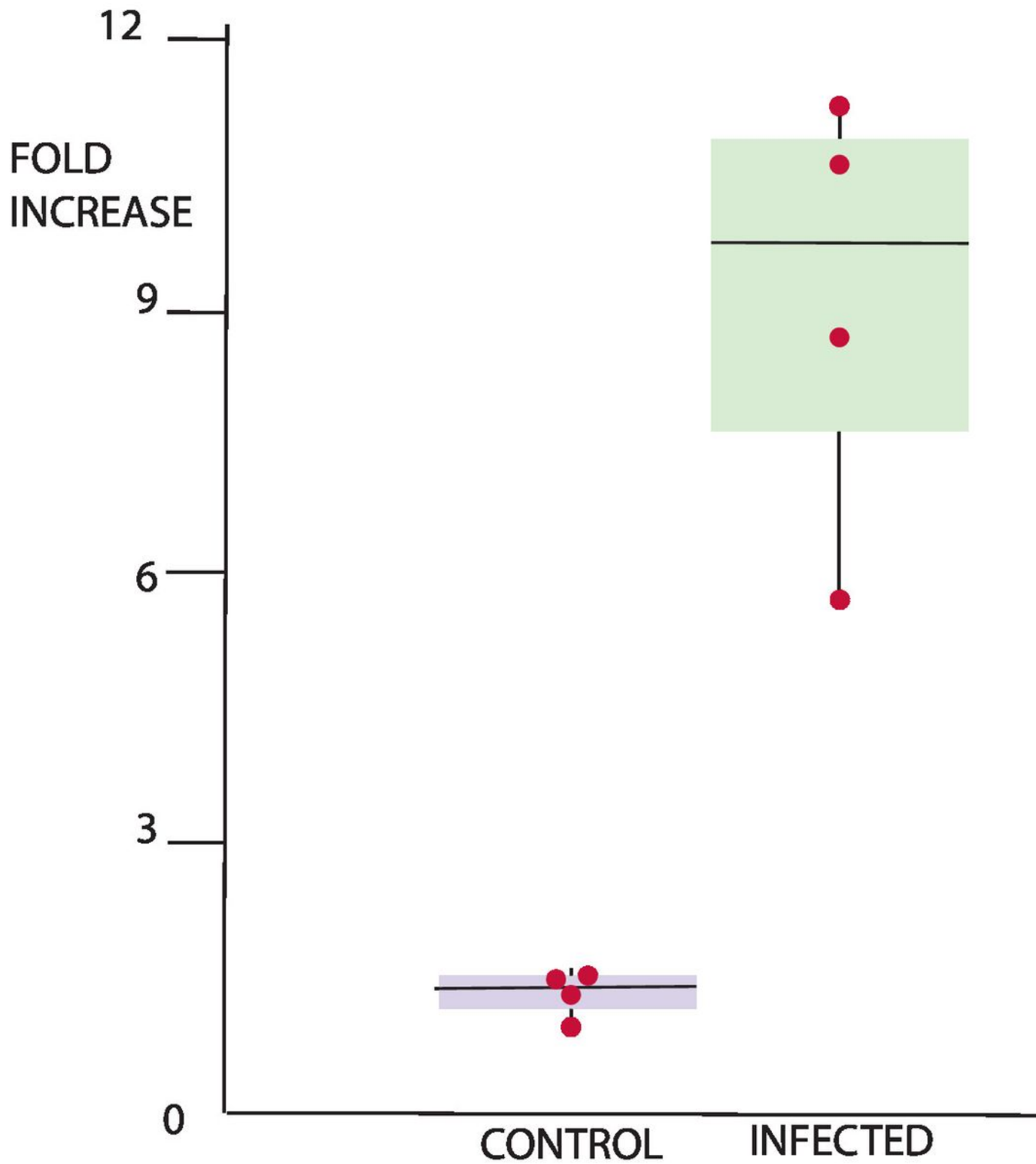


Figure 3

The relative expression of NPY RNA in the individual brains of eight Bornavirus-infected and non-infected cockatiels using the same quantitative RT-PCR assay.

