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News and reviews

The role of Neuropeptide Y in fear conditioning and extinction

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ABSTRACT

While anxiety disorders are the brain disorders with the highest prevalence and constitute a major burden for society, a considerable number of affected people are still treated insufficiently. Thus, in an attempt to identify potential new anxiolytic drug targets, neuropeptides have gained considerable attention in recent years. Compared to classical neurotransmitters they often have a regionally restricted distribution and may bind to several distinct receptor subtypes. Neuropeptide Y (NPY) is a highly conserved neuropeptide that is specifically concentrated in limbic brain areas and signals via at least 5 different G-protein-coupled receptors. It is involved in a variety of physiological processes including the modulation of emotional-affective behaviors. An anxiolytic and stress-reducing property of NPY is supported by many preclinical studies. Whether NPY may also interact with processing of learned fear and fear extinction is comparatively unknown. However, this has considerable relevance since pathological, inappropriate and generalized fear expression and impaired fear extinction are hallmarks of human post-traumatic stress disorder and a major reason for its treatment-resistance. Recent evidence from different laboratories emphasizes a fear-reducing role of NPY, predominantly mediated by exogenous NPY acting on Y1 receptors. Since a reduction of fear expression was also observed in Y1 receptor knockout mice, other Y receptors may be equally important. By acting on Y2 receptors, NPY promotes fear extinction and generates a long-term suppression of fear, two important preconditions that could support cognitive behavioral therapies in human patients. A similar effect has been demonstrated for the closely related pancreatic polypeptide (PP) when acting on Y4 receptors. Preliminary evidence suggests that NPY modulates fear in particular by activation of Y1 and Y2 receptors in the basolateral and central amygdala, respectively. In the basolateral amygdala, NPY signaling activates inhibitory G protein-coupled inwardly-rectifying potassium channels or suppresses hyperpolarization-induced I(h) currents in a Y1 receptor-dependent fashion, favoring a general suppression of neuronal activity. A more complex situation has been described for the central extended amygdala, where NPY reduces the frequency of inhibitory and excitatory postsynaptic currents. In particular the inhibition of long-range central amygdala output neurons may result in a Y2 receptor-dependent suppression of fear. The role of NPY in processes of learned fear and fear extinction is, however, only beginning to emerge, and multiple questions regarding the relevance of endogenous NPY and different receptor subtypes remain elusive. Y2 receptors may be of particular interest for future studies, since they are the most prominent Y receptor subtype in the human brain and thus among the most promising therapeutic drug targets when translating preclinical evidence to potential new therapies for human anxiety disorders.

Abbreviations: [D-Arg25]hNPY, Y1 receptor agonist; [D-Arg25]hNPY, Y1 receptor agonist; AAV vector, Adeno-associated viral vector; AStr, Amygdala-striatal transition zone; ATP, Adenosine triphosphate; BAC, Bed nucleus of the anterior commissure; BF, Basal forebrain; BIBO 3304, Y1 receptor antagonist; BIBP 3226, Y1 receptor antagonist; BIIE 0246, Y2 receptor antagonist; BLA, Basolateral amygdala; BNST, Bed nucleus of the stria terminalis; CA1, CA3, Cornu amonis 1, 3; cAMP, Cyclic adenosine monophosphate; CCK, Cholecystokinin; CEA, Central amygdala; CEc, Centro-capsular amygdala; CEl, Centro-lateral amygdala; CEm, Centro-medial amygdala; CNS, Central nervous system; CR, Calretinin; CS, Conditioned stimulus; CSF, Cerebrospinal fluid; D-Trp^{32} NPY, Y5 receptor agonist; D1R, Dopamine 1 receptor; DG, Dentate gyrus; DPPIV, Dipeptidylpeptidase IV; EN, Entorhinal cortex; Epac, Exchange protein activated by cyclic adenosine monophosphate; F7P34NPY, Y1 receptor agonist; GABA, Gamma-amino butyric acid; GIRK, G protein-coupled inwardly-rectifying potassium; HC, Hippocampus; HPA, Hypothalamic pituitary adrenal; icv, Intracerebroventricular; IL, Infralimbic cortex; Im, Main intercalated nucleus; ip, Intraperitoneal; IR, Immunoreactivity; JNJ-31,020,028, Brain-penetrant Y2 receptor antagonist; L152,804, Y5 receptor antagonist; LC, Locus coeruleus; Leu^{31}Pro^{34}-NPY, Y1 (Y4, Y5) receptor agonist; LTP, Long-term potentiation; MEA, Medial amygdala; NPY, Neuropeptide Y; NPY$_{13\text{-}36}$, Y2 receptor agonist; NPY$_{3\text{-}36}$, Y2/Y5 receptor agonist; NPYKO, Y1KO, Y2KO, Y4KO, NPY, Y1, Y2, Y4 receptor KO mouse; PAG, Periaqueductal gray; PBN, Parabrachial nucleus; PC-1, PC-2, Prohormone convertase1, 2; Pir, Piriform cortex; PKA, Proteinkinase A; PL, Prelimbic cortex; PP, Pancreatic polypeptide; PTSD, Post traumatic stress disorder; PV, Parvalbumin; PYY, Peptide YY; PYY$_{3\text{-}36}$, Y2/Y5 receptor agonist; RT, Reticular nucleus of the thalamus; SST, Somatostatin; st, Stria terminalis; US, Unconditioned stimulus; VIP, Vasoactive intestinal peptide; Y-28 (Des-AA$^{11\text{–}18}$ [Cys^{7,21}, d-Lys^{9} (Ac), d-His^{26}, Pro^{34}]-NPY), Y1 receptor agonist; Y-36 ([d-Arg^{25}, d-His^{26}]-NPY), Y1 receptor agonist; Y1R, Y2R, Y4R, Y5R, y6R, NPY Y1, Y2, Y4, Y5, y6 receptor.

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1. Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that belongs to a family of biologically active peptides comprising NPY, peptide YY (PYY) and pancreatic polypeptide (PP) (El-Salhy et al., 1982; Tatemoto et al., 1982). Since they provide bidirectional communication between gut and brain, they belong to the family of gut–brain peptides (Holzer et al., 2012). The peptides are translated from the respective mRNAs as pre-pro-peptides. Their signal peptide targets them to the endoplasmic reticulum and is proteolytically removed. The pro-peptides are then packaged in the Golgi apparatus into large-dense core vesicles and, in neurons, transported to the axon terminals. Within large-dense-core vesicles, pro-peptides are proteolytically processed by prohormone convertases (PC-1 and PC-2), carboxypeptidase E/H and by peptidylglycine alpha-amidating monooxygenase to the mature amidated peptide ($NPY_{1\text{-}36}$). NPY is released upon sustained depolarization together with classical neurotransmitters by exocytosis (Brakch et al., 1997; Hokfelt, 1991).

In the peripheral nervous system, NPY is expressed predominantly in enteric neurons in the adrenal medulla and in postganglionic sympathetic neurons, co-localizing there primarily with noradrenaline and ATP (Lundberg et al., 1990). However, the majority of NPY is located within the central nervous system (CNS) where it is considered to be the most abundant neuropeptide. In the CNS, NPY is expressed predominantly in the cortex, hippocampus, amygdala, basal ganglia and hypothalamus (Fig. 1A and B) (Fetissov et al., 2004; Morris, 1989; Pelletier et al., 1984). Furthermore, NPY has been detected in distinct brain nuclei of the brain stem, including the periaqueductal gray, locus coeruleus and dorsal raphe (Yamazoe et al., 1985). Using immunohistochemical markers it has been demonstrated that NPY in the cortex, hippocampus and amygdala is expressed in GABAergic neurons co-localizing often with somatostatin (SST) and to a lower extent with parvalbumin and nitric oxide (Karagiannis et al., 2009; McDonald, 1989; Somogyi et al., 2012; Tricoire et al., 2010). In addition, NPY-like immunoreactivity (NPY-IR) has been identified in long fiber tracts, such as the corpus callosum and stria terminalis (Allen et al., 1983; Tasan et al., 2010). Mechanic transection of the stria terminalis resulted in reduced NPY-IR in the bed nucleus of the stria terminalis (BNST), basal forebrain and hypothalamus, suggesting expression of NPY also in projection neurons (Allen et al., 1984). NPY is generally expressed in dispersed interneurons (Fig. 1A). However, there are distinct brain nuclei that consist of a particularly high density of NPY neurons: the arcuate nucleus of the hypothalamus (Arc, Fig. 1A), the bed nucleus of the anterior commissure (BAC, Fig. 2A), the reticular nucleus of the thalamus (RT, Fig. 2B) (Morris, 1989), and the main intercalated nucleus of the amygdala (Im, Fig. 2C) (Wood et al., 2015).

The other two members of the NPY family, peptide YY (PYY) and pancreatic polypeptide (PP) are absent from the CNS but are predominantly expressed in L-cells of the gut and in F-cells of the pancreatic islets, respectively. Upon release, they may act either locally within the gastrointestinal tract and pancreas or may be transported by the blood circulation and bind to Y receptors at distinct brain areas with a partially open blood–brain barrier, such as the hypothalamus or area postrema (Holzer et al., 2012).

Physiologically, NPY is involved in a variety of distinct processes, such as energy homeostasis, appetite, processing of pain, seizure suppression and importantly also in the modulation of emotional-affective behaviors (Hokfelt et al., 2008; Lee and Herzog, 2009; Lin et al., 2004; Loh et al., 2015; Sperk et al., 2007; Vezzani and Sperk, 2004). These effects are mediated by at least 5 different G-protein coupled receptors (Y1, Y2, Y4, Y5 and y6) (Michel et al., 1998). While NPY displays equally high affinities for all 5 receptors, PP preferentially recognizes Y4 receptors, while the cleavage products $PYY_{3\text{-}36}$ and $NPY_{3\text{-}36}$ bind to Y2 and Y5 receptors. However, in the CNS the predominant receptors are Y1 and Y2 receptors, which are distributed throughout the brain with a particular enrichment in limbic brain areas, such as hippocampus, amygdala, and hypothalamus but also in the different layers of the cerebral cortex (Fig. 1C-F) (Dumont et al., 1993, 1996). It is noteworthy that, although Y1 and Y2 receptors are often expressed in the same brain areas, they display a highly complementary distribution, probably reflecting the postsynaptic and presynaptic localization of Y1 and Y2 receptors, respectively (Fig. 1C–F). On the other hand, Y4, Y5 and also y6 receptors are only apparent in defined brain areas, generally representing a minor fraction of Y receptors in the CNS (Dumont et al., 1993, 1998; Yulyaningsih et al., 2014).

