Drugs for Relapse Prevention of Alcoholism
Edited by R. Spanagel and K. Mann

Neuropeptide Y antagonists: a perspective

Markus Heilig[1] and Todd E. Thiele[2]

[1] *Department of NEUROTEC, Karolinska Institute, M57 Huddinge University Hospital, S-14186, Stockholm, Sweden*
[2] *Department of Psychology, University of North Carolina, Davie Hall, CB# 3270, Chapel Hill, NC 27599–3270, USA*

Introduction

Over the last 15 years, a role has been firmly established for NPY as an endogenous anti-stress system, potentially also implicating this system in the pathophysiology of anxiety and depressive disorders (recently reviewed in, e.g., [1, 2]). More recently, converging evidence from genetically modified animals, pharmacological studies and human observations has indicated that endogenous NPY signalling is involved in regulation of voluntary alcohol intake, in particular in states of abnormally high drinking, and that targeting this system may offer an attractive mechanism for relapse prevention in alcoholism. In this chapter, we introduce the brain NPY system and its biology, and review the findings supporting a role for NPY receptor ligands in future treatment of alcoholism.

Basic biology of the central NPY system

NPY, named so because of its exclusively neuronal expression, and its terminal tyrosine (Y in the 1-letter aa code), is a 36 amino acid (aa) peptide with a C-terminal amide-group [3, 4]. It belongs to a family of peptides related to pancreatic polypeptide (PP; [5]). NPY is one of the most highly conserved neuroendocrine peptides known [6], which implies an important functional role. NPY-like peptides all consist of an N-terminal polyproline helix (residues 1–8) and an amphiphilic α-helix (residues 15–30), connected with a β-turn, creating a hairpin-like loop [7]. The preproNPY gene encodes a simple 97 aa precursor [8], which contains a 28 aa signal peptide and a 69 aa prohormone. Mature NPY (36 aa) is here flanked at its C-terminus by 33 amino acids, three of which are a motif necessary for NPY amidation, critical for virtually all actions of NPY. The peptide formed by the remaining 30 amino acids of the precursor has been named CPON (C flanking peptide of NPY). Although it also shows a high degree of sequence conservation, its function remains unknown [6].

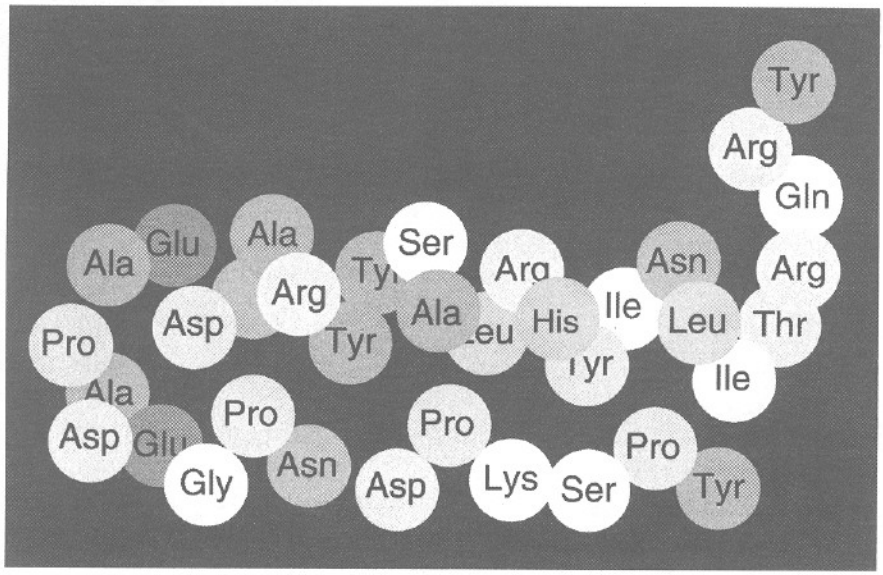

Figure 1. Structure of the NPY molecule. The hairpin loop structure is characteristic of the family of NPY related peptides. The full 1–36 a.a. sequence is required for activation of Y1 receptors, while shorter, truncated fragments are capable of activation of other receptor subtypes.

