ARCHIVAL REPORT

Neuropeptide Y Receptor Gene Expression in the Primate Amygdala Predicts Anxious Temperament and Brain Metabolism

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Background: Anxious temperament (AT) is identifiable early in life and predicts the later development of anxiety disorders and depression. Neuropeptide Y (NPY) is a putative endogenous anxiolytic neurotransmitter that adaptively regulates responses to stress and might confer resilience to stress-related psychopathology. With a well-validated nonhuman primate model of AT, we examined expression of the NPY system in the central nucleus (Ce) of the amygdala, a critical neural substrate for extreme anxiety.

Methods: In 24 young rhesus monkeys, we measured Ce messenger RNA (mRNA) levels of all members of the NPY system that are detectable in the Ce with quantitative real time polymerase chain reaction. We then examined the relationship between these mRNA levels and both AT expression and brain metabolism.

Results: Lower mRNA levels of neuropeptide Y receptor 1 (*NPY1R*) and *NPY5R* but not *NPY* or *NPY2R* in the Ce predicted elevated AT; mRNA levels for *NPY1R* and *NPY5R* in the motor cortex were not related to AT. In situ hybridization analysis provided for the first time a detailed description of *NPY1R* and *NPY5R* mRNA distribution in the rhesus amygdala and associated regions. Lastly, mRNA levels for these two receptors in the Ce predicted metabolic activity in several regions that have the capacity to regulate the Ce.

Conclusions: Decreased NPY signaling in the Ce might contribute to the altered metabolic activity that is a component of the neural substrate underlying AT. This suggests that enhancement of NPY signaling might reduce the risk to develop psychopathology.

Key Words: Anxiety, behavioral inhibition, depression, prefrontal cortex, rhesus macaque, stress

Anxious temperament (AT) is a dispositional trait that, when present early in life, increases the risk for the subsequent development of anxiety and depressive disorders (1–3). We have established a nonhuman primate model of childhood AT, facilitating the identification of the neural mechanisms underlying the development of early life anxiety. In rhesus monkeys, AT is assessed as a composite of threat-induced freezing behavior, inhibition of vocalizations, and increased plasma cortisol levels (4,5). We previously demonstrated that metabolic activity in the central nucleus (Ce) of the amygdala, indexed with high-resolution [^{18}F]-fluorodeoxyglucose positron emission tomography (FDG-PET), strongly predicts individual differences in AT (6,7). Moreover, we demonstrated a mechanistic role for the Ce as selective lesions decrease AT (8). To understand the molecular mechanisms in the Ce that underlie the at-risk AT phenotype, we recently performed a transcriptome-wide search for AT-related messenger RNA (mRNA) expression within the Ce (9). Among our significant results, we found that increased levels of neuropeptide Y receptor 1 (*NPY1R*) mRNA predict decreased levels of AT. This finding is of particular interest because of the hypothesized role of neuropeptide Y (NPY) in anxiety-like responding and as a resilience factor for stress-related psychopathology. The current study expands on our *NPY1R* mRNA finding by identifying a similar relationship between *NPY5R* mRNA levels in the Ce and AT. We also provide the first detailed description of *NPY1R* and *NPY5R* mRNA distribution in the primate amygdala and associated regions. Lastly, we demonstrate a relationship between *NPY1R* and *NPY5R* mRNA levels in the Ce and metabolic activity in cortical brain regions that have the ability to regulate the Ce.

There is growing evidence that NPY, a 36-amino acid peptide, is a stress-modulating resilience factor (10–12). Moreover, alterations in NPY signaling have been linked to anxiety, depressive, eating, and substance-abuse disorders (10,13,14). Neuropeptide Y is widely expressed in the brain, with high levels present in several brain regions that play a role in modulating the response to potential threat, including the amygdala and hippocampus (10). Work in rodents demonstrates that NPY participates in the regulation of anxiety-like responses (15–21) and has marked anti-stress effects (10,22).

The NPY family includes NPY, which is expressed in the central and peripheral nervous systems as well as pancreatic polypeptide and peptide YY (PYY), which are expressed in the gut (23). The actions of these peptides are mediated by several G protein-coupled, seven-transmembrane domain receptors, including: Y_1, Y_2, Y_4, Y_5, and y_6 (24). Although the *NPY6R* gene is functional in rabbits and mice, it is absent in rats and considered a pseudogene in primates and pigs (25–27). Receptor signaling is mediated by pertussis toxin-sensitive $G_{i/o}$ proteins and, depending on the cell-type in which they are expressed, can inhibit cyclic adenosine monophosphate formation, alter intracellular calcium ion mobilization, and modulate calcium ion and potassium ion channels (28). There is considerable evidence linking Y_1, Y_2, and Y_5 to the

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effects that central NPY exerts on anxiety-like responding (29–32). The anxiolytic responses to NPY administration are thought to be mediated in part by Y_1 and, to a lesser extent, Y_5 in the amygdala (20,33), hippocampus (15), septum (34), and locus coeruleus (35). In contrast, activation of Y_2 is thought to produce anxiogenic-like responses (30,36–38).

To assess the contribution of the NPY system to early-life AT in primates, we focused on mRNA expression levels in the Ce, a key component of the AT neural circuit. Our aim was to extend our earlier microarray findings by examining relations between expression levels of all members of the NPY system in the Ce, AT, and brain metabolism, indexed with FDG-PET. To understand the selectivity of the effects of the Ce NPY system in relation to AT, we also assessed the relationship between NPY system gene expression in a region that is not a core component of the neural circuit underlying AT, the motor cortex (6,7). Moreover, because there is no detailed description of *NPY1R* and *NPY5R* mRNA levels in either the human or nonhuman primate amygdala, we also used in situ hybridization to characterize expression patterns for *NPY1R* and *NPY5R* mRNA across the major amygdala nuclei and adjacent brain regions. Lastly, to define potential neural circuits that underlie the influences of the NPY system on AT, we looked at metabolic activity throughout the brain to identify regions outside of the Ce where variation in metabolism is correlated with *NPY1R* or *NPY5R* mRNA levels in the Ce.