A considerable amount of literature supports an anxiolytic and antidepressant-like function of NPY and has been extensively reviewed previously (Bowers et al., 2012; Gilpin, 2012; Heilig, 2004; Wu et al., 2011). These effects are predominantly mediated by activation of Y1

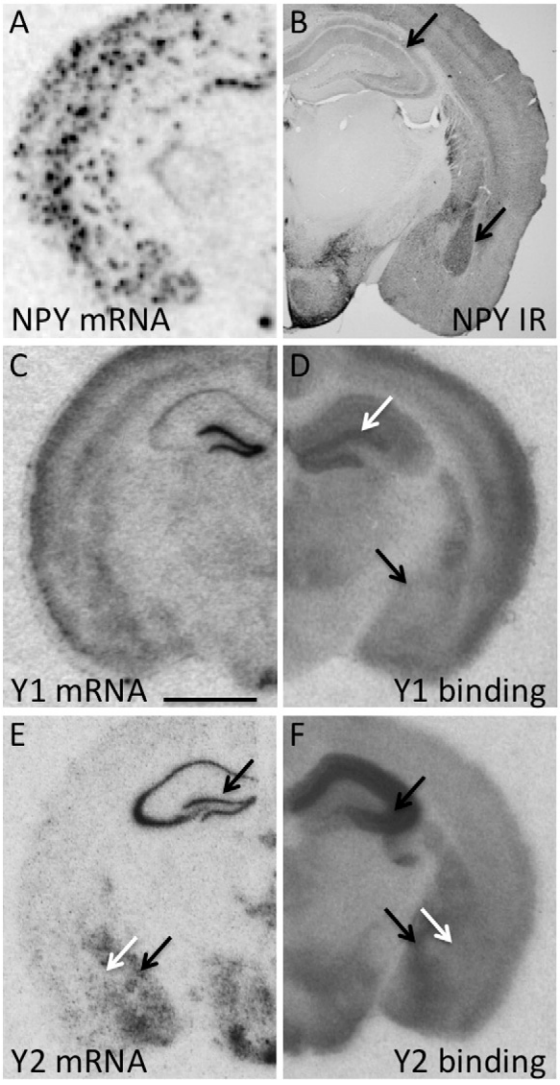

Fig. 1. Distribution of NPY, Y1 and Y2 receptors in the mouse brain. (A) Autoradiograph of NPY mRNA distribution in a coronal section of a mouse brain, (B) photomicrograph depicting NPY-immunoreactivity (NPY IR) on a coronal mouse brain section (arrow indicates the particularly high expression in the hippocampus and amygdala). (C) Autoradiographs of an in situ hybridization and (D) receptor binding using [^{125}I] Leu^{31}Pro^{34} PYY depicting Y1 receptor mRNA and protein, respectively (note the high levels of Y1 mRNA in the dentate granule cell layer and the corresponding Y1 receptor binding in the molecular layer, suggesting expression on soma and dendrites). (E) Autoradiograph of an in situ hybridization and (F) receptor binding for Y2 receptors ([^{125}I] PYY$_{3\text{-}36}$) on coronal mouse brain sections. Note the expression of Y2 mRNA in the granule cell layer and Y2 receptor binding in CA3, indicating pre-synaptic localization. Arrows in (E) indicate high Y2 mRNA expression in hippocampus and central amygdala (black arrows) and moderate expression in the basolateral amygdala (white arrow). Scale bar 2 mm.

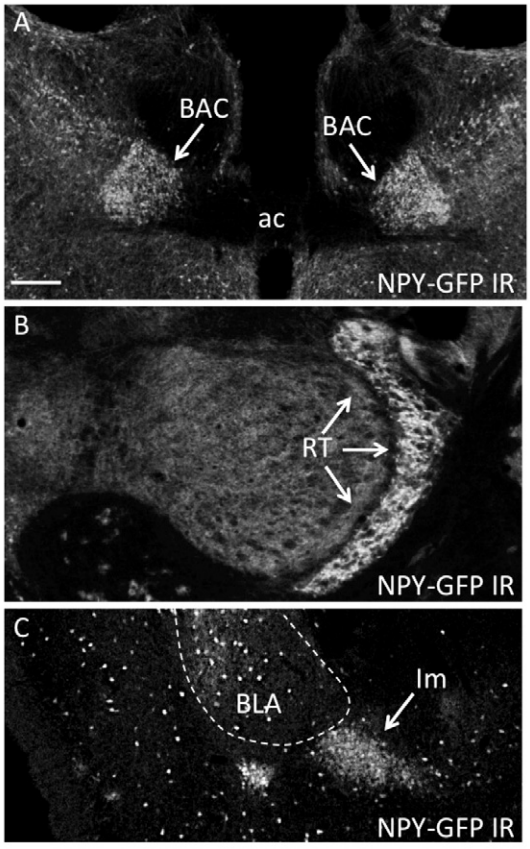

Fig. 2. Dense accumulation of NPY in specific brain areas in a NPY-GFP mouse. (A) Photomicrographs of immunohistochemistry for NPY-GFP (NPY-GFP IR) depicting a dense accumulation of NPY-GFP in the bed nucleus of the anterior commissure (BAC), (B) in the reticular nucleus of the thalamus (RT) and (C) in the main intercalated nucleus (Im). All three brain areas contain high-densities of low-level NPY expressing neurons. Scale bar 200 μm.

receptors, whereas presynaptic Y2 receptors are postulated to be anxiogenic. Furthermore, recent reviews also summarize the role of NPY in stress-related psychiatric disorders (Enman et al., 2015; Sah and Geracioti, 2013).

In the present review we will focus on the role of NPY in animal models of learned fear and fear extinction and related human pathologies. Furthermore, to provide a tentative framework for the underlying mechanisms, we will summarize the electrophysiological and neurobiochemical data as well as the connectivity of NPY and Y receptor containing neurons specifically in limbic brain areas that are relevant for fear learning.

2. NPY, fear and depression in humans

2.1. The NPY system in the human brain

Over the past three decades, NPY has been identified as a major neurochemical component of the stress response, coordinating cardiovascular, neuroendocrine, immune and metabolic functions (Heilig, 2004; Rasmusson et al., 2010). Such a functional diversity can only be carried out by a widespread distribution in the central and peripheral nervous systems. A considerable overlap in brain NPY-expression was observed between different species, including the human brain. The highest levels of NPY-IR and NPY mRNA have been demonstrated in the human dentate gyrus with somewhat lower expression in the amygdala, basal ganglia and cortex (Adrian et al., 1983; Caberlotto et al., 2000). On the other hand, species differences have been observed regarding NPY receptor distribution and density (Dumont et al., 1998). In the rodent brain (mouse and rat), Y1 and Y2 receptors are most abundant, with Y1 receptors being primarily expressed in the superficial layers of the cerebral cortex (Fig. 1C and D) and in the molecular layer of the dentate gyrus and Y2 receptors display a particular enrichment in the *strata oriens* and *radiatum* of the hippocampal formation (Fig. 1E and F) as well as in the septum. In contrast, receptor binding performed on post mortem human brain tissue revealed an apparent paucity of Y1 receptor binding sites (determined by [^{125}I] Leu^{31}Pro^{34}-PYY), with notable expression in the dentate gyrus but relative absence from cortical areas. Interestingly, considerably higher expression of Y2 receptors (determined by [^{125}I] PYY$_{3\text{-}36}$) were observed in the superficial and middle layers of the human cortex, two brain areas that are generally populated

by Y1 receptors in the rodent brain (Fig. 1D) (Jacques et al., 1997). Furthermore, it was suggested that NPY mRNA co-localizes with Y2, but not Y1 receptor mRNA in the human cortex, hippocampus, amygdala and striatum (Caberlotto et al., 2000), while substantial evidence for a co-localization of NPY and Y2 receptors in the rodent brain is still lacking (Stanic et al., 2011; Wood et al., 2015). Although comparing fresh snap-frozen tissue from rodents with post mortem tissue from human subjects that was frozen with a delay of up to 23 h may bear potential pitfalls, the relative abundance of Y2 receptors and the paucity of Y1 receptors in human brain tissue has to be taken into consideration when extrapolating the contribution of individual Y receptor subtypes in fear-related behavior from rodent to human. Similarly, the presence of NPY in limbic regions of the human brain may indicate a role in emotional processing, but again, translation from localization to function or from preclinical models to humans is not without caveats.

2.2. Role of NPY in pathological fear in humans — focus on post-traumatic stress disorder

NPY may, however, adapt the whole organism to stressful, potentially life-threatening conditions and maintain the psychological and physiological integrity, similarly in rodents (Cohen et al., 2012) and humans (Wu et al., 2013), by orchestrating resilience to traumatic events (Wu et al., 2013). While most people are resilient to traumatic events and recover within hours or days, some never recover completely or even develop a permanent state of heightened fear and anxiety, commonly referred to as Post-Traumatic Stress Disorder (PTSD) (Kessler et al., 2005). PTSD is characterized by emotional numbing and avoidance of the traumatic memories, hyper-arousal and flashbacks with a tendency towards fear generalization. Unspecific cues associated with the initial traumatic event can then evoke a context-inappropriate fear response even in the absence of actual danger. The development of PTSD has been linked to dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system, and both are modulated by NPY (Heilig, 2004; Hirsch and Zukowska, 2012; Rasmusson et al., 2010). Thus, investigating inter-individual differences in disease susceptibility in human patients may help to unravel the underlying mechanisms and the relevance of NPY in PTSD. Indeed, it has been suggested that polymorphisms in the NPY gene predict individual differences in stress response and emotionality. Thus, genetically induced lower NPY expression may result in higher emotion-induced activation of the amygdala (Zhou et al., 2008, but see also Cotton et al. (2009)), development of major depression (Mickey et al., 2011), as well as lower NPY plasma levels (Xu et al., 2012, but see also Nishi et al. (2014)). Furthermore, a functional NPY variation was suggested to cause lower NPY mRNA expression in the amygdala and reduced cerebrospinal fluid (CSF)-NPY levels in higher primates exposed to aversive situations (Lindell et al., 2010). The relevance of NPY levels in CSF and plasma for emotional processing in the CNS is definitely limited and probably reflects predominantly sympathetic nervous system activity and release from the adrenal gland. However, genetic polymorphisms may affect NPY processing in the CNS and periphery in a similar manner, suggesting that the CSF/plasma levels could parallel an altered NPY system in the brain.

2.3. Response of NPY plasma levels to sympathetic stimulation, stress, and post traumatic stress disorder in humans

Since NPY is contained in sympathetic neurons and in the adrenal medulla together with noradrenaline and adrenaline, respectively, stress exposure may also increase NPY plasma levels. For instance, intravenous administration of the adrenergic α2-receptor antagonist, yohimbine, in healthy subjects was suggested to augment noradrenaline and NPY release from sympathetic neurons resulting in increased NPY plasma levels (Rasmusson et al., 1998). Similarly, after exposing healthy subjects to uncontrollable stress, NPY plasma levels increased together with cortisol and noradrenaline (Morgan et al., 2001; Rasmusson et al., 2010). As mentioned above, plasma NPY mainly serves as an indicator for sympathetic activity and correlations between plasma and CSF concentrations are also relatively weak (Baker et al., 2013). NPY in the plasma may, however, also interact with Y receptors in brain areas that are partially open to the blood–brain barrier, such as the arcuate nucleus of the hypothalamus or the area postrema. Since neurons of the arcuate nucleus project not only to other hypothalamic nuclei but also to the amygdala and to the BNST (Betley et al., 2013), plasma NPY may play an indirect role in the integration of emotional, autonomic, and metabolic functions (Akabayashi et al., 1994; Heilig, 1995). In line with this, intravenous infusion of NPY decreased HPA-axis activation resulting in reduced cortisol levels, suggesting that also NPY in the plasma may have important implications for regulating emotional states (Antonijevic et al., 2000). Compared to healthy controls, PTSD patients displayed lower NPY levels in the plasma (Rasmusson et al., 2010; Yehuda et al., 2006) and CSF (Sah et al., 2009, 2014) already under baseline conditions. Whether these changes are related to the disorder (Sah et al., 2014) or to an extreme stress exposure per se (Morgan et al., 2003) remains to be demonstrated. However, NPY levels seem to recover during remission from PTSD (Yehuda et al., 2006) indicating that NPY may serve as a resilience factor in PTSD or at least as a potential biomarker.