NPY-positive neurons are abundant in the central nervous system (CNS), including brain areas involved in emotionality [9–11]. Within the cerebral cortex and forebrain nuclei, NPY is mainly present in inhibitory interneurons. Coexistence of NPY with somatostatin and nitric oxide synthase is common in cortex and striatum. In addition, within the cortex and amygdala, NPY is extensively colocalized with GABA [11]. NPY-positive neurons in the hypothalamus, brainstem and spinal cord are more heterogeneous. An important NPY-ergic pathway from the arcuate nucleus to the ipsilateral paraventricular nucleus of the hypothalamus has been described [12] and is involved in feeding effects of NPY. More recently, a co-existence was also reported of NPY and agouti-gene-related protein (AGRP) in arcuate hypothalamic neurons. Among these, numerous cells project to telencephalic brain areas involved in emotionality, such as the amygdala complex [13].

Pharmacological experiments followed by molecular cloning have revealed a diversity of the NPY receptor family. NPY receptors cloned to date all belong to the superfamily of G-protein coupled receptors. The NPY-Y_1 receptor, one of the subtypes implicated in feeding effects of NPY [14–16], requires the intact NPY sequence for recognition and activation, and appears to be the subtype mediating anti-stress actions of NPY [17–21]. The Y_2 receptor subtype is also activated by C-terminal fragments of NPY such as NPY_{13-36} [22, 23]. A high number of Y_2 sites is found within the hippocampus. Activation of Y_2 receptors within this structure has been shown to suppress hippocampal

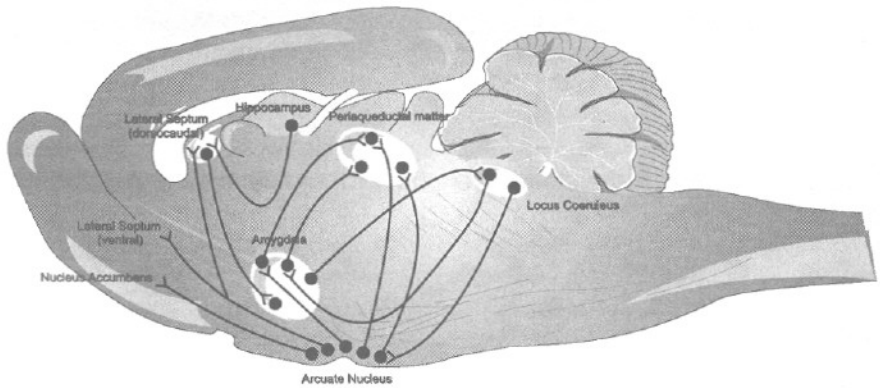

Figure 2. Brain NPY circuitry potentially related to regulation of alcohol intake.

glutamatergic transmission through presynaptic mechanisms [24, 25]. Behavioral consequences of Y_2 signalling in this area are unclear. Although the existence of a Y_3 receptor has been postulated on the basis of pharmacological experiments [26], this has not been confirmed by molecular studies [27]. Furthermore, a receptor termed Y_4 has been cloned, but appears to preferentially bind PP, and is therefore more appropriately referred to as a PP receptor [28, 29]. Finally, a Y_5 receptor with restricted hypothalamic expression has been cloned, and postulated to mediate the profound effects of NPY on feeding [30]. Subsequent work indicates that the Y_5 receptor probably shares this role with the Y_1 subtype.

NPY in EtOH-responses and EtOH seeking behavior

Central actions of NPY and alcohol show similarities

Studies carried out shortly following its isolation indicated shared properties between NPY and several classes of sedative compounds, including alcohol, benzodiazepines and barbiturates [31]. It was subsequently noted that icv infusion of NPY, as well as a Y_1 receptor agonist, produced electrophysiological and behavioral profiles similar to those induced by anxiolytic drugs such as EtOH and benzodiazepines [32]. Additionally, icv infusion of NPY and peripheral administration of EtOH to rats produced identical effects on event-related potential (ERP) profiles in response to auditory stimuli, both in cortex and amygdala. The effects of NPY and EtOH were additive [33]. Following 10–15 weeks of withdrawal from chronic exposure to EtOH vapor, icv infusion of NPY significantly decreased the amplitude of the N1 component of ERP in the amygdala of withdrawn Wistar rats when compared to controls, indicating that EtOH withdrawal augments brain sensitivity to NPY [34].