Methods and Materials

Overview

Methods were similar to those previously described in detail and are only briefly summarized here (7,12). A detailed description of the subjects as well as select methods that were not employed in prior work by our group is provided in Supplement 1. We assessed individual differences in the AT phenotype and brain metabolic activity with the well-validated, widely used No-Eye Contact condition of the human intruder paradigm and high-resolution FDG-PET. The AT phenotype was defined as a composite score of behavioral (increased freezing and decreased coo vocalizations) and hormonal measures (increased plasma cortisol) in response to the mildly threatening No-Eye Contact challenge. A magnetic resonance imaging scan was acquired in a separate session to aid in image registration. At the end of the study, subjects were sacrificed, and brain tissue was obtained from the Ce and motor cortex for RNA extraction and quantification by microarray analysis and quantitative real-time polymerase chain reaction (qRT-PCR). A series of regression models was used to test relations between AT and mRNA expression levels for members of the NPY family that were detectable in the Ce (*NPY*, *NPY1R*, *NPY2R*, and *NPY5R*); it was not possible to reliably quantify pancreatic polypeptide, *PYY*, or *NPY4R*. In cases where expression levels predicted AT (*NPY1R* and *NPY5R*), we used in situ hybridization to assess the pattern of expression across the amygdala and neighboring regions. We also used whole-brain FDG-PET to test whether mRNA expression levels in the Ce (ex vivo) are correlated with metabolism in distal regions of the brain (in vivo).

Results

Elevated *NPY1R* and *NPY5R* mRNA Levels in the Ce Selectively Predict Decreased AT

We previously demonstrated that metabolic activity in the rhesus Ce strongly predicts individual differences in AT (7) (Figure 1A,B,C) and that the *NPY1R* was one of 139 genes that had mRNA expression levels in the Ce as determined by microarray analysis that predicted significant variation in the AT phenotype (false discovery rate $q < .05$) (9). Given the known role of the NPY system in anxiety-like responding, in the present study we sought to define the relationship between AT, Ce metabolism, and the expression of all members of the NPY family of genes. As shown in Figures 1D and 1E, analyses of qRT-PCR-determined gene expression levels in the Ce revealed significant negative correlations between AT and both *NPY1R* ($t = -2.28$; $p = .035$) and *NPY5R* ($t = -2.55$; $p = .020$). Interestingly, there was a trend for *NPY1R* and *NPY5R* mRNA levels to be correlated with each other in the Ce ($r = .35$, $p = .11$). In contrast, AT was unrelated to variation in the expression of *NPY* ($t = .6$, $p = .553$) and *NPY2R* ($t = -1.56$, $p = .135$) in the Ce. It was not possible to reliably quantify *PYY* or *NPY4R*, likely due to very low levels of expression in the Ce. The *NPY6R* mRNA was not included in our analysis, because it is considered a nonfunctional pseudogene in primates (27). Collectively, these results replicate our published gene chip finding on the *NPY1R* and extend these results to the *NPY5R* by demonstrating an anatomically selective relationship between mRNA expression levels in the Ce and AT expression.

To assess the regional selectivity of the correlation between *NPY1R* mRNA and AT as well as *NPY5R* mRNA and AT, we examined mRNA expression levels for these two genes in a region of the motor cortex that is not a core component of the neural substrate underlying AT (7). Gene expression analysis with qRT-PCR did not reveal a significant correlation between AT and motor cortex expression levels for either *NPY1R* mRNA ($t = .18$, $p = .856$) or *NPY5R* mRNA ($t = -1.2$, $p = .245$). Additionally, there was no significant association between *NPY1R* mRNA levels in the motor cortex and Ce ($r = .37$, $p = .084$) or *NPY5R* mRNA levels in the motor cortex and Ce ($r = .18$, $p = .41$). The motor cortex is a brain region involved in the expression of locomotion (9), and highlighting the anxiety-specific nature of these results, there was no significant correlation between motor cortex *NPY1R* or *NPY5R* mRNA levels and locomotion (*NPY1R*: $t = .05$, $p = .957$; and *NPY5R*: $t = .23$, $p = .824$).

Expression Pattern for *NPY1R* and *NPY5R* mRNA in the Nonhuman Primate Amygdala and Neighboring Regions

To assess the regional expression of *NPY1R* and *NPY5R* mRNA in the rhesus amygdala, in situ hybridization was performed with tissues slices obtained through the same region of the Ce that was used for PCR analysis in the other hemisphere. The pattern of *NPY1R* and *NPY5R* hybridization signals are shown in Figure 2. For *NPY1R* mRNA, hybridization signals are seen throughout the entire extent of the amygdala. Qualitatively, the highest levels of expression are found in the lateral and medial nuclei, amygdalopiriform cortex transition area and the ventral cortical amygdala nucleus. Moderate levels of expression are seen in the Ce and parvicellular division of the basomedial nucleus. For the *NPY5R* mRNA, a diffuse signal throughout the extent of the amygdala was observed. This signal was considerably weaker than the *NPY1R* signal, necessitating a significantly longer exposure time on the phosphor screen (13 days vs. 1 day for *NPY1R* mRNA). However, it is evident that there are relatively high levels of expression in the medial amygdala, moderate levels of expression in the lateral amygdala, and relatively low levels of expression in the Ce (Figure 2). Interestingly, because half the samples were obtained from each hemisphere, it was possible to use our qRT-PCR data to assess hemispheric differences in expression within the Ce. The levels of *NPY1R* mRNA did not differ between hemispheres (left .80 $\pm$ .08; $n = 11$ vs. right .67 $\pm$ .04; $n = 12$; $t = 1.47$; $p = .16$), but *NPY5R* mRNA levels were 20% higher in the left hemisphere