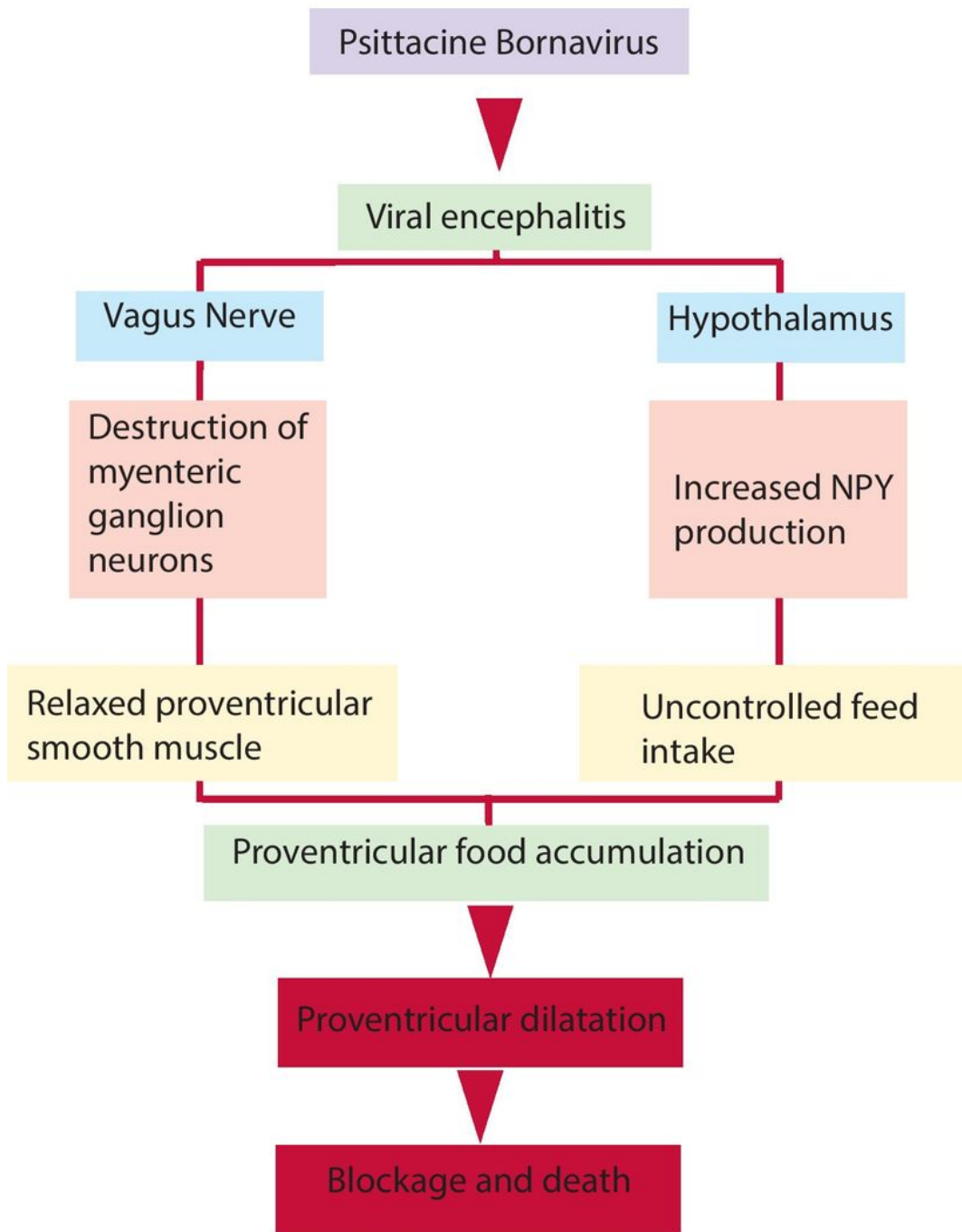


Figure 4

A suggested pathogenesis of proventricular dilatation disease. The dual effects of neuronal damage that permits proventricular relaxation together with overproduction of neuropeptide Y results in a failure in satiation signaling and overfilling of the proventriculus.

Supplementary Files

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- [S1.upregulatedgene.xls](#)