Together, these data suggest that electrophysiological responses to EtOH and EtOH withdrawal may be mediated, in part, by NPY signalling. A related study comparing the P and NP rats showed opposite electrophysiological activity in the amygdala following icv infusion of NPY [35]; this observation, together with those showing low NPY levels in P rats [36] strongly suggest that altered NPY signalling in the amygdala of P rats contributes to their high alcohol drinking.

Additional similarities between NPY and conventional sedatives, including alcohol are suggested by anti-convulsant actions of NPY [37, 38], mutual substitution of NPY and EtOH with regard to electrophysiological effects [33] and potentiation by NPY of barbiturate-induced sleep [39, 40]. The latter study mapped out NPY effects on sedation to the posterior hypothalamus, an area involved in the regulation of sleep–wake cycles. Y_1 mediation of NPY-induced sedation was demonstrated using Y_1-receptor knockouts. In contrast, anti-convulsant actions of NPY appear to be mediated through Y_5-receptors [41].

EtOH administration modulates central NPY signalling

EtOH administration influences NPY signalling. Relative to control animals that received an isocaloric diet as their sole source of calories, Long-Evans rats given access to a diet containing 6% EtOH for 12 weeks showed significant increases of NPY levels in the arcuate and ventromedial nuclei of the hypothalamus, the median eminence, and the suprachiasmatic nucleus [42]. Additionally, peripheral injection of 1.5 and 3.5 g/kg EtOH caused activation of NPY-containing neurons in the ventrolateral medulla of Long-Evans rats [43]. Wistar rats exposed to EtOH vapor for 14 h/day showed no differences in brain NPY expression after 7 weeks of exposure, but showed increased NPY expression in the hypothalamus 7 weeks after withdrawal from EtOH [44]. On the other hand, EtOH administration and withdrawal from EtOH have also been found to reduce NPY signalling. NPY mRNA levels in the arcuate nucleus of the hypothalamus were decreased when Sprague-Dawley rats were given a single peripheral injection of a 1.0 g/kg dose of EtOH [45]. More recently, Sprague-Dawley rats examined 24 h after withdrawal from a diet containing 9% EtOH (after 15 days of exposure) showed decreased NPY immunoreactivity in the cingulate gyrus, various regions of the cortex, the central and medial nuclei of the amygdala, and the paraventricular and arcuate nuclei of the hypothalamus [46]. Thus, withdrawal from EtOH is associated with reduced central NPY signalling. Consistent with this observation, a recent report found that icv infusion of NPY significantly attenuated EtOH withdrawal responses in Wistar rats [47]. The discrepancies between these studies (that is, either increases or decreases of NPY levels) may be related to rat strain differences, method of EtOH administration, technique for assessing NPY levels, or an interaction between these factors. An indication which might be helpful in relating these findings to the human clinical situation is a recent cDNA

microarray study which examined the expression of approximately 10,000 genes in the frontal cortex and motor cortex of alcoholics and matched control samples. One of the most intriguing observations was that brain tissue from alcoholics had significantly lower NPY expression than brain tissue from controls [48]. Some caution in the interpretation is, however, warranted, since in this type of study it is unclear if low NPY levels were the result of chronic alcohol use, or reflect a pre-existing, genetically encoded susceptibility factors for triggering the disease.