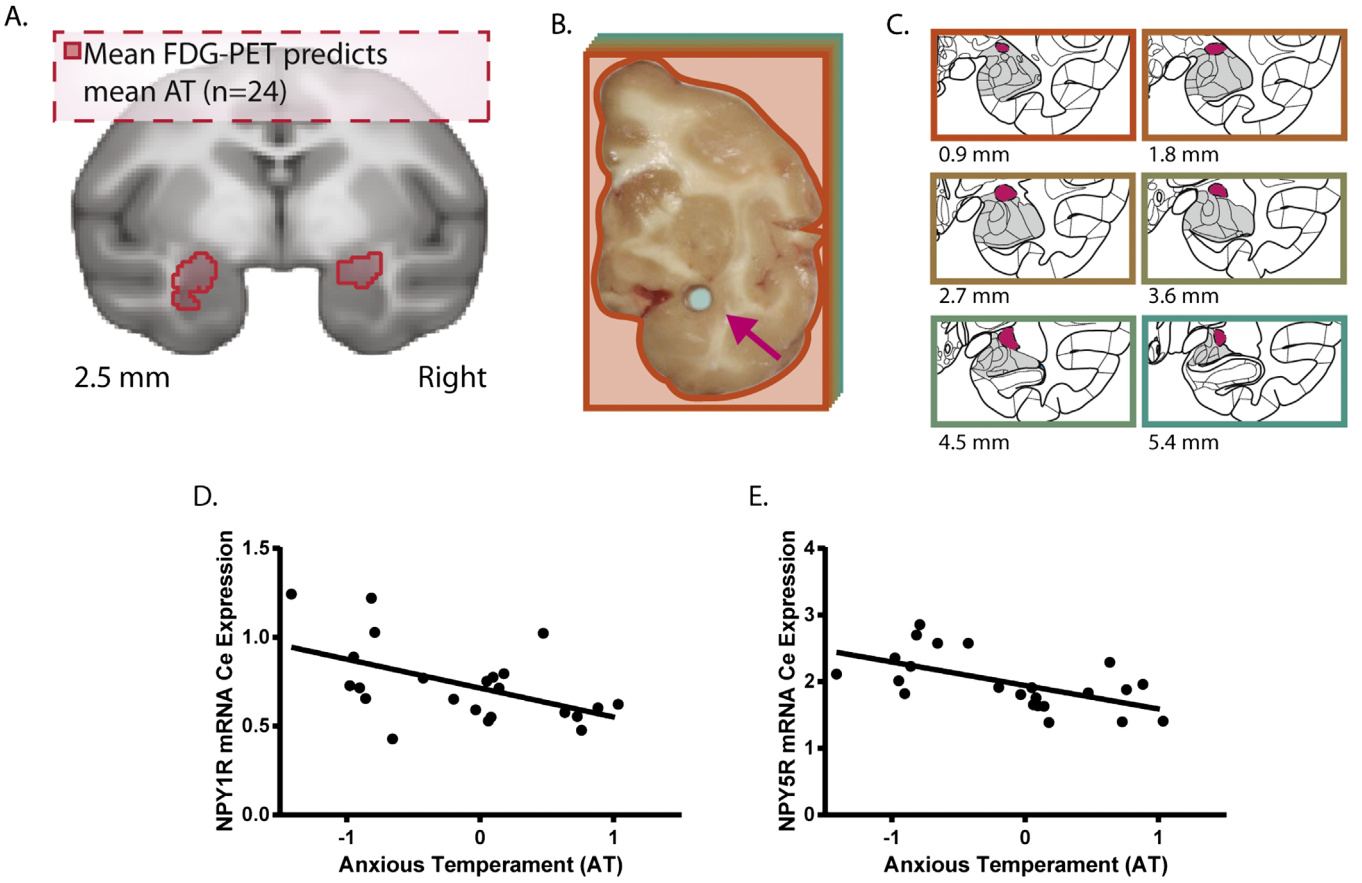

Figure 1. Higher levels of anxious temperament (AT) are associated with reduced *NPY1R* and *NPY5R* messenger RNA (mRNA) expression in the primate central nucleus of the amygdala (Ce) as assessed by quantitative real-time polymerase chain reaction (qRT-PCR). **(A)** Metabolic activity in the Ce strongly predicts variation in AT. The bilateral regions identified by the red trace correspond to the 95% confidence interval for the maximal voxel-wise correlation between amygdala metabolic activity and AT. **(B)** The functionally defined location of the Ce punch (see Methods and Materials). **(C)** Atlas plates corresponding to the 3-mm tissue punch [reprinted from a published atlas (66) with permission from Elsevier, copyright 2009]. The Ce is depicted in red, and the numbers indicate distance posterior to the anterior commissure. **(D, E)** Correlational analyses between AT and *NPY1R* and *NPY5R* mRNA levels determined by qRT-PCR analysis. Scatter plots depicting the significant correlations between AT and the expression of *NPY1R* ($t = -2.28$, $p = .035$, $n = 23$) and of *NPY5R* ($t = -2.55$, $p = .020$, $n = 23$) as detected by qRT-PCR. **(A, B, C)** Adapted with permission from our previously published figure (7,66). FDG-PET, [^{18}F]-fluorodeoxyglucose positron emission tomography.

compared with the right hemisphere (left 2.18 ± .14; $n = 11$ vs. right 1.81 ± .08; $n = 12$; $t = 2.34$; $p = .029$).

These tissue sections provided us with the opportunity to examine mRNA expression patterns in other brain regions that are present in the same anterior/posterior plane as the amygdala. For *NPY1R* mRNA, the strongest signals are present throughout the cortex. These cortical signals tended to be laminar-specific, and in general the signals were strongest in the superficial and deep cortical layers and less intense in the middle layers, although this pattern was less evident in the temporal cortex. In addition, the cortical signals were most intense in the ventral half of the tissue section and include the somatosensory cortex, superior temporal sulcus, insular cortex, temporal cortex, and entorhinal cortex. There is also significant expression in the anterior cingulate and ventral medial region of the head of the caudate nucleus. Although the full extent of the claustrum contained in this section has moderate levels of *NPY1R* expression, the strongest expression is seen at the ventral tip and represents some of the strongest expression in the entire section. There was also significant expression seen in several midline structures, including the stria terminalis, retrochiasmatic part of the supraoptic nucleus, arcuate nucleus, anterior paraventricular region of the thalamus, and the septo-hippocampal nucleus. For *NPY5R* mRNA, there is a diffuse signal throughout the extent of the section including regions of the temporal cortex and somatosensory cortex that was less laminar-specific in comparison with *NPY1R* mRNA. There are also signals in the ventral tip of the claustrum, internal capsule, and a band across the central portion of the putamen. The most intense mRNA signals are in midline structures including the optic tract, retrochiasm of the supraoptic nucleus, and the medial division of the arcuate nucleus.