Taken together, these studies indicate that NPY can act as a stress buffer in response to traumatic events mainly by reducing noradrenergic hyperactivity and that NPY levels loosely parallel the disease course in PTSD patients. Although PTSD is a complex disease, individual aspects may be recapitulated in animal models. Thus, *Pavlovian* fear conditioning may mimic the etiology and maintenance of PTSD ("Fear acquisition") and may also serve as a model for cognitive behavioral therapy ("Fear extinction"), reflecting the underlying principles of exposure therapy in human patients (Jovanovic and Ressler, 2010; Milad et al., 2009). Thus, comparing animal models with the human disease may increase our understanding of the biological mechanisms underlying pathological fear, opening new possibilities of interfering with the disease course.

3. Neuropeptide Y and pancreatic polypeptide in fear and fear extinction

3.1. Fear conditioning and extinction

Anxiety is generally described as a diffuse and unpleasant apprehension of a forthcoming threat involving predominantly an imagined, potential danger (Barlow, 2002). In contrast, fear is considered to be an emotional response towards a clearly identifiable, real and immediate threat that is essentially based on previous learning processes (Marks, 1987). However, anxiety and fear are intimately connected, may cause each other and human anxiety disorders are characterized by variable degrees of both. Experimentally, fear and anxiety can be studied by different behavioral paradigms. Fear learning involves distinct learning processes requiring the disentanglement of learning and memory from the accompanying emotional aspects. However, fear can be investigated in a highly controlled setting consisting of clearly defined consecutive phases. Thus, fear is generally investigated by *Pavlovian fear conditioning*: a simple form of associative learning that involves the repetitive pairing of an initially neutral stimulus (conditioned stimulus, CS), such as a tone with an aversive stimulus, typically a mild electric foot shock (unconditioned stimulus, US) (LeDoux, 2000). The successful fear learning is then measured by the emotional response (freezing behavior, alterations in blood pressure and heart rate or increased skin conductance) after exposure to the tone alone. A consecutive fear extinction training consisting of repetitive presentations of the CS in the absence of a foot-shock usually results in a reduction of the fear response (an overview of the fear and extinction pathway in the amygdala is depicted in Fig. 3B and Fig. 4 and respective figure legends). *Extinction*

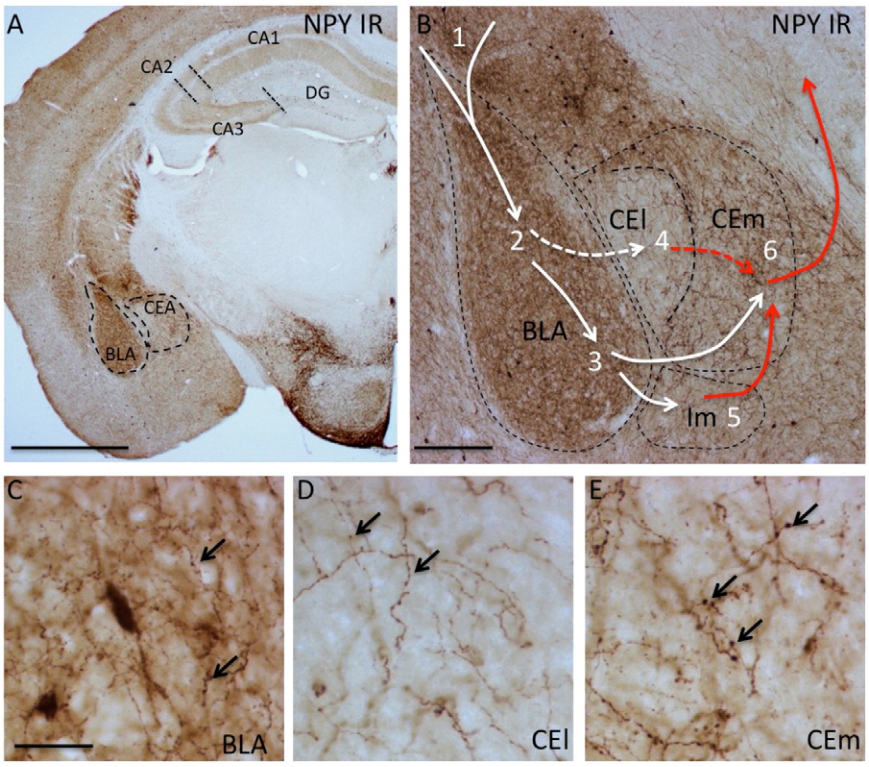

Fig. 3. High levels of NPY in the fear circuitry of the amygdala. (A) Photomicrograph depicting NPY-IR in a coronal section of a mouse brain depicting strong labeling of NPY-NPY IR in the basolateral (BLA) and central amygdala (CEA), (B) higher magnification of NPY-IR in the amygdala with arrows indicating the fear circuit: (1) thalamic and cortical inputs are transmitting fear related stimuli to neurons of the lateral amygdala (2), which are in turn projecting the BLA (3) that is known for its reciprocal connections with the prefrontal cortex (prelimbic: fear; infralimbic: extinction) and hippocampus (context fear). Projections from the lateral amygdala (2) are targeting, via dorsomedial intercalated neurons (dashed arrow), the centrolateral amygdala (CEl, 4) and BLA neurons project directly (fear neurons) or indirectly (extinction neurons) via the main intercalated nucleus (Im, 5) to the centromedial amygdala (CEm, 6), the main output nucleus of the amygdala with efferent connections to the bed nucleus of the stria terminalis, hypothalamus and brain stem. (C) Higher magnification depicting NPY-IR in the BLA, (D) CEl and (E) CEm. Note the dense labeling of thin axons in the BLA, faint fiber labeling in the CEl and distinct axons with big varicosities in the CEm (black arrows). Scale bars in A: 2 mm, B: 200 μm and C-E: 20 μm.

learning generates a competing memory that is generally assumed to be the underlying principle of human exposure therapy (Quirk and Mueller, 2008). The experimental properties of fear and extinction learning are highly controlled and largely independent of the prevailing motivational state of the tested subject. An advantage of these controllable settings is that different drugs or molecules that are suspected to modify the processing of fear conditioning and extinction can be applied in a controlled manner during the different experimental phases. Thus, the obtained results may provide valuable information relevant for treatment of human anxiety disorders.

3.2. Role of NPY in fear conditioning and extinction

A role of NPY in emotional memory processes was first postulated by Flood et al. (1987) whereby NPY affected learning and memory in a complex time and dose-dependent manner with an overall enhancement of retention in a T-maze foot-shock avoidance and a step-down passive avoidance training in mice (Flood et al., 1987). This effect was likely mediated by Y2 receptors in the dorsal but not in the ventral hippocampus (Cleary et al., 1994; Flood et al., 1989; Flood and Morley, 1989). However, since the test settings were rather complex, a clear conclusion regarding the effect of NPY on memory in general and the isolation of an anxiolytic and possibly also fear-reducing component is rather difficult to draw.

Early evidence that NPY modifies, in particular, fear learning comes from the work of Broqua et al. (1995). In this study, the authors suggested that intracerebroventricular (icv) injections of NPY and PYY inhibited fear-potentiated startle in rats. Since the Y1 receptor-preferring agonist $Leu^{31}Pro^{34}$-NPY produced a similar response as NPY, the effects were likely mediated by Y1 receptors. However, $NPY_{2\text{-}36}$ (a mixed Y2/Y5 agonist) but not $NPY_{13\text{-}36}$ (a more specific Y2 agonist) was also effective, suggesting the involvement of additional Y receptors (Broqua et al., 1995). All drugs were applied icv, indicating that the involved brain areas may be close to the ventricular system, however, an exact localization of this effect is still open.

Furthermore, transgenic rats over-expressing NPY displayed absent fear suppression in a punished drinking test and impaired spatial memory acquisition in the *Morris* water maze, suggesting a potential amnestic effect of NPY on emotional learning processes (Thorsell et al., 2000). Alternatively, a higher stress-buffer may drive NPY over-expressing rats to tolerate increased numbers of punished drinking episodes. Neurochemical alterations included a marked increase of NPY mRNA in the CA1 of these rats that was accompanied by a down-regulation of Y1 receptors but not of Y2 receptor binding sites in all hippocampal subfields. These data shift the Y2 receptor in the dorsal hippocampus into focus as a possible candidate for suppression of spatial and potentially also fear-related memories. On the other hand, germ-line over-expression of a neuropeptide (i.e. NPY) may alter the establishment of synaptic contacts during pre- and postnatal development leading to additional changes that may have remained undetected in this study.

Using contextual and cued fear conditioning, Karlsson et al. (2005) suggested that icv administration of NPY prior to training reduced context (0.5 nmol) and cued (1 nmol) freezing behavior in C57BL/6J mice when tested 24 h later under drug free conditions (Karlsson et al., 2005). Although post-US/CS freezing levels, examined immediately after fear acquisition were reduced by approximately 20%, this change was not significant. However, it may indicate that the action of NPY already influenced the acquisition of fear. Cued fear conditioning depends predominantly on the amygdala, whereas contextual fear is also modulated by the hippocampal formation in conjunction with the basolateral

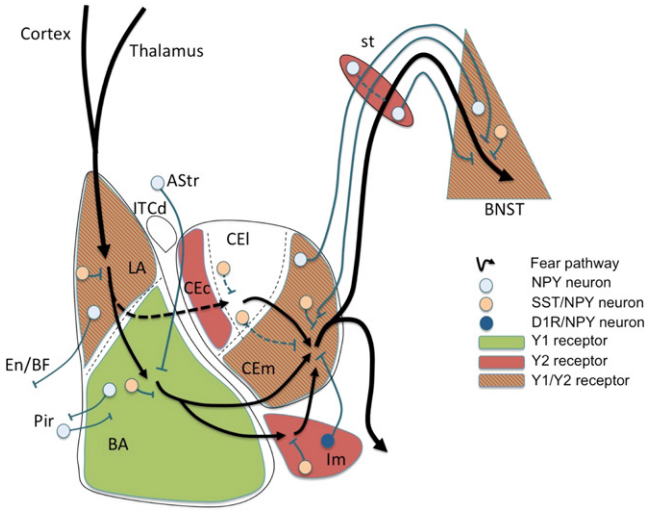

Fig. 4. Proposed fear circuit in the amygdala and potential interaction points of identified NPY neurons. Cortical and thalamic projections conveying somatosensory (US) and auditory (CS) information are targeting the lateral amygdala (LA), a major location of initial synaptic plasticity. SST/NPY/GABA neurons may inhibit pyramidal neurons by activation of Y1 and Y2 receptors reducing CS-related activation and fear learning. Similarly SST/NPY neurons in the basolateral nucleus (BLA) may have additional Y1 receptor-dependent inhibiting effects. The role of NPYergic afferent and efferent projections of the basolateral complex (LA and BLA) is not yet clear. Fear-related stimuli reach the central amygdala (CEA) also via mediodorsal intercalated neurons (ITCd to the centrolateral subdivision, CEl) or directly via projections from the BLA to the centromedial subdivision (CEm). NPY neurons may modulate afferent and efferent signals locally in the CEl or via inhibitory connections between CEl and CEm, probably by Y2 receptors. However, the main processing occurs in the CEm (expressing Y1 and Y2 receptors) by (1) local inhibitory SST/NPY/GABA neurons, (2) by NPY/GABA afferents originating from the BNST and (3) by NPY axon terminals originating from neurons of the main intercalated nucleus (Im). The latter projection consisting of NPY/D1R/GABA neurons in the Im is considered to play an essential role in fear extinction by providing a marked increase in feed-forward inhibition from the BLA to the CEm. Lastly, efferents from the CEm may be further modulated by NPY along the stria terminalis (st) or even in the bed nucleus of the stria terminalis (BNST), both by local interneurons and by bi-directional projections connecting the CEm with the BNST. (AStr, amygdalastriatal transition zone, En, entorhinal cortex, Pir, pririfrom cortex, BF, basal forebrain, CEc, capsular subdivision of the central amygdala nucleus).

amygdala (BLA) (Maren et al., 2013; Pape and Pare, 2010). As the icv application of NPY precludes a clear anatomical correlation, further studies are needed to elucidate the fear-reducing function of NPY in different limbic brain areas.