Genetic models suggest involvement of NPY in alcohol drinking

The first genetic evidence linking NPY to alcoholism came from studies involving rats selectively bred for high alcohol drinking. Quantitative trait locus (QTL) analysis identified a region of chromosome 4 that significantly correlated with differences in alcohol drinking between the Indiana alcohol-preferring (P) and alcohol-nonpreferring (NP) rats. This chromosomal region includes the NPY precursor gene [49, 50]. Interestingly, another transcript differentially expressed between P and NP rats is also encoded by a gene in this region, suggesting the possibility of a commonly regulated haplotype block [51]. Subsequent research found that P rats had low levels of NPY in the amygdala, frontal cortex, and hippocampus relative to NP rats, but higher levels of NPY in the hypothalamus and cingulate cortex [36, 44]. High alcohol-drinking (HAD) rats, bred by a similar strategy as that used to generate the P rats, also had low levels of NPY in the amygdala compared with low alcohol-drinking (LAD) rats, and had lower levels of NPY in hypothalamic nuclei [36]. Hwang et al. concluded that the high alcohol drinking by the P and HAD rats are best explained by low levels of NPY in the amygdala. It should be noted, however, that QTL analyses with HAD and LAD rats failed to confirm a role for the NPY precursor gene [52]. More recently, alcohol-avoiding, ANA (Alko Non-Alcohol) line of rats was found to have high NPY mRNA in the hippocampal Cornu Ammonis (CA) region and the dentate gyrus when compared with the alcohol-preferring, AA (Alko Alcohol) line and nonselected Wistar rats. Additionally, NPY Y_2 receptor mRNA was reduced in the AA line, suggesting a role for the Y_2 receptor in modulating alcohol drinking [53].

The studies reviewed above provide suggestive, but only correlative evidence for a role of NPY transmission in regulation of alcohol drinking. Two complementary lines of intervention studies provide data supporting the notion that this relation is in fact causal. The first of these is based on genetic manipulations in rodents, leading to absent or excessive expression of NPY, or selective inactivation NPY receptor subtypes. Voluntary EtOH consumption and resistance to the sedative effects of EtOH were inversely related to NPY levels in knockout and transgenic mice [54]. These initial results were obtained in a mixed C57BL/6Jx129/SvEv genetic background. These mice also react with an exaggerated locomotor response to EtOH administration. It has subse-

quently been found that EtOH-associated phenotypes are partly dependent on the genetic background. Neither the resistance to sedative effects nor the potentiation of locomotor stimulation were found in an inbred 129/SvEv background, where the differences in voluntary EtOH consumption were also less marked. However, at the highest concentration of EtOH tested, 20%, increased voluntary consumption was found also in this background [55].

Regionality of central NPY overexpression appears to be crucial for modulation of EtOH drinking. This is highlighted by data from transgenic rats selectively overexpressing NPY in CA1 and CA2 regions of the hippocampus. Relative to control animals, these subjects were resistant to anxiety provoked by restraint-stress, and showed impairment of spatial memory acquisition. However, the NPY transgenic rats showed normal voluntary EtOH drinking [56].

Data from Y_1 receptor knockout mice ($Y_1^{-/-}$) provide further support for a role of the NPY system, and point to receptor mediation of NPY effects on EtOH intake. Except for slightly diminished food intake and the development of late-onset obesity due to low energy expenditure, these animals show normal gross phenotypic features [57]. However, $Y_1^{-/-}$ mice showed increased consumption of solutions containing 3%, 6%, and 10% (v/v) EtOH but displayed normal consumption of sucrose and quinine solutions. Furthermore, $Y_1^{-/-}$ mice were less sensitive to the sedative effects of 3.5 and 4.0 g EtOH/kg as measured by more rapid recovery from EtOH-induced sleep, even though plasma EtOH levels did not differ significantly between the genotypes following a 3.5 g/kg dose. Finally, male $Y_1^{-/-}$ mice showed normal EtOH-induced ataxia on a rotarod test following administration of a 2.5 g/kg dose [58].

The Y_2 receptor is a presynaptic autoreceptor and inhibits NPY release [59, 60]. Mutant mice lacking the Y_2 receptor ($Y_2^{-/-}$) have been shown to have increased food intake, body weight, and fat production but have a normal response to NPY-induced food intake [61]. It was hypothesized that if presynaptic Y_2 receptors are involved with modulating voluntary EtOH consumption and sensitivity, the $Y_2^{-/-}$ mice should exhibit EtOH-related phenotypes opposite to those found with the $Y_1^{-/-}$ mice. Thus, an absence of presynaptic inhibition of NPY release in $Y_2^{-/-}$ mice would augment NPY signalling, rendering mice with a similar phenotype as NPY overexpressing mice. Relative to wild-type ($Y_2^{+/+}$) mice, the $Y_2^{-/-}$ mice drank significantly less of solutions containing 3% and 6% EtOH, and had significantly lower EtOH preference at each concentration tested. On the other hand, $Y_2^{-/-}$ mice showed normal consumption of solutions containing either sucrose or quinine, normal time to recover from EtOH-induced sedation following 3.0 or 3.5 g/kg doses, and normal metabolism of EtOH following injection of a 3.0 g/kg dose [62].