Assessing the Relationship Between *NPY1R* and *NPY5R* Gene Expression and Metabolism Throughout the Brain

We used whole-brain voxel-wise regressions to identify brain regions where *NPY1R* or *NPY5R* mRNA levels significantly predict metabolism ($p < .005$, uncorrected) (for detailed results, see Tables 1 and 2). As shown in Figure 3, individuals with higher levels of *NPY1R* mRNA in the Ce were characterized by increased metabolism in the right dorsolateral prefrontal cortex (dlPFC) and decreased metabolism in the pregenual anterior cingulate cortex (pgACC). As shown in Figure 4, individuals with higher levels of *NPY5R* mRNA in the Ce were characterized by increased metabolism in the dorsal prefrontal cortex (dPFC; area 8). In terms of Ce metabolic activity, the mean FDG-PET signal extracted from the 95%

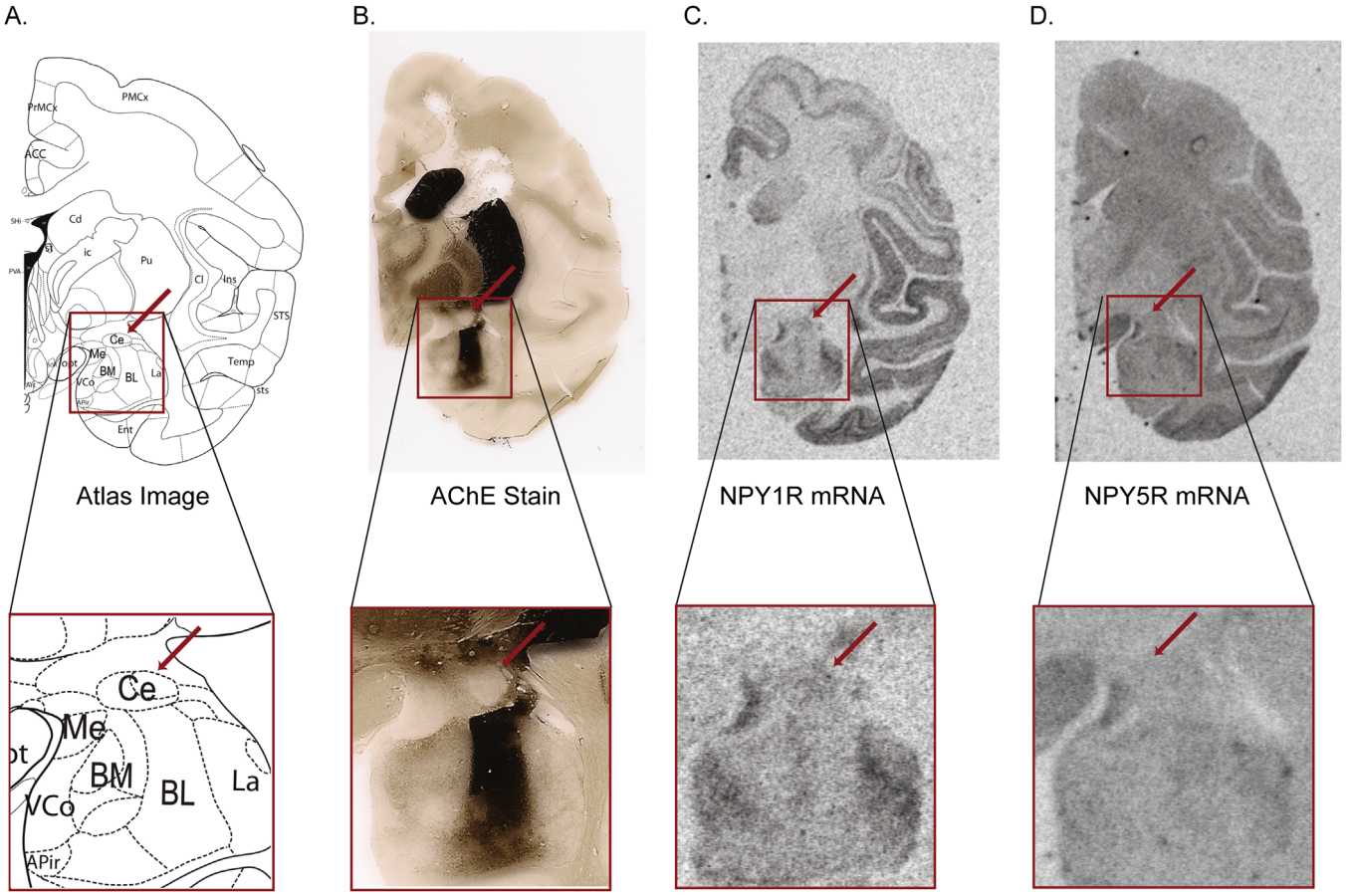

Figure 2. *NPY1R* messenger RNA (mRNA) and *NPY5R* mRNA expression in the amygdala region assessed by in situ hybridization. **(A)** Atlas image of the rhesus brain at the level of 2.25 mm posterior to the anterior commissure (−5.85 mm bregma) to identify regions discussed in the Results section [reprinted from a published atlas (66) with permission from Elsevier, copyright 2009]. **(B)** Acetylcholinesterase (AChE) stain of an adjacent section used to identify the structure of the rhesus amygdala. **(C)** Signal for *NPY1R* mRNA. **(D)** Signal for *NPY5R* mRNA in coronal brain sections at the level of the amygdala. The red arrow indicates the location of the central amygdala (Ce). ACC, anterior cingulate cortex; APir, amygdalopiriform cortex; Arc, arcuate nucleus; BL, basolateral amygdala; BM, basomedial amygdala; Cd, caudate nucleus; Cl, claustrum; Ent, entorhinal cortex; ic, internal capsule; Ins, insular cortex; La, lateral amygdala; Me, medial amygdala; opt, optic tract; PMCx, primary motor cortex; PrMCx, premotor cortex; Pu, putamen; PVA, anterior paraventricular region of the thalamus; SHi, septo-hippocampal nucleus; SOR, retrochiasm of the supraoptic nucleus; st, stria terminalis; STS, somatosensory cortex; sts, superior temporal sulcus; Temp, temporal cortex; VCo, ventral cortical amygdala nucleus.

confidence interval most predictive of AT (Figure 1) showed a weak trend toward a significant negative correlation with *NPY5R* mRNA levels as assessed by qRT-PCR analysis ($t = -1.63$, $p = .12$). There was no significant correlation with *NPY1R* mRNA ($t = -.01$, $p = .99$).