A fear-reducing effect was corroborated by an NPY-induced suppression of tachycardia during fear recall in an auditory fear conditioning paradigm in mice. The CS-induced tachycardia was also reduced by icv injection of the Y1 receptor agonist [D-Arg25]hNPY while the Y1 receptor antagonist BIBO 3304 (icv) was able to block the effects of icv NPY injections. These results indicate that tachycardia induced by aversive stimuli can be suppressed by exogenous NPY, probably by a Y1 receptor-dependent mechanism (Tovote et al., 2004). The involvement of other Y receptors and the role of endogenous NPY were, however, not addressed in this study.

The above findings are in agreement with the work of Gutman et al. (2008) testing the effect of NPY in the fear potentiated-startle paradigm in rats. They suggested that exogenously applied NPY, either icv or locally in the BLA, reduces the expression of conditioned fear, but not when injected locally into the medial amygdala (MEA). Interestingly, injection of the Y1 receptor antagonist BIBO 3304 into the BLA had no effect, suggesting that endogenous NPY in the BLA has no fear-reducing properties in this paradigm.

Furthermore icv injection of NPY facilitated the extinction of fear-potentiated startle while injection of the Y1 antagonist BIBO 3304 alone into the BLA blocked fear extinction (Gutman et al., 2008). Collectively, these data suggest that NPY reduces the expression of fear probably independent of Y1 receptors or alternatively that only exogenous application of NPY may reduce fear expression. On the other hand, endogenous NPY may facilitate fear extinction learning and recall likely by activation of Y1 receptors in the BLA (see Table 1).

In addition to classical pharmacological manipulations, genetic deletions of specific receptors or combining pharmacology and receptor knockout mice are attractive alternative strategies for studying the role of NPY in fear processing. Interestingly, fear conditioning experiments performed in Y1 receptor knockout (Y1KO) mice do not necessarily support a central role of Y1 receptors in the modulation of

Table 1
Summary of the role of NPY and Y receptors in different phases of fear conditioning and extinction.

	NPY	PP	Y1 activation	Y2 activation	Y4 activation	Y5	References
Context fear acquisition	Reduction, (icv, NPY)	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	Reduction (icv, LP-NPY)	n.d.	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	n.d.	Karlsson et al., 2005; Lach and de Lima, 2013, Verma et al., 2015a,c
Context fear consolidation	Reduction, (icv, NPY)	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	Reduction (icv, LP-NPY	n.d.	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	n.d.	Lach and de Lima, 2013, Verma et al. (2015a,c)
Context fear expression/recall	Reduction, (icv, NPY)	n.d.	Reduction (icv, LP-NPY)	n.d.	No effect (Y4KO mouse)	n.d.	Karlsson et al., 2005; Gutman et al. 2008; Lach and de Lima, 2013, Verma et al., 2015a,c
Context fear extinction	Facilitated, (icv, NPY)	n.d.	No effect, (icv, LP-NPY)	n.d.	Facilitated (Y4KO mouse)	n.d.	Lach and de Lima, 2013, Verma et al., 2015a
CS fear acquisition	Facilitated, (icv, NPY)	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	Reduction (Y1Y2KO mouse)	Reduction (Y1Y2KO mouse)	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36}) & Y4KO mouse	n.d.	Karlsson et al., 2005; Verma et al. 2012, Verma et al., 2015a,c
CS fear consolidation	n.d.	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	n.d.	n.d.	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	n.d.	Verma et al., 2015a,c
CS fear expression/recall	Reduction, (icv, BLA, NPY)	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36})	Reduction (icv, BLA)	Reduction (CEA/AAV-NPY $_{3-36}$, $Y2^{lox/lox}$)	No effect, (ip, [K^{30}(PEG2)]hPP_{2-36}) & Y4KO mouse	n.d.	Tovote et al., 2004; Gutman et al. 2008; Verma et al., 2015a,b,c
CS fear extinction	Facilitated, (icv, NPY)	Facilitated, (ip, [K^{30}(PEG2)]hPP_{2-36})	Facilitated (BIBO3304, BLA)	Facilitated (CEA/AAV-NPY $_{3-36}$, $Y2^{lox/lox}$)	Facilitated (ip, [K^{30}(PEG2)]hPP_{2-36}) & Y4KO mouse	n.d.	Gutman et al. 2008; Verma et al. 2012, Verma et al., 2015a,b,c
CS fear discrimination	Improved, (NPYKO mouse)	n.d.	No effect	Improved (Y2KO mouse)	No effect (Y4KO mouse)	n.d.	Verma et al. 2012
Fear re-emergence	n.d.	Reduced, (ip, [K^{30}(PEG2)]hPP_{2-36})	n.d.	Reduced (CEA/AAV-NPY $_{3-36}$)	n.d.	n.d.	Verma et al., 2015a,b,c

n.d., not done.

conditioned fear. More specifically, Y1KO mice did not display altered fear expression during fear acquisition and during context or cue-related fear recall, as consistently reported by independent groups (Fendt et al., 2009; Karlsson et al., 2008; Verma et al., 2012). This may be explained by the irrelevance of endogenous NPY, suggested by Gutman et al. (2008) or by the involvement of other Y receptors. On the other hand, germ-line deletion of Y1 receptors may provoke adaptations during pre- and postnatal development masking a potential role of Y1 receptors. It is therefore important to mention that the fear-reducing effect of intra-amygdala injections of NPY was neither recapitulated by application of the specific Y1 receptor agonists Y-28 (Des-AA$^{11-18}$ [Cys^{7,21}, d-Lys^{9} (Ac), d-His^{26}, Pro^{34}]-NPY) and Y-36 ([d-Arg^{25}, d-His^{26}]-NPY) nor blocked by concomitant injections of the Y1 receptor antagonist BIBO 3304 in mice. Consequently, injection of NPY into the amygdala of Y1KO mice still produced a robust decrease in fear expression, suggesting the involvement of other NPY receptors in the amygdala and/or other brain areas in the modulation of learned fear behavior (Fendt et al., 2009).

The role of NPY in contextual fear conditioning and extinction was investigated by icv injections of NPY or the Y1 receptor-preferring agonist Leu^{31}Pro^{34}-NPY during context fear acquisition, consolidation and extinction (Lach and de Lima, 2013). Low doses of NPY (3 pmol) and Leu^{31}Pro^{34}-NPY (1 pmol) were selected to avoid confounding factors such as altered locomotor activity or reduced states of anxiety. NPY as well as Leu^{31}Pro^{34}-NPY inhibited freezing behavior when administered icv in the acquisition or consolidation phase. These effects were blocked by pretreatment with the Y1 receptor antagonist, BIBO 3304, suggesting a suppression of contextual fear consolidation by a Y1 receptor-mediated mechanism. Interestingly, when injected in the extinction phase, only NPY but not Leu^{31}Pro^{34}-NPY reduced contextual freezing (Lach and de Lima, 2013). These results demonstrate a robust fear-inhibiting effect of exogenous NPY on contextual fear conditioning in rats, a response that is mediated, at least in part, by Y1 receptors (see Table 1). The fact that only NPY, but not a Y1 receptor agonist reduced fear during extinction learning, however, suggests again an interplay with additional Y receptors. As contextual fear conditioning is dependent on hippocampal processes, these data suggest that NPY may have distinct functions mediated by different receptors depending on the brain areas targeted. However, as the data were obtained by icv injections, a more detailed investigation taking into account different specific injection sites is still required.

Recent evidence concerning the role of the different Y receptors comes from the experiments of Verma et al. (2012) by investigating KO mice lacking NPY and different Y receptors in a differential fear conditioning and extinction paradigm (Verma et al., 2012). NPYKO mice displayed dramatically accelerated acquisition and an increased expression of CS-induced conditioned fear. On the other hand, Y1KO mice exhibited only a modestly accelerated fear acquisition, similar to the changes described by Fendt et al. (2009), whereas Y2KO mice were similar to controls. Interestingly, the strong phenotype seen in NPYKO mice was only reproduced in mice lacking both Y1 and Y2 receptors. In addition, NPYKO mice displayed impaired fear extinction. This behavior was recapitulated again only in Y1/Y2 receptor double KO mice (see Table 1). These data indicate that both, Y1 and Y2 receptors are acting synergistically to reduce the acquisition and expression of conditioned fear while on the other hand promoting fear extinction. It also suggests that postsynaptic Y1 and presynaptic Y2 receptors serve a similar purpose in processes of conditioned fear and that they may in fact compensate for each other.

Furthermore, an important finding from these experiments (Verma et al., 2012) was the inability of Y2KO mice to differentiate between two conditioned auditory stimuli, one that was repetitively paired with an unconditioned stimulus (CS+) and a second one that was never paired (CS−). The inability to discriminate fear-related stimuli from similar but innocuous stimuli points towards a generalization of conditioned fear. In fact, activation of Y2 receptors may counteract such generalization tendencies. Importantly, generalized fear is a phenomenon characteristic for certain types of human anxiety disorders, such as PTSD, and a main reason for the difficulty to achieve long-term suppression of fear by psychotherapy. Thus, further studies are needed to address a possible role of Y2 receptors in preventing fear generalization and to identify the involved neuronal networks.

Interestingly, the fear-reducing effect of NPY reported by Gutman et al. (2008) was bigger when NPY was applied icv compared to intra-BLA injections and was probably not purely Y1 receptor-mediated. These data indicate a contribution of other Y receptors and/or additional brain areas, as suggested by the experiments of Lach and De Lima (2013). Evidence supporting a role of Y2 receptors in learned fear was provided by a recent study demonstrating that viral-vector-mediated local over-expression of the Y2-preferring agonist NPY$_{3-36}$ in the central amygdala reduces the expression of conditioned fear and promotes fear extinction (Verma et al., 2015b). Importantly, peripheral injection of the brain-penetrant Y2 receptor antagonist JNJ-31020028 blocked the fear–reducing and extinction-promoting effect of viral vector-induced over-expression of NPY$_{3-36}$. In line with this, site-specific local deletion of Y2 receptors in the CEA of conditional Y2 receptor KO mice increased the expression of conditioned fear and delayed fear extinction (see Table 1), suggesting also a role of endogenous NPY acting on Y2 receptors (Verma et al., 2015b). In this regard it is important to mention that local over-expression of NPY$_{3-36}$ in the CEA also produced a permanent suppression of conditioned fear as evidenced by reduced spontaneous recovery (re-emergence of fear over time) and re-instatement of extinguished fear (re-emergence of fear by stress exposure). Both parameters, spontaneous recovery and re-instatement, were, however, increased when the mice were pre-treated with ip injections of the Y2 receptor antagonist JNJ-31020028 before extinction training (Verma et al., 2015b). These findings have important implications for the translation of experimental data into clinics. In particular, the labile nature of fear extinction memories in general, and the frequently observed transient effect of exposure therapy in human patients constitute a limiting factor for successful treatment of human anxiety-disorders. A limitation of the study was, however, the long-term over-expression and deletion of NPY$_{3-36}$ and Y2 receptors, respectively that may have triggered adaptations actually responsible for the altered fear behavior. Thus, future studies combining acute local drug injections and Y receptor KO mice may shed more light on the role of Y2 receptors in the modulation of conditioned fear.