Mutant mice lacking the NPY Y_5 receptor ($Y_5^{-/-}$) show late-onset obesity and increased food intake, have reduced sensitivity to NPY, and are seizure-prone [41]. When given access to solutions containing EtOH, $Y_5^{-/-}$ mice drank normal amounts of 3, 6, 10, and 20% (v/v) EtOH, but had increased sleep time following administration of 2.5 or 3.0 g EtOH/kg. However, the $Y_5^{-/-}$ mice also

showed high plasma EtOH levels relative to wild-type mice following injection of a 3.0 g/kg dose [63].

Taken together, evidence from genetic animal models implies that low NPY signalling can promote high voluntary EtOH drinking while upregulation of NPY signalling can be protective against excessive consumption. Data from NPY receptor knockout mice suggest that voluntary consumption of EtOH is modulated by the Y_1 and Y_2 receptors, and that EtOH-induced sedation is modulated by Y_1, and perhaps Y_5, receptors. These results are consistent with several recent findings. First, like $Y_2^{-/-}$ mice which drink low amounts of EtOH, rats self-administer less EtOH following central infusion of a Y_2 receptor antagonist [64] (see below). Second, $Y_1^{-/-}$ mice are resistant to the sedative effects of EtOH, and recent studies found that $Y_1^{-/-}$ mice are resistant to sodium pentobarbital-induced sleep [39, 40].

NPY, alcoholism and human genetics

The human preproNPY gene is polymorphic. Most attention this far has been attracted by a thymidine (T) to cytosine (C) single nucleotide polymorphism (SNP) that is present at the 1128 position of the human *NPY* gene, resulting in a leucine-to-proline substitution (Leu7Pro) in the signal peptide of preproNPY [65]. Individuals with the *Leu7/Pro7* genotype have an average of 42% higher maximal increases of plasma NPY in response to physiological stress when compared with *Leu7/Leu7* individuals [66]. Interestingly, Finnish men with the Pro7 substitution reported 34% higher average alcohol consumption when compared to men not having this polymorphism [67]. It should be noted that consumption levels in this study were reported from non-dependent subjects, and the reported consumption levels were low, averaging app. 70 g/week. The relevance of these data for alcohol dependence is thus unclear. A subsequent study carried out in European–American men with carefully diagnosed alcoholism reported a 5–5.5% Pro7 allele frequency, while non-alcoholics had a Pro7 allele frequency of only 2.0%, leading to a statistically significant association between genotype and diagnosis [68]. However, results are mixed. A lower rather than higher frequency of the Pro7 allele has been reported in type 2 alcoholics compared to controls [69], while a more recent study found no difference of Pro7 allele frequency between diagnosed Caucasian alcoholics and ethnically matched controls from Finland and Sweden [70]. Furthermore, a meta-analysis performed in the latter study found that while the Pro7 allele frequencies in alcoholics were similar in each report, the allele frequencies in nonalcoholic control groups were very different between studies. This issue remains unresolved at present. Despite ambitious attempts to exclude this possibility by Lappalainen and colleagues, the discrepant results might be related to ethnic stratification. The Pro7 allele differs in frequency between ethnic groups, and is, e.g., entirely absent in Asians [71, 72].

The preproNPY gene is polymorphic also at other positions. Among these, an SNP within a trk-B consensus sequence in the promoter region (–399C/T) is clearly functional, as C-containing alleles in this position confer higher transcriptional activity in neuronal cells. Both alleles have high frequencies in populations examined, and preliminary associations have been suggested both for schizophrenia [73] and treatment-refractory depression [94]. It is at present unknown whether this polymorphism plays a role in alcohol dependence of subtypes thereof.