Discussion

The current work links the NPY system to AT by examining the expression of NPY system genes in the Ce. Specifically, we

Table 1. *NPY1R*-Related Brain Regions

Direction of Relationship	Hemisphere	Cluster	Cluster Volume (mm^2)	Local Maxima Within Region	*t*	*p*	Location Relative to Anterior Commissure (mm)		
							x	y	z
Positive	Right	Dorsolateral prefrontal cortex	78.8574	Area 46/area 47	6.67	5.42×10^{-6}	13.750	23.750	8.750
Negative	Right	Thalamus/caudate	51.0254	Anterodorsal thalamus/caudate	−6.37	9.22×10^{-6}	4.375	−5.000	7.500
	Left	Pregenual anterior cingulate	73.9746	Area 32	−6.75	4.69×10^{-6}	−2.500	16.875	7.500
	Right	Motor cortex	31.0059	Area 4	−5.55	4.44×10^{-5}	0.625	−2.500	23.750

Regions with a significant correlation ($p < .05$, two-tailed uncorrected) between metabolic activity and *NPY1R* messenger RNA expression as assessed by quantitative real-time polymerase chain reaction.

Table 2. *NPY5R*-Related Brain Regions

Direction of Relationship	Hemisphere	Cluster	Cluster Volume (mm^2)	Local Maxima Within Region	*t*	*p*	Location Relative to Anterior Commissure (mm) x	y	z
Positive	Left	Dorsal prefrontal cortex	24.1699	Area 8	4.52	3.51×10^{-4}	−7.500	11.875	18.125
	Left	Visual cortex	23.4375	V1	4.66	2.64×10^{-4}	−2.500	−43.750	2.500
	Left	Dorsal prefrontal cortex	27.0996	Area 8	5.03	1.24×10^{-4}	−11.875	5.625	12.500
	Left	Premotor cortex	51.2695	Area 6	5.24	8.08×10^{-5}	−8.125	−1.250	21.250
	Right	Motor cortex	15.8691	Area 4	5.85	2.47×10^{-5}	13.750	−1.875	21.250
Negative	Right	Thalamus	78.3691	Anterior ventral thalamus	−8.07	4.96×10^{-7}	4.375	−3.750	3.750
	Left	Somatosensory cortex	13.6719	Area S2	−4.67	2.54×10^{-4}	−16.875	−8.125	8.125
	Right	Putamen	17.5781	Putamen	−4.17	7.22×10^{-4}	15.000	−8.125	4.375
	Left	Posterior cingulate	8.5449	Area 30/area 29	−4.15	7.58×10^{-4}	−3.125	−13.750	8.750

Regions with a significant correlation ($p < .05$, two-tailed uncorrected) between metabolic activity and *NPY5R* messenger RNA expression as assessed by quantitative real-time polymerase chain reaction.

demonstrate that individuals with increased expression of *NPY1R* or *NPY5R* mRNA in the Ce are characterized by lower levels of the anxious phenotype. We expanded on these findings to describe the distribution of *NPY1R* and *NPY5R* mRNA in the rhesus amygdala and surrounding regions. Lastly, we identify several brain regions where metabolic activity is predicted by the Ce expression of the *NPY1R* or *NPY5R* genes.

The inverse relationship between AT and *NPY1R* and *NPY5R* mRNA levels suggests that lower anxiety levels are accompanied by increased NPY receptor expression in the Ce, which is consistent with studies indicating that Y_1 and Y_5 mediate the anxiolytic-like effects of NPY in the brain (29,31,32). Because extreme and stable AT is a risk factor for the development of psychopathology, the current results are in agreement with the postulated role of the NPY neurotransmitter system as a resilience factor that decreases the risk to develop stress-related psychopathology (39). It should be noted that the studies reported here assessed NPY receptor mRNA levels; confirmation of the mRNA findings at the protein level would provide further confidence in the results. Although there is often strong agreement between variations in mRNA and protein levels, ultimately our findings need to be confirmed at the protein level with immunodetection or in vitro autoradiography.

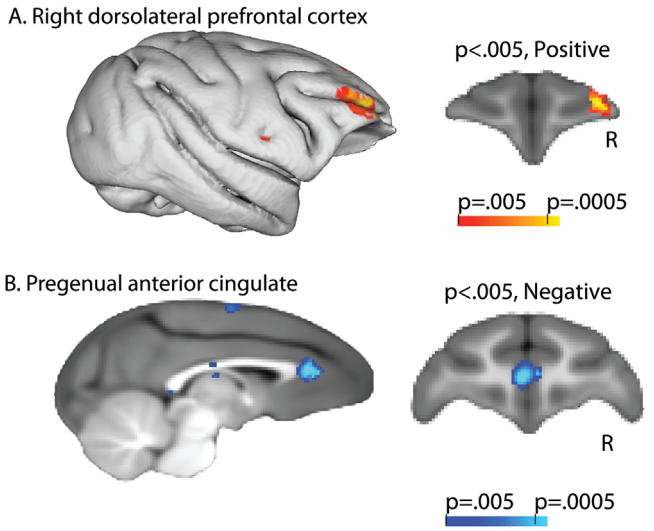

Figure 3. The Ce *NPY1R* mRNA levels predicted metabolism in the prefrontal and cingulate cortices. Voxel-wise analysis revealed that Ce *NPY1R* mRNA levels predicted **(A)** increased metabolism in the right (R) dorsolateral prefrontal cortex and **(B)** decreased metabolism in the pregenual anterior cingulate. Color variation represents level of statistical significance as defined in horizontal color bars with shades of red through yellow for positive correlations and shades of blue for negative correlations. Other abbreviations as in Figure 1.

We failed to find a significant relationship—consistent with a Ce-specific regulation of NPY-receptor gene expression in AT—between AT and *NPY1R* and *NPY5R* mRNA expression in motor cortex, which is not a core component of the AT circuit. The levels of *NPY1R* and *NPY5R* mRNA in the motor cortex were not correlated with locomotion even though motor cortex metabolic activity was correlated with locomotion, consistent with an AT-specific relationship. Moreover, *NPY1R* and *NPY5R* mRNA levels in the motor cortex were not significantly correlated with expression in the Ce. These results indicate that the relationship between *NPY1R* and *NPY5R* mRNA levels and AT is not general across brain regions and that the correlations are not simply nonspecific markers for brain metabolism or other behaviors.