Most recently, Verma et al. (2015a,c), also demonstrated an important role of Y4 receptors in the extinction of fear. In the central nervous system, Y4 receptors are expressed in specific brain areas, such as the hypothalamus and brain stem (Tasan et al., 2009). They are targeted by PP originating from F-cells of the pancreas. Verma et al. (2015) subjected Y4KO mice to a differential fear conditioning paradigm followed by contextual and cued fear testing and fear extinction. Importantly, while Y4KO mice displayed unchanged fear acquisition and recall, the extinction of contextual and cued fear was significantly delayed or completely impaired, respectively (see Table 1). Interestingly, the impaired fear extinction was associated with a loss of synaptic plasticity in an amygdala microcircuit that was rescued by short-term fasting (Verma et al., 2015c). In fact, also NPY can activate Y4 receptors in the CNS and NPY may even be the predominant ligand. However, in a follow-up study Verma et al. (2015a) demonstrated that peripheral injection of a long-acting Y4 receptor agonist specifically promoted fear extinction under fasted but not under fed conditions (Verma et al., 2015a). These data highlight the close relationship between fear and feeding and put, in particular, gut-brain related peptides, such as NPY, PYY and PP, into focus for adapting emotional behaviors to restore internal homeostatic balance.

An interesting and novel strategy of how to interfere with the NPY system was recently provided by Canneva et al. (2015). In their study, the group investigated functionally deficient dipeptidylpeptidase IV (DPP4) congenic rats in *Pavlovian* fear conditioning and extinction (Canneva et al., 2015). DPP4 is involved in the degradation of NPY, but

also other biologically active peptides. While acquisition and expression of fear was unchanged, short-term fear extinction was facilitated in mutant rats. This was associated with altered NPY levels in the CSF and reduced mRNA expression in the amygdala for Y1, Y2 and Y5 receptors but not of NPY itself. The extent to which CSF or plasma levels reflect the conditions in the CNS may be questionable and is still under debate. However, the reductions in Y1, Y2 and Y5 mRNA levels in the amygdala indeed suggest an altered NPY turnover. Importantly, prolonged and high NPY levels may be one explanation for the facilitated fear extinction. Since these rats harbor a germ-line mutation, adaptations during embryonic or postnatal development may be equally plausible. In addition, DPP4 has broad substrate specificity and NPY is only one of several potential substrates. Importantly, DPP4 inhibitors are already approved drugs for treating type 2 diabetes. Thus, this potentially attractive approach warrants further studies to demonstrate that fear processing may be indeed modulated by interfering with degradation enzymes for NPY.

Taken together, the above-mentioned experimental data emphasize the involvement of multiple Y receptors in the modulation of conditioned fear, and more specifically they highlight the importance of Y2 and Y4 receptors as important partners of Y1 receptors in the extinction of learned fear (see Table 1). The role of Y5 receptors as an additional target has been discussed in many of the above-mentioned studies, however, experimental data supporting a role of Y5 receptors in conditioned fear are still lacking.

Local injections of NPY suggest that the resulting suppression of fear is mediated at least in part in the BLA. The role of endogenous NPY acting through Y1 receptors in the suppression of fear is, however, still not convincingly defined. On the other hand, experiments using local injections of AAV vectors suggest that Y2 receptors in the CEA suppress fear expression while promoting fear extinction. Different brain structures, such as amygdala and hippocampus, are distinctly involved in fear and extinction learning and NPY may interfere there with multiple pathways and receptors. However, only 3 studies have used site-restricted injections into the amygdala (Fendt et al., 2009), BLA (Gutman et al., 2008) and CEA (Verma et al., 2015b). As most of the other studies employed icv injections, the role of NPY in other fear-relevant brain areas is essentially unknown. It becomes more and more apparent that apart from Y1 receptors, also other Y receptors, and in particular Y2 receptors, are involved in fear conditioning and extinction. A wealth of literature suggests an anxiolytic action of postsynaptic Y1 receptors and an anxiogenic-like action after activating presynaptic Y2 receptors. This concept may be questioned, since anxiolysis and improved stress-coping abilities were lost after backcrossing Y2KO mice to a C57BL/6J background (Tschenett et al., 2003; Zambello et al., 2011). Overall, the evolutionary utility of an anxiolytic neuropeptide that essentially acts as a stress buffer and reduces anxiety by one receptor while at the same time increasing anxiety by another receptor is difficult to reconcile. Under physiological conditions, endogenous NPY is released and will activate all available Y receptors in a similar fashion. Regarding fear and fear extinction, it rather seems that individual Y receptor subtypes exhibit a high degree of synergy collectively directed towards a reduction of fear expression. In particular, Y2 receptors in the CEA may adapt inhibitory and excitatory afferents originating from diverse extinction-related brain areas, including the BLA and the main intercalated nucleus, but also cortical structures. Clearly further studies are needed to elucidate those amygdala inputs that are modulated by NPY and Y2 receptors.

3.3. The role of NPY and its receptors in spatial learning

When looking at the impact of a neuromodulator on emotional learning processes of fear and fear extinction, one has to consider also its activity in memory formation in general or ideally on learning processes that are largely independent of emotional components.

Among the first studies investigating the role of NPY on memory formation and retention were those by Flood and coworkers (Flood et al., 1989). The authors infused 0.05 to 1 μg (0.03 to 0.5 nmol) of NPY into the rostral and caudal hippocampus, septum, amygdala, striatum and thalamus of mice and tested the animals for memory retention of foot shock avoidance training in a T-maze. NPY improved retention when injected into the septum or the rostral hippocampus but impaired retention when applied to the caudal hippocampus or amygdala. It had no effects when injected into the striatum or thalamus (Flood et al., 1989). Injection of an NPY antibody into the same brain regions produced opposite effects. Since the injections of NPY or the NPY antibody were performed after training, NPY interfered probably with the memory consolidation process. This is also supported by NPY injections 24 h after training, in which no memory-altering effects were observed. On the other hand, icv injections of higher doses of NPY (3 and 10 μg) 30 min before testing, impaired short-term memory in a conditioned discrimination task involving a food reward in rats (Cleary et al., 1994). The test systems used, however, were relatively complex and included components of both, spatial and emotional memory.

Thorsell et al. (2000) developed a transgenic rat-line over-expressing NPY in particular in the CA1 sector of the hippocampus (see above). These rats displayed a pronounced impairment in acquisition and potentially also retrieval of spatial memory in the *Morris* water maze (Thorsell et al., 2000), a testing paradigm in which rodents have to find a hidden platform in a water-filled pool by orienting using external cues (D'Hooge and De Deyn, 2001). The hippocampus is crucially involved in spatial memory processes and NPY is contained in numerous GABAergic interneurons of the dentate gyrus and the hippocampus proper (Sperk et al., 2007). In line with these results, rats overexpressing NPY in the hippocampus by injecting locally an adeno-associated viral vector (AAV-NPY) displayed impaired spatial learning during the acquisition of a two-platform discrimination version of the *Morris* water maze (Sorensen et al., 2008a). The authors also reported impaired long-term potentiation (LTP is an electrophysiological correlate of synaptic plasticity associated with learning and memory) in ex vivo hippocampal slices from AAV-NPY injected rats, while basal neurotransmission appeared unaffected.

In support of a potential role of Y1 receptors, NPY and the Y1 receptor agonist [Leu^{31}, Pro^{34}] NPY decreased the latency of colchicine treated rats to find the platform in a *Morris* water maze, whereas the Y1 receptor antagonist BIBP3226 produced an opposite effect (Rangani et al., 2012).

Furthermore, a recent study found that peripheral injection the Y2 receptor agonist PYY_{3-36} (1 and 20 μg/kg) significantly prolonged the latency to reach the platform in a matching-to-position version of the *Morris* water maze. Whether peripherally injected PYY_{3-36} may indeed enter the brain parenchyma (Nonaka et al., 2003) or target only Y2 receptors in brain areas that are partially open to the blood–brain barrier (Dumont et al., 2007) remains to be demonstrated. Furthermore, PYY_{3-36} is also a potent activator of Y5 receptors, however, a role of Y5 receptors in emotional or non-emotional learning and memory has not been addressed so far.

In a complementary approach, Redrobe et al. (2004) tested Y2 receptor germ-line knockout (Y2KO) mice in a *Morris* water maze. While memory acquisition in Y2KO mice was similar to controls, memory retention, tested on day 5 when no platform was present, was markedly impaired in Y2KO mice (Redrobe et al., 2004). Since spatial learning and retrieval in the *Morris* water maze is largely dependent on the hippocampal circuitry (Rolls, 1999), the authors concluded that hippocampal functions were compromised in Y2KO mice (Redrobe et al., 2004).

Taken together, there is a clear trend towards an impairment of spatial memory consolidation and probably also acquisition and retrieval by exogenous NPY acting on Y2 receptors. Y2KO mice may have the advantage that they allow an insight into the action of endogenous NPY on Y2 receptors. However, the apparent discrepancy between the results obtained in Y2KO mice and the experiments using pharmacology or viral vectors, might have several other reasons: 1) permanent lack of

Y2 receptors during pre- and postnatal development has been demonstrated to alter synaptic activity (Wood et al., 2015), 2) differences in genetic background are significantly influencing the testing results of Y2KO mice in the same behavioral approach (Redrobe et al., 2003; Tschenett et al., 2003; Zambello et al., 2011), 3) in addition, compared to semi-aquatic rats, mice have an inborn aversion towards water, and enforced swimming in the *Morris* water-maze includes a strong emotional, challenge (D'Hooge and De Deyn, 2001; Harrison et al., 2009). Thus, interpreting *Morris* water maze performance in mice as purely memory-related without considering emotional components may harbor several pitfalls.

An approach that should have minimal emotional influence on memory performance is the object recognition test. This test is based on spontaneous, differential exploration of a familiar versus a novel object (Ennaceur and Delacour, 1988) and is supposed to reflect hippocampal, and in particular, cortical processing (Buckmaster et al., 2004; Clark et al., 2000). Y2KO mice displayed impaired consolidation of object recognition memory (6 h) (Painsipp et al., 2008; Redrobe et al., 2004) whereas short-term memory tested after 1 h was still intact. However, experiments using NPY pharmacology in this test are yet not available.

In conclusion, a predominant amnestic-like role of NPY has been proposed for non-emotional learning. Especially in spatial learning, NPY may exert a modest inhibitory role on memory consolidation and retrieval, likely mediated by activation of Y2 receptors in the dorsal hippocampus. Such a memory-impairing function of Y2 receptors may in part underlie the reduced expression of conditioned fear, however, it does not entirely explain the extinction-promoting effect of Y2 receptors or the long-term suppression of fear. Thus, even though Y2 receptors seem to interfere with the formation of spatial learning, they also direct emotional memories towards reduced fear expression.

4. Underlying mechanisms

4.1. Distribution and connectivity of NPY neurons in the amygdala complex

NPY is considered to be the most abundant neuropeptide in the rodent and human brain. Importantly, high levels are found in the amygdala, hippocampus and cerebral cortex (Fig. 1A and B) (Allen et al., 1983; Gustafson et al., 1986; Morris, 1989), brain areas that are also fundamentally involved in the modulation of conditioned fear (Pape and Pare, 2010; Sah et al., 2003). Although the basic organization of the fear/survival circuit has been well described (LeDoux, 2000; Pare et al., 2004), the relevance of individual neurotransmitters and neuromodulators, such as NPY, and their effects on local microcircuits are only poorly understood. Essentially, thalamic and cortical inputs (Fig. 3B-1) target the lateral amygdala (LA, Fig. 3B-2), where the unconditioned stimulus and the conditioned stimulus converge, generating an initial associative fear memory. Furthermore, the BLA (Fig. 3B-3) seems to propagate transitions between different fear states and also reciprocally connects to the hippocampus and prefrontal cortex conveying information about fear context and/or fear extinction memories (Herry et al., 2008). The BLA (Fig. 3B-3) and LA (Fig. 3B-2) project directly or indirectly, via the intercalated cell masses (Fig. 3B-5), to the CEA (Fig. 3B-6), which then forms the main efferent route of the amygdala complex (Pape and Pare, 2010).