Finally, a C to T substitution at position 5671, mapping to exon 3 of the *NPY* gene, has been described in a Japanese population. Although there was no association between genotype and a diagnosis of alcohol dependence, it was reported that the T-allele was found in a significantly higher frequency in alcoholic patients experiencing seizures [72]. Since this SNP is synonymous (i.e., it does not encode an amino acid substitution), its role is unclear. One possibility is that it is in linkage dysequilibrium with other, functional polymorphisms.

NPY and alcoholism: pharmacological mechanisms and strategies

Genetic modifications are powerful and highly selective tools, but have known limitations, in particular related to issues of genetic background, and compensatory mechanisms which can be activated in constitutive overexpressors or knockouts [74]. In the case of NPY and alcohol, however, genetic and neurochemical evidence is largely supported by emerging pharmacological studies. These have used icv infusion of NPY and other NPY receptor ligands to determine if NPY signalling regulates voluntary EtOH consumption, and thus directly point to possible future applications in the clinic. To correctly interpret the results of these studies, it is crucial to understand a basic fact of experimental alcohol research: laboratory rodents, and in particular genetically heterogeneous rats commonly used in pharmacological experiments, do not voluntarily consume sufficient amounts of EtOH to achieve pharmacological effects. Instead, they are likely to drink for other types of motivation, such as caloric content. Modifications of this baseline consumption are of little relevance for developing clinical treatments. On the other hand, states of excessive drinking can be induced either by genetic selection or behavioral manipulations [75]. This induced, excessive drinking component is selectively affected by clinically effective drugs (see, e.g. [76, 77]), and therefore the appropriate target for candidate anticraving/relapse-preventive drugs.

The general picture which has emerged against the background of this distinction is that exogenous NPY does not reliably regulate basal voluntary EtOH drinking and may even slightly increase it under normal conditions. In contrast, potentiation of NPY signalling potently suppresses EtOH drinking in states of excessive intake. Thus, in the first attempt with Golden Hamsters, icv infusion of NPY did not reliably alter drinking of a 5% EtOH solution [78].

More recently, Wistar rats were given icv infusion of various doses of NPY ranging from 2.5 to 15.0 μg in a within-subjects design. While 5.0 μg of NPY significantly increased consumption of a sucrose solution, none of the doses tested altered alcohol intake [79]. Similarly, neither third ventricle infusion of NPY nor direct infusion of NPY into the amygdala altered EtOH drinking in Wistar rats [80, 81]. In fact, direct infusion of femtomolar doses of NPY into the paraventricular nucleus of the (PVN) hypothalamus increased consumption of alcohol in Long-Evans rats, an effect which was blocked by pretreatment with the Y_1 receptor antagonist BIBP 3226 [82]. The PVN is known to mediate appetite effects of NPY, and this finding likely indicates that in "normal" rats, effects of NPY on EtOH consumption primarily reflect appetite modulation, since EtOH in addition to being an intoxicating agent is also a caloric nutrient. Recently, a report found that amygdalar infusion of BIBP 3226 decreased ethanol self-administration in Long-Evans rats [83], an effect that may be unrelated to the pharmacological effects of ethanol by this moderate alcohol-consuming strain.

On the other hand, icv infusion of both 5.0 and 10.0 μg doses of NPY significantly reduced voluntary consumption of an 8% EtOH solution in alcohol-preferring P rats; in these experiments, it again did not alter EtOH drinking of non-preferring NP or outbred Wistar rats [84]. The suppressing effect of NPY on EtOH intake in P rats is even more pronounced after a sequence of continuous access followed by a deprivation phase, a procedure known to increase the motivation to consume EtOH for its reinforcing properties [85]. More recently, the ability of NPY to selectively suppress excessive EtOH drinking was confirmed in an interesting manner in another genetically selected high-preferring line, HAD rats [86]. In this study, icv administration of NPY increased sucrose self-administration in both HAD and low-preferring LAD rats, but selectively suppressed EtOH-self-administration in the HAD line only. Interestingly, this was found despite the observation that the well-known anti-anxiety actions of NPY (1) were identical in the two lines. This indicates that, although altered emotionality may contribute to regulation of EtOH intake by NPY, the latter can also be modulated independently of the former. Recently, amygdalar infusion of a PKA inhibitor increased anxiety and ethanol drinking by Sprague Dawley rats, and caused local reductions of NPY levels. Elevated levels of anxiety and ethanol drinking were rescued by amygdalar co-administration of NPY. Consistent with the above observations, NPY did not affect ethanol consumption by rats not treated with the PKA inhibitor and which had normal (i.e., non-elevated) ethanol consumption [87].