It is interesting that mRNA levels for both *NPY1R* and *NPY5R* show an inverse correlation with AT. In humans, the genes for these two receptors are located on the same region of chromosome 4 in opposite orientations and share common transcriptional control regions (40). A search of the rhesus genome reveals a similar organization with these two genes located on chromosome 5 in opposite orientations. It is possible that in the Ce there are shared transcription factors that control the amount of expression of these two genes, which is consistent with the observed trend for a correlation between *NPY1R* and *NPY5R* mRNA levels in the Ce. Understanding the regulation of the transcription factors that control expression of these two genes in the Ce sets the stage for identifying novel targets for pharmacological manipulation of NPY receptor expression in relation to anxiety.

It is possible that the variations in NPY receptor mRNA levels are determined by DNA sequence variation in the promoter

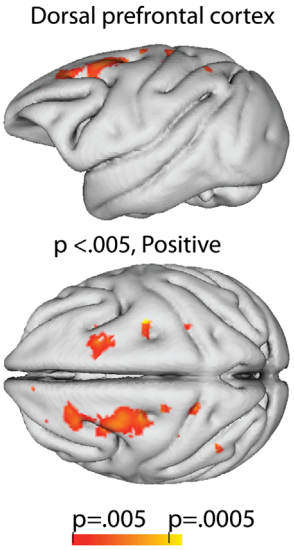

Figure 4. Central amygdala *NPY5R* messenger RNA levels predicted increased metabolism in the dorsal prefrontal cortex. Voxel-wise analysis revealed that central amygdala *NPY5R* messenger RNA levels predicted increased metabolism in the dorsal prefrontal cortex. Color variation represents level of statistical significance as defined in horizontal color bar.

region of the *NPY1R* and *NPY5R* genes. In fact, there is evidence that a polymorphism in the promoter region of the human NPY gene (single nucleotide polymorphism rs16147) regulates the level of NPY mRNA and protein in the brain and is associated with differences in stress responsiveness and anxiety symptoms (41,42). Moreover, polymorphisms in the *NPY1R* and *NPY5R* genes have been linked to drug addiction and variations in diet (43–45). In future studies it will be of value to determine whether these variations exist in the rhesus monkey and whether they influence the function of the brain NPY system in relation to anxiety.

The rhesus monkey model of extreme AT is associated with persistently elevated levels of anxiety as well as chronically elevated Ce metabolism (7). Although NPY receptor expression is not related to Ce metabolism in this study, it is possible that the decreased levels of NPY receptor expression impair NPY signaling, resulting in extreme AT. Conversely, there might be an increase in NPY signaling in an attempt to compensate for the extreme AT phenotype that then results in downregulation of Ce *NPY1R* and *NPY5R* expression.

Few previous reports have described the role of the Y_5 in anxiety-like responding, in part, because of the lack of selective ligands that differentiate between the various NPY receptor subtypes. Furthermore, outside of hypothalamic regions, Y_5 is expressed at significantly lower levels compared with the Y_1 receptor. The present study highlights the important role of the Y_5 in AT and anxiety in general, suggesting that future studies aimed at more fully delineating the role of the Y_5 in the anxiety-like responding are likely to prove fruitful.

This study is the first to provide a detailed description of the expression of *NPY1R* and *NPY5R* mRNA in the amygdala and neighboring regions in nonhuman primates. The signal for *NPY1R* mRNA was strong and widespread in several cortical areas, consistent with prior reports in humans (46–48). Although there are no published studies describing the mRNA distribution of *NPY1R* in the rhesus brain, a PET and in vitro autoradiography study employing ^{18}F-Y1-973 revealed patterns of receptor localization that are in general agreement with our observations (49). The widespread distribution throughout the cortex is also seen in the rat and mouse brain where *Npy1r* mRNA is detectable in essentially all cortical fields (50). In all of these species, there is a layer specific pattern to the *NPY1R* mRNA expression with the layers of highest expression varying between cortical regions and between species. Regarding the expression pattern of *NPY1R* mRNA in the amygdala, only limited information is available for the primate brain, with moderate expression levels being reported in the human amygdala, but the report did not assess the differential distribution across the various amygdala nuclei (47,48). In the rat amygdala, the highest mRNA expression is seen in the amygdalohippocampal transition area, amgydala-piriform transition area, anterior basomedial nucleus and posteroventral medial nucleus, while a small number of intensely labelled cells are present in the Ce, and weakly labeled cells were scattered throughout the basomedial, basolateral, and lateral nuclei. Similar mRNA expression patterns are seen in the mouse (50–52). The mRNA expression patterns are in general agreement with two detailed studies examining Y_1 immunoreactivity in rat brain (51,53). The expression pattern in the rodent amygdala is very similar to that described in the present study for the rhesus amygdala and suggests there is significant cross-species conservation in the expression of the *NPY1R* transcript.

The expression of the *NPY5R* mRNA as determined by in situ hybridization tended to be less robust and more localized to hypothalamic and amygdala regions compared with the *NPY1R* mRNA expression. There have been several published reports describing the mRNA distribution of *NPY5R* in the rodent brain (54–58). The results from these studies are in general agreement with the mRNA expression pattern reported here and previously reported in the human brain (58,59). There were regions of common expression between the *NPY1R* and *NPY5R*, and these included the Ce and lateral and medial nuclei of the amygdala as well as supraoptic nucleus, arcuate nucleus, and temporal and somatosensory cortex. This is consistent with studies in the rat brain, where the presence of *Npy5r* mRNA always corresponded with the presence of *Npy1r* mRNA but not vice versa (54). This might arise from the overlapping structure of these genes on the chromosome and the shared transcriptional control elements. The significantly higher Ce expression of *NPY5R* mRNA in the left hemisphere compared with the right is noteworthy in light of evidence describing hemispheric difference in the control and expression of emotion via the amygdala (60).

In terms of *NPY1R* and *NPY5R* mRNAs in relation to metabolic activity of the Ce region, there was no significant correlation with glucose metabolism in the Ce. Nevertheless, whole-brain voxel-wise analyses revealed several other regions where *NPY1R* or *NPY5R* mRNA expression predicted metabolism. Brain regions that had metabolic activity that were significantly correlated with *NPY1R* expression included the dlPFC and pgACC. Similarly, for the *NPY5R* mRNA, regions with metabolic activity that significantly positively correlated with mRNA levels in the Ce included the dPFC. These prefrontal cortical regions have previously been shown to be part of the circuit that regulates the activity of the amygdala (61,62). Thus, our data suggest that *NPY1R* and *NPY5R* mRNA levels in the Ce might be regulated by the influences of prefrontal cortex on the *NPY1R*- and *NPY5R*-expressing neurons. Alternatively, *NPY1R*- or *NPY5R*-expressing Ce neurons could modulate metabolism in these brain regions via direct or indirect mechanisms.