4.1.1. NPY in the basolateral amygdala (BLA)

Since NPY is expressed in neurons of several amygdala nuclei (Fig. 3A and B), it is ideally positioned to influence different steps of fear and extinction processing. The BLA is considered to have a cortex-like structure consisting of glutamatergic principal neurons (PN) and local GABAergic neurons providing a constant suppression of PN activity. About 15% of these GABAergic neurons in the BLA were suggested to be immunopositive for NPY and more than 80% of BLA-NPY neurons also express GABA (McDonald, 1989). According to the expression of immunohistochemical markers for calcium-binding proteins and different neuropeptides, inhibitory BLA-neurons can be divided into several different subpopulations: These include parvalbumin (PV) neurons, somatostatin (SST) neurons, large cholecystokinin (CCK) neurons and small bipolar neurons containing various amounts of vasoactive intestinal polypeptide (VIP), CCK and calretinin (CR) (McDonald and Mascagni, 2001, 2002). In the BLA, NPY is generally co-expressed in the group of SST neurons, where virtually all NPY cells co-express SST (McDonald, 1989). More than 80% of these SST/NPY neurons in the BLA form symmetrical synapses at distal dendrites and dendritic spines of CaMKIIalpha-positive pyramidal neurons and their axon terminals were often seen in close proximity to asymmetrical (excitatory) synapses (Muller et al., 2007). This configuration may allow SST/NPY neurons to efficiently control the impact of inputs to their target cells. Indeed, it has been suggested that during auditory CS presentation PV-positive neurons inhibit SST neurons of the BLA, enabling associative fear learning (Wolff et al., 2014). Thus, activation of SST neurons, and probably also concomitantly released NPY, may reduce fear learning and decrease auditory responses in BLA principal neurons (Fig. 4). Furthermore, neurogliaform cells and the related Ivy cells are considered to be the most abundant types of interneurons in several brain structures, including the cortex, hippocampus and interestingly also the BLA (Fuentealba et al., 2008; Klausberger and Somogyi, 2008; Manko et al., 2012). These neurons innervate the dendrites of pyramidal neurons and evoke a slow GABAergic inhibition. Importantly, neurogliaform and Ivy cells also contain considerable amounts of NPY. It has been proposed that in the BLA these neurons form, more likely, non-synaptic appositions than real synaptic contacts, indicating that they may act via volume transmission releasing GABA and also NPY to provide slow and diffuse inhibition of BLA network activity. It is thus conceivable that upon emotional arousal, activation of neurogliaform and Ivy cells may result in a reduction of fear expression during acquisition, recall and fear extinction, an effect that may be even prolonged by concomitant NPY release. Recent evidence suggests the additional presence of long-range non-pyramidal NPY neurons in the LA and BLA projecting to the basal forebrain (McDonald et al., 2012) and entorhinal cortex (Fig. 4) (McDonald and Zaric, 2015a). On the other hand, a relatively small number of GABAergic, SST/NPY neurons in the amygdala-striatal transition zone and in the entorhinal area are also targeting the BLA (Fig. 4) (McDonald and Zaric, 2015b). The physiological relevance of these NPY projection neurons is, however, still unclear. Taken together, NPY in the BLA may predominantly originate from local interneurons (most of them also contain SST) but also from afferent projections, and NPY is also expressed in BLA efferent projections (Fig. 4).

4.1.2. NPY in the central amygdala (CEA)

The CEA, consisting of a lateral (CEl), medial (CEm) and capsular (CEc) subdivision, is the main output nucleus of the amygdala and projects to the BNST, hypothalamus, basal forebrain and brain stem (Fig. 5) (LeDoux et al., 1988). Ample evidence suggests that the CEA is most relevant for fear expression. In fact, the CEA is far from being a passive output relay, but forms in itself a highly elaborated micro-network and is capable of establishing diverse forms of plasticity (Ciocchi et al., 2010; Haubensak et al., 2010). In contrast to the BLA, the CEA is considered to be a striatal-like structure (Fig. 3C–E) (McDonald, 1982a, 1982b). Early studies have found that NPY is predominantly expressed in the medial subdivision of the CEA, but only scattered neurons were found in the CEc, and CEl. Most of the NPY neurons in the CEl did not co-localize with SST (Gustafson, Card, & Moore, 1986; McDonald, 1989). Furthermore, the NPY-IR in the CEm was predominantly originating from local sources since different neurotoxic lesions of catecholaminergic nuclei, such as the locus coeruleus and the ventral tegmental area did not reduce NPY fiber staining in the CEm (Gustafson et al., 1986).

A recent study using NPY-GFP mice has demonstrated that NPY in the CEA is contained in SST neurons and to a lesser extent in CR neurons, in particular in the CEm (Wood et al., 2015). In the same study it has been demonstrated that NPY neurons of the CEm connect also to the BNST and vice versa, NPY neurons of the BNST project to the CEm.

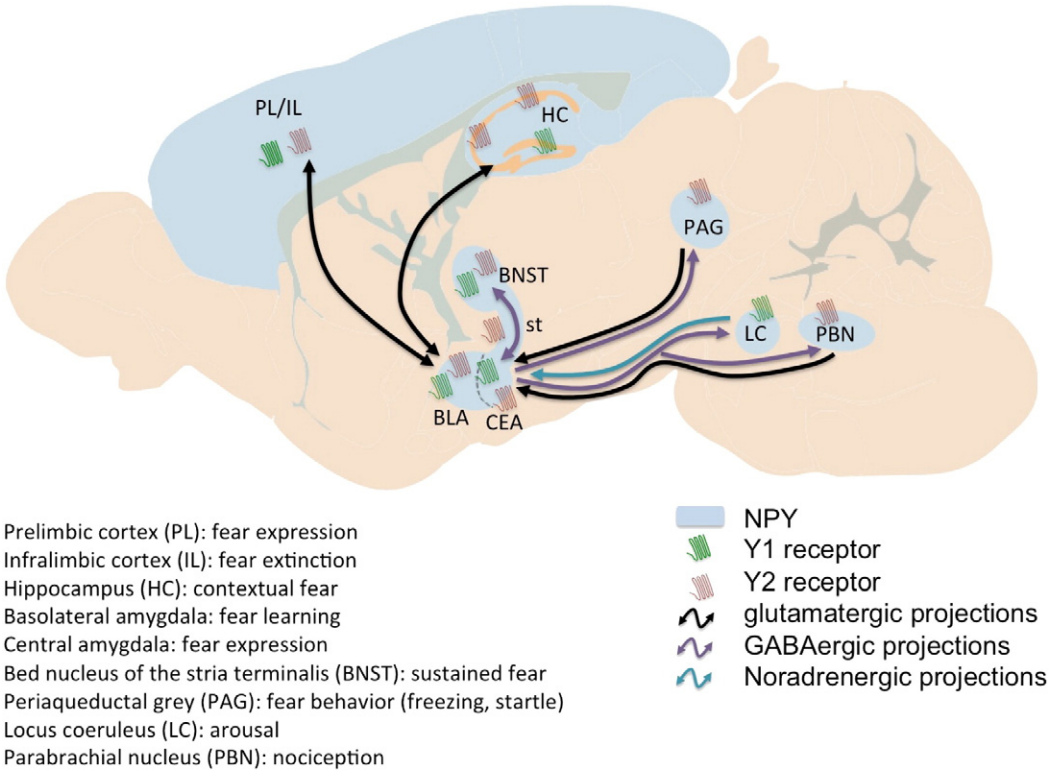

Fig. 5. Schematic illustration of the main fear pathways with the expression of NPY, Y1 and Y2 receptors at different levels. Reciprocal connections between BLA and prefrontal cortex (PL, prelimbic, IL, infralimbic) as well as hippocampus (HC) are important for fear expression, contextual modulation, and fear extinction allowing the transition between different fear states. The central amygdala nucleus (CEA) initiates a fear response by projecting via the stria terminalis (st) and the bed nucleus of the stria terminalis (BNST) or directly to the periaqueductal gray (PAG, causing a behavioral response), locus coeruleus (LC, arousal) and parabrachial nucleus (PBN, nociception), all of which are populated by NPY and Y receptor expressing neurons.

Furthermore, NPY is highly expressed in the main intercalated nucleus (Im) (Fig. 2C), an amygdala cell cluster fundamentally involved in the extinction of conditioned fear (Likhtik et al., 2008). In the Im, at least two types of NPY neurons have been described (Fig. 4). Type one expresses high levels of NPY and co-localizes with SST and type two co-expresses dopamine D1 receptors (D1R) and has significantly lower levels of NPY, both on protein as well as on mRNA levels (Wood et al., 2015). Thus, an extinction promoting effect may be mediated by NPY released from D1R-expressing neurons of the Im targeting the CEm (Fig. 4). Indeed, recent evidence suggests that a marked increase in feed-forward inhibition from the BLA to the CEm via medioventral intercalated neurons is an electrophysiological correlate for the extinction of conditioned fear (Amano et al., 2010; Verma et al., 2015c). It is tempting to speculate that the low basal levels of NPY observed in D1R-positive neurons of the Im can be up-regulated upon fear extinction learning and thus represent a cornerstone for long-term suppression of fear. A central role of NPY in fear extinction is, however, supported by the complete absence of fear extinction in NPYKO mice (Verma et al., 2012). In this context it is noteworthy that there are two additional brain areas with similar NPY-expression patterns as observed in the Im: the reticular nucleus of the thalamus (RT, Fig. 2B) (Morris, 1989) and the bed nucleus of the anterior commissure (BAC, Fig. 2A), both consist of very dense accumulations of NPY neurons that express considerably lower levels of NPY than generally observed in NPY-neurons of the rest of the brain. Interestingly, immunotoxin-mediated ablation of BAC neurons that exclusively target the medial habenula resulted in higher fear responses and increased fear memory (Yamaguchi et al., 2013). However, the role of the dense accumulation of low-level NPY in these neurons remains to be determined.

In summary, NPY in the amygdala cannot only modulate afferent and efferent connections when released from local circuit neurons, but NPY is also contained in afferent and efferent GABAergic projections of the amygdala (Fig. 4). Furthermore, NPY is involved in the micro-network of inter-nuclear projections that connect different amygdala nuclei or subdivisions of the same nucleus, some of which have been identified as being central for fear extinction. Finally, there are several brain regions with neurons expressing a relatively low level of NPY with still unknown physiological relevance (Fig. 2).

4.2. Distribution of Y receptors in amygdala complex

Y1 receptors: In the amygdala complex, Y1 receptor mRNA has been described in the BLA and LA as well as in the CEA (Fig. 1C). Immunohistochemical data indicate that Y1 receptors are expressed by cell bodies and dendrites (Kopp et al., 2002). A high concentration of Y1 receptor protein was apparent in the LA and in particular in the CEm. Although recent evidence suggests the expression of Y1 receptors in BLA pyramidal neurons further studies are needed to refine these findings (Giesbrecht et al., 2010; Rostkowski et al., 2009). The identity of Y1 receptor expressing neurons in the CEA has not been addressed so far.