Of particular relevance for development of NPY-based pharmacological treatments of alcohol dependence, EtOH self-administration has also been examined following central administration of the selective NPY-Y_2 antagonist BIIE0246. This compound is known to potentiate the release of endogenous NPY [59], an indirect approach which may circumvent the difficulties inherent in developing an agonist for NPY-Y_1 receptors. Initial experiments with BIIE0246 were carried out using regular rats, but under conditions of limited

access operant self-administration which do produce significant blood alcohol concentrations. Icv administration of BIIE0246 dose-dependently suppressed self-administration. Interestingly, in follow-up experiments using rats with a history of dependence induced according to a recently published model [76], doses of BIIE0246 which were subthreshold in non-dependent animals were effective in suppressing self-administration in subjects with a history of dependence (Thorsell et al., in preparation). The same dissociation was observed using antisense mediated inhibition ("knockdown") of Y_2 receptor expression. Thus, Y_2 antagonism appears to offer an attractive strategy, which might selectively target states of excessive EtOH consumption.

Finally, it should be noted that peripheral administration of a Y_5 receptor antagonist delayed the onset of ethanol-reinforced responding but did not alter the amount of ethanol consumed by C57BL/6 mice in a 16-h session [88]. These findings, and the observation that Y_5 receptor knockout mice show normal ethanol consumption [63], do not provide a strong case for the Y_5 receptor in the modulation of ethanol consumption.

NPY and alcohol: conclusions and future directions

Research over more than 15 years has implicated NPY in mechanisms of emotionality and stress, identifying it as a potential therapeutic target for novel treatments in anxiety disorders and depression [1, 89–91]. More recent evidence identifies the NPY system as a highly interesting treatment target in alcoholism. In summary, EtOH and NPY have similar effects on brain electrophysiological activity, while CNS responses to EtOH involve central NPY signalling, as evidenced by altered central NPY levels and expression following administration of EtOH and EtOH withdrawal. Low NPY signalling in animal models predisposes to high EtOH drinking, while central administration of NPY selectively reduces excessive EtOH drinking but not drinking in 'normal' unselected animals. Activation of NPY Y_1 receptors appears to mediate NPY's suppression of excessive drinking; blockade or inactivation of Y_2 receptors leads to the same functional outcome, presumably through removal of Y_2-mediated presynapic inhibition of endogenous NPY release.

It can be hypothesized that central NPY activity is recruited in response to EtOH consumption, and that this NPY activation serves as a protective feedback mechanism to prevent high EtOH drinking. Animals with abnormally low NPY levels would not benefit from this feedback protection and drink excessive quantities of EtOH. Such a mechanism could also explain excessive drinking in alcoholics with low brain NPY expression. The regulatory role of NPY for regulation of excessive EtOH might in part also be related to effects of NPY on emotionality and stress responses. While adaptive in the short term, activation of these systems imposes an allostatic load on the organism if present over prolonged periods [92]. Negative emotionality and dysregulated stress responses are important factors in the development of dependence, and in one

of its hallmarks, relapse [93]. NPY counteracts and buffers negative emotionality and stress responses [1, 89], and these effects of potentiating NPY transmission may be beneficial in the treatment of alcoholism.

Thus available data suggest that drugs targeting central NPY systems may become useful therapeutic agents in alcoholism. Agonists aimed at the Y_1 or, perhaps more realistically, antagonists of Y_2 receptors are particularly promising candidates. NPY-targeting drugs might turn out to be most useful in alcoholism with co-morbid anxiety and/or depression.

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