It is very relevant that variations in Ce NPY receptor mRNA expression that are associated with AT are also associated with

variations in the metabolic activity of the pgACC and right dlPFC. This is because alterations in both of these brain regions have been associated with anxiety disorders. For example, generalized anxiety disorder has been linked to impaired functional connectivity between the pgACC and the amygdala (63). In addition, adolescents with generalized anxiety disorder show greater activation to fearful faces in a distributed network centered on the anterior cingulate cortex (64). With regard to the right dlPFC, high-frequency (10-Hz) repetitive transcranial magnetic stimulation of this region has been shown to decrease anxiety symptoms in posttraumatic stress disorder (65).

In conclusion, *NPY1R* and *NPY5R* mRNA expression levels within the Ce are negatively correlated with AT and predict altered metabolic activity in prefrontal regions that are thought to regulate the amygdala. Higher levels of expression of these receptor subtypes would be expected to increase the capacity for NPY signaling in this region. The NPY in the amygdala has been hypothesized to suppress anxiety-like responding, and NPY has a putative role as a resilience factor. It is possible that children that express more NPY receptors in the Ce region might have a lowered anxiety response to threatening situations that might protect against the development of stress-related psychopathologies such as anxiety and depression. Moreover, these data suggest a potential link between prefrontal metabolic mechanisms and Ce molecular mechanisms that might underlie resilience. Future studies aimed at genetic manipulation of the NPY system in the Ce in rodent as well as primate species will provide additional evidence to support this hypothesis. Because the present findings are directly relevant to at-risk early anxious dispositions, treatment strategies targeting the NPY system might have therapeutic benefit in the prevention of stress-related anxiety disorders in at-risk children. It will also be of interest to determine whether behavioral treatments that promote resilience impact the NPY system.

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Neuropeptide Y Receptor Gene Expression in the Primate Amygdala Predicts Anxious Temperament and Brain Metabolism

Supplemental Information

Supplemental Methods

All subjects (*N* = 238) were indexed for anxious temperament (AT) using a combination of behavioral and hormonal measures, and brain metabolic activity was subsequently assessed using [^{18}F]-fluorodeoxyglucose - positron emission tomography (FDG-PET) as described in detail below. A subset of animals (*n* = 24) were chosen for quantification of gene expression levels for the neuropeptide Y (NPY) family of genes. Robust regression analysis was used to test relations between AT and mRNA expression levels for the NPY family members that were detectable in the central nucleus of the amygdala (Ce). For those genes that had expression levels that predicted levels of AT (*NPY1R* and *NPY5R*), *in situ* hybridization analysis was used to define expression patterns in the amygdala and neighboring regions. Lastly, whole brain FDG-PET was used to determine if Ce gene expression levels for NPY1R or NPY5R predicted brain metabolic activity at distal sites.

Subjects

Behavior and brain metabolism were initially characterized in 238 young rhesus monkeys (*Macaca mulatta*) injected with FDG and exposed for 30-minutes to the No-Eye Contact (NEC) condition (described below) that elicits the AT phenotype. The details of the imaging of these monkeys have been previously described (1-3). At the time of behavioral testing/FDG-PET scans, the mean age was 2.41 years (SD = 0.92 years; 51.3% female), which is considered to be peripubertal. Animal housing and experimental procedures were in accordance with institutional guidelines. Monkeys were mother-reared and paired-housed at the

Wisconsin National Primate Research Center or Harlow Center for Biological Psychology. From this larger sample, 24 male monkeys were assessed two additional times using FDG-PET in the NEC condition for a total of three assessments over a period of 6 to 18 months (for additional details, see Ref. (3)). Between the second and third assessment, half of the animals were relocated every 5 days over a period of 3 weeks; the other half of the animals remained in their home cages. Relocation did not have any significant effects on behavior or physiology (3). The separation between the second and third assessments varied from 27 to 77 days with an average of 50 days and standard deviation of 10 days, and varied in duration from the initial assessment as previously described (3). Housing and experimental procedures complied with the animal care and use guidelines of the United States National Institutes of Health and were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee.

Behavioral Assessment

During the NEC challenge, a human intruder enters the test room and presents his or her profile to the monkey while avoiding eye contact (4). Behavior was unobtrusively assessed via a closed-circuit television system by an experienced rater (4). Freezing was defined as a period of ≥ 3 sec of tense body posture, no vocalizations, and no locomotion except for slow movements of the head. Coo vocalizations were defined as audible calls made by rounding and pursing the lips with an initial increase and subsequent decrease in frequency and intensity. Locomotion was defined as one or more full movements at any speed in any direction, including such behavior as dropping from ceiling to floor.

Cortisol Assessment

Following 30 min exposure to the NEC challenge, animals were anesthetized and blood was collected. Blood sampling occurred between 08:45 and 14:45 hours, and approximately 6

min elapsed between the end of the NEC and blood collection. Plasma cortisol levels were quantified using the DPC Coat-a-count assay (Siemens, Los Angeles, CA). Samples were diluted 8-fold prior to being measured in duplicate, and the average ED_{80} and ED_{20} of the assay were 1.1 µg/dL and 36.9 µg/dL, respectively. The inter-assay and intra-assay coefficients of variation were 6.6% and 4.0%, respectively.

Computing Anxious Temperament Composite

AT is a composite of behavioral (freezing and cooing) and hormonal measures (cortisol) (1, 2, 5). Each appropriately-transformed measure [-1 x cooing^{½}, cortisol, $\log_e$(freezing)] was residualized to remove variance linearly predicted by age and, in the case of cortisol, time-of-day. Cooing values were reflected to ensure consistent signs across the three component measures. The mean of the *Z*-transformed standardized measures obtained during the three assessments was used to compute average AT for each subject (1, 5).