Y2 receptors: In the amygdala, Y2 receptors are predominantly expressed in the medial subdivision of the central nucleus and in the medial amygdala (Fig. 1E and F). Little expression has been observed in the LA and BLA (Stanic et al., 2006). Similar to NPY, also Y2 receptors have been described in the stria terminalis (Tasan et al., 2010). This indicates that projections from the amygdala that target the BNST and other forebrain structures are also expressing Y2 receptors and may be thus potentially modulated along the whole extent of the stria terminals. Indeed, local deletion of Y2 receptors in the central nucleus of the amygdala resulted also in a reduction of [^{125}I]PYY_{3-36} receptor binding in target areas, such as BNST, *nucleus accumbens* shell and *locus coeruleus* (Tasan et al., 2010). In the CEA, Y2 receptors are highly expressed in the medial and in the capsular subdivisions but only weakly in the centrolateral part (Stanic et al., 2006; Wood et al., 2015). Y2 receptor-expressing

neurons are co-localizing there with GABA, but not with Y1 receptors or NPY as shown by dual-immunohistochemistry in colchicine treated mice (Stanic et al., 2011). In a recent study, expression of Y2 receptors was also apparent in intercalated neurons of the amygdala. In particular, Y2 receptor labeling was demonstrated between the BLA and CEA connecting there the dorsomedial cluster of the intercalated neurons with the main intercalated nucleus (Wood et al., 2015). This connection between different ITC clusters has been implicated in fear extinction previously (Pare et al., 2004) and NPY may have a supportive role by activation of Y2 receptors. Thus, Y2 receptors are expressed along fiber tracts of the extended amygdala (Fig. 4) and their putative presynaptic localization may reduce the frequency of neurotransmitter release and dampen network activity (discussed in detail below). Importantly, Y2 receptors in the amygdala did not co-localize with NPY neurons (Stanic et al., 2011; Wood et al., 2015) precluding a direct release-inhibiting activity on NPY neurons. Y2 receptors may rather function as hetero-receptors on long-fiber tracts and NPY may be released from adjacent interneurons upon strong and repetitive stimulations. Thus, a Y2 receptor-dependent effect will be apparent under stressful conditions and volume transmission of NPY may produce a long-lasting inhibition.

4.3. Electrophysiology of NPY in the extended amygdala

The amygdala complex consists of various nuclei with a predominantly unidirectional information-flow from the LA and BLA nuclei as major input stations to the CEA as a major output (Fig. 3B). Because of their high similarity in terms of neuronal populations and connectivity, the CEA, the BNST, *substantia inominata* and the *nucleus accumbens shell* are summarized as the so-called extended amygdala. As mentioned above, the CEA and BNST are reciprocally connected independently by NPY and Y2 receptor containing neurons providing a framework for the modulation of synaptic activity (Fig. 5).

Bed nucleus of the stria terminalis: Recent studies utilizing stimulations of specific projections by optogenetics have demonstrated that the BNST and its projections to several brain regions play a critical role in modulating specific physiological aspects of fear and anxiety (Jennings et al., 2013; Kim et al., 2013). It is well known that NPY and NPY receptors are expressed in the BNST and stria terminalis (Kash and Winder, 2006; Tasan et al., 2010; Wood et al., 2015) and are thus well positioned to modify fear and anxiety.

Consistent with the finding that NPY reduces excitatory and inhibitory synaptic transmission in the hippocampus, often by presynaptic Y2 receptors and reduced presynaptic calcium influx (see Electrophysiology in the Hippocampus below), activation of the Y2 receptor in the BNST suppresses GABAergic transmission by a similar mechanism (Kash and Winder, 2006; McCall et al., 2013). Using the Y2 receptor agonist and antagonist, $NPY_{13\text{-}36}$ and BIIE0246, respectively, Kash and Winder (2006) demonstrated that Y2 receptor activation reduces the amplitude of evoked inhibitory postsynaptic currents in ventrolateral BNST neurons while Y1 and Y5 receptor agonists and antagonists had no effect. Interestingly, chronic stress reduces the ability of NPY to suppress evoked inhibitory postsynaptic currents in DBA/2J mice, but not in C57BL/6J mice, suggesting that stress can alter NPY signaling and that this may depend on genetic background (Pleil et al., 2012). Postsynaptic NPY receptors have also been detected in the BNST. Specifically, NPY causes hyperpolarization of the resting membrane potential in a subset of dorsolateral BNST neurons by blocking the hyperpolarization-activated current (Ih): an effect blocked by co-application of the Y1 and Y5 receptor antagonists, BIBP-3226 and L-152,804, respectively (Ide et al., 2013). Although many studies have found that NPY typically reduces synaptic transmission, it is interesting to note that NPY can also enhance inhibitory transmission. Pleil et al. (2015) demonstrated that the Y1 receptor agonist, $Leu^{31}Pro^{34}$ NPY increases the frequency of miniature inhibitory postsynaptic currents in BNST neurons probably by increasing the surface expression of postsynaptic $GABA_A$ receptors (Pleil et al., 2015).

Although relatively few studies have investigated the effects of NPY in the BNST, it is clear that NPY may cause a decrease or increase in inhibitory input to BNST neurons depending on whether NPY activates pre- or postsynaptic receptors, respectively. Identifying the neuronal populations that are modulated by Y1 and Y2 receptors and understanding the following downstream projections and their relevance for fear expression may shed more light on the role of NPY in the BNST.

Central amygdala: Moving to the central amygdala, Gilpin et al. (2011) found that while ethanol increases inhibitory transmission by increasing presynaptic GABA release probability, this could be occluded by NPY-mediated activation of the Y2 receptor (Gilpin et al., 2011). Interestingly, application of the Y2 receptor antagonist, BIIE0246, increased the amplitude of evoked inhibitory postsynaptic potentials but NPY application did not modify miniature inhibitory postsynaptic currents, together suggesting that NPY may tonically inhibit GABAergic transmission in the central amygdala. On the other hand, Wood et al. (2015) found that the Y2 receptor agonist, $PYY_{3\text{-}36}$, reduces the frequency of both spontaneous inhibitory and excitatory postsynaptic currents in neurons specifically in the medial subdivision of the central amygdala (CEm) and that NPY- and Y2 receptor knockout mice exhibit elevated inhibitory transmission. These latter results are consistent with a tonic activation of Y2 receptors on GABAergic synapses targeting neurons of the CEm, however, application of the Y2 receptor antagonist, JNJ 31020028, did not increase inhibitory neurotransmission, suggesting an important role for NPY receptors during development. Evoked inhibitory postsynaptic currents in response to electrical stimulation of the medial input to the CEm, likely arising from the BNST, were attenuated by $PYY_{3\text{-}36}$, providing evidence that BNST projections to the CEm express the Y2 receptor. Although, at first glance, the studies by Gilpin et al. (2011) and Wood et al. (2015) may appear to have opposing results, the differences observed are probably due to differences in cell selection for recordings or time of day that they were performed (i.e., CEA versus CEm, and dark versus light phase, respectively). Regardless, these studies provide evidence that NPY signaling, particularly via the Y2 receptor, modifies synaptic transmission in the central amygdala and may present a therapeutic target in the treatment of anxiety and fear-related disorders.

Basolateral amygdala: Although the BLA is essential for fear learning and contains considerable amounts of NPY and Y receptors, only a handful of studies have investigated how NPY influences neurotransmission in the BLA using electrophysiological recordings. These studies provide important insights into how NPY signaling alters BLA function and fear processing. As in the other brain regions discussed, NPY signaling via the Y1 receptor leads to a reduction in intrinsic excitability of neurons in the BLA. Sosulina et al. (2008) found that, specifically in the lateral amygdala, NPY application activated Y1 receptor-coupled G protein-coupled inwardly-rectifying potassium (GIRK) channels causing hyperpolarization of the resting membrane potential and attenuated action potential firing (Sosulina et al., 2008). Similar results have been reported, more generally, in the BLA, where NPY hyperpolarizes the resting membrane potential of pyramidal neurons and decreases their intrinsic excitability (detected by an increase in rheobase, i.e., the minimal amount of current required to evoke action potentials (Giesbrecht et al., 2010)). The effect of NPY was replicated using the Y1 receptor-selective agonist, F7P34NPY, and was blocked by the Y1 receptor antagonist, BIBO3304. Similar to findings in the BNST, the effect of NPY was attributable to suppression of a tonic hyperpolarization-activated current (Ih). As would be expected, NPY can dampen network activity in the BLA as compound postsynaptic potentials (generated using a high-potassium model) are attenuated by NPY (Chung and Moore, 2009).

The Y1 receptor has also been shown to alter excitatory and inhibitory synaptic transmission in BLA pyramidal neurons. Specifically, activation of Gi/o-coupled Y1 receptors reduces NMDA receptor-mediated excitatory postsynaptic currents and increases $GABA_A$ receptor-mediated inhibitory postsynaptic currents by reducing the activity of exchange protein activated by cAMP (Epac) and Protein Kinase A

(PKA), respectively (Molosh et al., 2013). It is worth highlighting that the Y1 receptor was also found to increase $GABA_A$ receptor-mediated inhibition in the BNST (Pleil et al., 2015), as this provides a fine example of the similarities in Y receptor function in different brain regions. Thus, these studies provide evidence that NPY likely exerts its potent fear-reducing effects when injected into the BLA by reducing the activity of BLA pyramidal neurons.

4.4. Distribution and connectivity of NPY neurons in the hippocampus

GABA/NPY interneurons are abundant in all parts of the hippocampal formation, including the dentate gyrus, sectors CA1 to CA3 of the hippocampus proper, the subiculum and the entorhinal cortex (Kohler et al., 1986; Morris, 1989). They display extensive co-localization with SST and form symmetric synapses on dendrites of pyramidal neurons (Kohler et al., 1987). In the dentate gyrus, NPY is expressed by multiple classes of morphologically distinct dentate interneurons and potentially also in dentate granule cells where NPY is up-regulated following epileptic seizures (Deller and Leranth, 1990; Sperk et al., 2007). The majority of NPY neurons of the dentate hilus are medium-sized multipolar and fusiform cells. They co-express SST and send their axons to granule cell dendrites in the outer molecular layer (Kohler et al., 1987; Kosaka et al., 1988). A second population of NPY interneurons comprises pyramidal shaped cells at the inner surface of the granule cell layer with long apical dendrites reaching also the outer molecular layer. Some small multipolar NPY-IR cells are located in the molecular layer (Deller and Leranth, 1990; Kohler et al., 1986). In the rat, about 2% of the NPY neurons also project to the contralateral dentate gyrus (Deller and Leranth, 1990). The main target cells of NPY neurons are, however, probably ipsilateral dentate granule cells on which they may exert a dendritic Y1 receptor- and an axonal Y2 receptor-mediated inhibition. Furthermore, NPY neurons are also targeting neurons located in the hilus. In the dentate gyrus, synaptic inputs to NPY neurons may originate from different sources, such as the entorhinal and commissural inputs and mossy fiber collaterals from dentate granule cells. Also extra-hippocampal projections from cholinergic neurons of the medial septum are targeting NPY neurons of the dentate gyrus (Milner et al., 1997, 1999). In CA3 and CA1, inputs to NPY neurons may originate from mossy fibers and Schaffer collaterals or the entorhinal cortex, respectively.

4.5. Distribution and connectivity of Y receptors in the hippocampus

Y1 receptors: The highest amounts of Y1 receptors are present on dentate granule cells with somewhat lower expression in the pyramidal cell layer of the CA regions and probably also on scattered interneurons of the *strata oriens* and *radiatum*. Immunohistochemical data suggest a predominantly postsynaptic expression of Y1 receptors in the hippocampus, supported by dendritic labeling of the molecular layer of the dentate gyrus and *strata oriens* and *radiatum* of the hippocampus proper (Dumont et al., 1996; Kopp et al., 2002). This is also corroborated by comparing in situ hybridization data and receptor binding using Y1 receptor selective ligands (Fig. 1C and D) (Dumont et al., 1996; Gobbi et al., 1998; Kofler et al., 1997; Larsen et al., 1993).