[^{18}F]-FDG-PET Acquisition

The procedure for high-resolution FDG-PET assessment of brain metabolic activity has previously been described in detail (1-3). Scanning was performed using a microPET P4 scanner (Concorde Microsystems, Inc., Knoxville, TN; (6)) with an intrinsic resolution of approximately 2-mm full-width at half-maximum.

Tissue and RNA Isolation

Twenty-four monkeys were sacrificed and brain tissue was obtained and cut into slabs prior to freezing for storage at -80°C. From each monkey, one hemisphere was dissected into 14.5 mm slabs in preparation for cryostat sectioning for *in situ* hybridization. The remaining hemisphere was dissected into 4.5 mm slabs for tissue punches. The hemisphere used for each procedure was counterbalanced across monkeys. At the start of the molecular analyses, tissue

slabs were subsequently thawed to obtain punches of the Ce region as previously described (3). In addition to the Ce, a control brain region corresponding to the primary motor cortex was obtained. The Ce region that was punched corresponds to the amygdalar FDG-PET signal that was most predictive of AT (Figures 1A and 1B in the main report), whereas the primary motor cortex is not a core component of the neural circuit that underlies AT (1). RNA was extracted from the Ce and motor cortex samples using Qiagen RNeasy plus mini kit (Valencia, CA).

Gene Expression Analysis

For quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis, the cDNA was reverse transcribed from the RNA using SuperScript Vilo (Life Technologies, Carlsbad, CA). The cDNA served as template for qRT-PCR using TaqMan probes and the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The TaqMan probe and primer sets were custom designed by Applied Biosystems to target the same regions targeted by the Affymetrix probe sets on the Affymetrix GeneChip rhesus macaque genome arrays. These regions corresponded to the following: NPY (GenBank #NM_001032814; bases 57-384; AffyID MmugDNA.43201.1.S1_at), peptide YY (GenBank #NM_001113958; bases 113-543; Affy ID MmugDNA.31444.1.S1_s_at), NPY receptor 1 (NPY1R) (GenBank # NM_001032833; bases 984-1152; Affy ID MmuSTS.3013.1.S1_at), NPY receptor 2 (NPY2R) (GenBank # NM_001032832; bases 595-1141; Affy ID MmuSTS.3014.1.S1_at), and NPY receptor 5 (NPY5R) (GenBank # NM_001032833; bases 949-1338; Affy ID MmuSTS.1973.1.S1_at). Because NPY receptor 4 (NPY4R) is not included on the Affymetrix GeneChip, the probe set was custom designed by Affymetrix to target the published sequences (GenBank # AY149475).

To minimize qRT-PCR assay variability, we used geNorm (7) to identify housekeeping genes for normalization that showed the least variability between samples for each brain region. In each case, we confirmed that the expression levels for these genes did not correlate with AT. The Ce expression levels of the NPY system genes were normalized to the expression level of

a housekeeping gene, succinate dehydrogenase complex, subunit A, (SDHA) flavoprotein variant using a custom-designed TaqMan probe set targeting bases 757-1091 (GenBank # XM_001094170). For each sample, the results for each member of the NPY system were divided by the levels of SDHA expression. For qRT-PCR performed on motor cortex tissue, NPY system gene expression was normalized to the geometric mean of the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (ActB) using TaqMan probes sets targeting bases 302-388 (GenBank # NM_001195426) and 830-952 (GenBank # NM_001033084), respectively.

In Situ NPY1R and NPY5R Assessment

Because NPY1R and NPY5R mRNA levels were associated with AT levels we assessed the expression pattern of these two mRNAs in the amygdala and surrounding tissue by radiolabeled *in situ* hybridization. The 530 bp rhesus NPY1R probe was amplified from rhesus amygdala cDNA using forward (5'GAGAGACTTGCAGTTCTTCTTTAACTTT 3') and reverse (5'TAATCTAATGGCAGTATTGGATGGCAAGT 3') PCR primers. The 373 bp rhesus NPY5R probe was amplified from rhesus amygdala cDNA using forward (5'CTGTAAGAAGTCAGCTCTCTTCATC 3') and reverse (5'CAGTGTATAAGGGACATTAAATCAGC3') PCR primers. The sequences were based on the Affymetrix probe sets MMUSTS.3013.1.S1_AT and MMUSTS.1973.1.S1_AT and were 96% and 99% identical to the human NPY1R and NPY5R, respectively (GenBank #s NM_000909 and NM_006174). The riboprobes were prepared and *in situ* hybridization was performed using previously published procedures (8). Phosphor screens were scanned using Typhoon 9410 Imaging System and the signal was quantified using ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ).

Acetylcholinesterase (AChE) Staining

AChE staining was used to identify the structural details of the amygdala nuclei. The method was based on a previously published procedure (9). The slides were then mounted using Distrene Plasticiser Xylene (DPX) mountant (Sigma-Aldrich, St. Louis, MO).

Statistical Analyses

Gene Expression Correlational Analysis. Gene expression analysis was performed as previously described (3). The primary analysis of interest was the relationship between variation in the mean level of AT across the three assessments and individual differences in gene expression levels, indexed using qRT-PCR. This was tested using robust regression techniques that attenuate the influence of high-leverage outliers, minimizing the likelihood that a small number of observations exerted disproportionate effects on the regression estimate (2, 10).

FDG-PET Statistical Analyses. Because NPY1R and NPY5R mRNA levels predicted AT, we assessed the relationship of these two signals to brain metabolism. Voxelwise robust regressions were performed between qRT-PCR-measured Ce and motor cortex NPY1R and NPY5R mRNA levels and mean FDG-PET across three assessments. These analyses were performed using an adaptation (11) of Fmristat (http://www.math.mcgill.ca/keith/fmristat/) (12, 13). Regressions were performed across the whole brain controlling for nuisance variation in mean-centered age, change in age across assessments, relocation, and voxelwise gray-matter probability. Separate regression analyses were performed between qRT-PCR-measured Ce and motor cortex NPY1R and NPY5R mRNA levels and the 95% confidence interval in the Ce region. This is the region within the Ce that, with 95% certainty contains the peak voxelwise correlation between FDG metabolism and AT. The 95% confidence intervals were calculated based on the entire set of 238 monkeys as previously described (1, 14). The mean of the three FDG-PET signals extracted from the 95% confidence intervals most predictive of AT were used

to perform regressions with NPY1R and NPY5R gene expression levels co-varying for mean-centered nuisance variance in age, change in age across assessments, and relocation.

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