Y2 receptors: Y2 receptor mRNA containing somata are predominately located in the granule cell layer of the dentate gyrus and in the pyramidal cell layer of the CA regions (Gustafson et al., 1997). On the other hand, Y2 receptor immunoreactivity and Y2 receptor binding is apparent in the *strata radiatum*, *oriens* and *lucidum* as well as in the hilus of the dentate gyrus (Dumont et al., 1996; Schwarzer et al., 1998; Stanic et al., 2006). Interestingly, dense Y2 receptor immunolabeling has been described in the granule cell layer of the ventral but not of the dorsal hippocampus (Stanic et al., 2006). The discrepancy between the distribution of Y2 receptor mRNA and Y2 receptor IR/receptor binding strongly supports a predominantly presynaptic localization of Y2 receptors within the hippocampal formation (Fig. 1E and F). Furthermore, since Y2 receptors seem to be present on glutamatergic principal neurons, release of NPY may result in a significant reduction of synaptic transmission in the tri-synaptic hippocampal pathway (Colmers et al., 1991). It is well known that hippocampal activity is correlated with contextual memory formation (Maren et al., 2013). Dentate granule cells are usually strongly inhibited and only a specific ensemble becomes activated upon encoding of new memories (Chawla et al., 2005; O'Keefe and Nadel, 1978). Recently, it has been suggested that newly born neurons of the dentate gyrus are essential for discriminative learning in a contextual fear conditioning task (Aimone et al., 2010). This is interesting in light of impaired fear discrimination in Y2KO mice (Verma et al., 2012) as it suggests that Y2 receptors may increase the contrast of synaptic encoding by inhibiting granule cell firing frequency in the mossy fiber pathway. Whether Y2 receptors are absent from newly born neurons, selectively increasing their activity and resulting in improved pattern separation remains to be demonstrated.

4.6. Electrophysiology of neuropeptide Y in the hippocampus

Solid evidence supports the role of the hippocampus in contextual fear learning (Ji and Maren, 2007; Maren et al., 2013). As discussed above, a few studies have investigated how modifying NPY signaling alters contextual fear memory, but none has directly targeted the hippocampus or combined electrophysiological analysis to investigate how synaptic transmission was altered. This is of course a big drawback considering the high expression of NPY itself and the even higher expression of the respective Y receptors in this brain area (Fig. 1). However, one can speculate as to how NPY signaling modifies fear processing in the hippocampus based upon the numerous studies characterizing how NPY modifies synaptic transmission in the different hippocampal subfields.

In fact, many of the initial studies to describe how NPY influences synaptic transmission were performed in the hippocampus. These studies demonstrated that NPY modulates synaptic transmission in the hippocampus at each level of the tri-synaptic loop: entorhinal cortex input to the dentate gyrus, dentate gyrus to CA3, CA3 to CA1 and subiculum. In general, electrophysiological recordings obtained from in vitro brain slice preparations from rodents and resected human tissue, have consistently found that bath application of NPY leads to a decrease in synaptic transmission via a presynaptic mechanism (Haas et al., 1987; Klapstein and Colmers, 1993). In region CA1 and CA3, NPY activates Y2 receptors that reduce glutamate release probability by limiting presynaptic influx of calcium ions via N and P/Q-type voltage dependent calcium channels (Colmers et al., 1987, 1988; El Bahh et al., 2005; Klapstein and Colmers, 1993; Qian et al., 1997; Sun and Miller, 1999; Weiser et al., 2000). Lower levels of calcium influx attenuate evoked excitatory postsynaptic potentials, and decrease the frequency of spontaneous excitatory postsynaptic currents to pyramidal neurons (McQuiston and Colmers, 1996). Thus, NPY signaling in the CA1 and CA3 regions significantly modifies particularly excitatory neurotransmission and can thereby influence synaptic plasticity. Sorensen et al. (2008a, 2008b) demonstrated that transgene expression of NPY in the hippocampus impairs long-term potentiation (LTP, a type of synaptic plasticity critical for learning and memory) at both Schaffer collateral-CA1 synapses and CA1-subiculum synapses, and impairs performance in the two-platform spatial discrimination water maze designed to assess hippocampal function. Furthermore, exogenous application of NPY prior to tetanic stimulation (high frequency stimulation used to induce LTP) blocks LTP induction by activating the Y2 receptor, and NPY administered several minutes after successful LTP induction attenuates evoked excitatory postsynaptic potentials back to baseline levels (Sorensen et al., 2008a, 2008b).

Excitatory synaptic transmission at mossy fiber-CA3 synapses is also attenuated by NPY application (Klapstein and Colmers, 1993). Guo et al. (2002) observed that NPY, the Y1 agonist $Leu^{31}Pro^{34}$ NPY, the Y2 agonist $NPY_{13\text{-}36}$ and the Y5 receptor-preferring agonist $D\text{-}Trp^{32}$ NPY all decreased the amplitude of field excitatory postsynaptic potentials in region CA3 (evoked by electrical stimulation of the mossy fiber

pathway). Importantly, in tissue obtained from temporal lobe epilepsy patients and in an animal model of epilepsy, Y1 receptor binding was significantly reduced (Furtinger et al., 2001; Kofler et al., 1997), suggesting rapid down-regulation upon repetitive, strong neuronal excitation, presumably by receptor internalization. The role of Y1 receptors in hippocampal circuitries is, however, poorly understood. Taken together, these studies provide a detailed characterization of how NPY alters synaptic transmission in region CA1 and CA3, particularly via the Y2 receptor, and shed light on how changes in synaptic transmission modify learning and memory.

Within the dentate gyrus, inhibitory cholecystokinin (CCK)-expressing basket cells, known to play a central role in generating γ-frequency oscillations (Freund, 2003), exhibit a reduction of excitatory and inhibitory input in response to NPY application in vitro. Ledri et al. (2011; 2012) found that NPY, via Y2 receptors, reduced the frequency of both excitatory and inhibitory postsynaptic currents in CCK-expressing basket cells, however, NPY treatment did not alter the intrinsic membrane properties (e.g., resting membrane potential, rheobase or action potential characteristics) of the basket cells, suggesting that CCK-expressing basket cells do not express postsynaptic NPY receptors but rather receive excitatory and inhibitory input from synapses containing functional Y2 receptors (Ledri et al., 2011, 2012). Interestingly, in mice subjected to repetitive seizures, NPY acted preferentially on synapses targeting the peri-somatic region of CCK-expressing basket cells, rather than more distant dendritic synapses, detected by specific changes in event kinetics. Thus, given the central role of CCK-expressing basket cells in governing network oscillations, the localization of NPY receptors along the somatodendritic axis of CCK-expressing basket cells may allow fine-tuning of network activity from the dentate gyrus to its postsynaptic targets.

Inhibitory hilar neurons extend their axons to the molecular layer where they modulate excitatory synaptic transmission from the perforant path to the granule cell layer of the dentate gyrus. Hilar neurons express postsynaptic Y1 receptors and activation of the Y1 receptor enhances the activity of GIRK channels, leading to membrane hyperpolarization and a reduction in spike frequency (Fu and van den Pol, 2007; Paredes et al., 2003). Interestingly, GABA may act excitatory in hilar NPY neurons of the mouse (Fu and van den Pol, 2007), potentially resulting in rapid synchronization of GABA, SST and NPY release and profound pre- and postsynaptic inhibition of excitatory hippocampal circuits.

Importantly, resected brain tissue taken from epileptic patients has provided evidence that NPY reduces excitatory transmission in the dentate gyrus and region CA1, via Y2 receptors, also in the human brain (Ledri et al., 2015; Patrylo et al., 1999). Furthermore, expression of NPY as well as Y1 and Y2 receptors undergo considerable plastic changes in the dentate gyrus and subiculum in animal models of epilepsy and in human tissue obtained by temporal lobe epilepsy surgery (Furtinger et al., 2001; Vezzani and Sperk, 2004).

Taken altogether, these studies show that NPY signaling has a prominent role in the hippocampus. Thus, pharmacological interventions of the NPY system here, using specific agonists and antagonists, could be used to alter hippocampal function thereby modifying learning and memory, possibly in relation to fear and anxiety disorders.

Considering the electrophysiological data obtained in the central extended amygdala, BLA and hippocampus altogether, it is tempting to conclude that the role of NPY is to reduce the activity of neurons. Broadly speaking, this is accomplished by presynaptic Y2 receptors that reduce excitatory transmission, and/or by postsynaptic Y1 receptors that reduce intrinsic excitability. However, a more complex situation exists whereby the hallmark NPY-mediated reduction of excitability is observed primarily in excitatory principal neurons, whereas NPY can increase inhibition, as observed in the BLA and BNST, or decrease inhibition, with or without a concomitant reduction in excitatory input, as observed in CCK-expressing basket cells in the hippocampus and GABAergic neurons in central extended amygdala. Furthermore, the functions of the Y4 and Y5 receptor are still poorly characterized. Thus, when considering therapeutic approaches that aim to modify fear processing by altering neurotransmission by NPY signaling, Y receptor distribution (i.e., pre- versus postsynaptic, excitatory versus inhibitory neurons) is an important consideration.

5. Conclusions and future directions

As outlined in this review, NPY modulates fear with a strong tendency towards reducing fear expression. Furthermore, NPY facilitates cued and contextual fear extinction, within-session and also the retrieval of extinction. Not surprisingly, it was suggested that these effects are generated in the BLA and CEA, two hotspots of fear processing. Since Y1 receptors are predominantly mediating the anxiolytic responses of NPY, they have been primarily addressed in models of learned fear. In the BLA, NPY colocalizes mostly with SST and hyperpolarizes principal neurons decreasing their intrinsic excitability by a Y1 receptor-mediated mechanism. Whether these NPY/SST neurons are reducing fear responses by inhibiting pyramidal neurons via direct synaptic contacts or if they are generally dampening synaptic activity by volume transmission, as suggested for the neurogliaform/Ivy cell groups, remains to be demonstrated. It seems, however, that fear and fear extinction rely also on other Y receptors that are acting synergistically with Y1 receptors. In particular, Y2 and also Y4 receptors are both involved in the extinction of fear. This may have important implications when developing novel therapeutic concepts for treating anxiety disorders, putting agonists and not antagonists for Y2 and Y4 receptors into focus. Given the general paucity of Y1 receptor expression in the human brain contrasting with high expression of Y2 receptors in the human cerebral cortex, a central role of Y2 receptors becomes more and more apparent when trying to translate experimental data from bench to bedside. Since Y2 receptors in the CEA may reduce inhibitory and excitatory synaptic transmission, characterization of the synaptic inputs and identification of the respective target neurons will increase our understanding of the involved microcircuits. From the available data it becomes clear that NPY modulates the fear circuitry at multiple successive access points, including the main intercalated nucleus, with its dense accumulation of NPY neurons, and the CEA with its reciprocal connections to the BNST. However, the role of Y1 receptors in the CEA or Y2 receptors in the BNST in fear conditioning and extinction as well as the underlying electrophysiological responses have not been investigated so far. Furthermore, while Y5 receptors have been proposed as possible modulators of conditioned fear, experimental evidence is not yet available. Behavioral and electrophysiological evidence of how NPY modifies the fear circuit is largely based on exogenous NPY application, leaving the role of endogenous NPY still open. Furthermore, additional studies are required to provide an overall concept of how NPY regulates amygdala activity at the level of microcircuits and to delineate the role of other brain areas, such as the hippocampus and cortical areas, all in relation to fear and fear extinction.

Conflict of interest

The authors declare no potential conflict of interest.

Acknowledgments

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