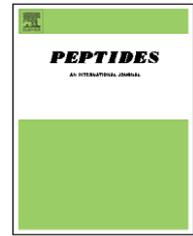


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## Review

# Neuropeptide Y in the recurrent mossy fiber pathway

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### ABSTRACT

In the epileptic brain, hippocampal dentate granule cells become synaptically interconnected through the sprouting of mossy fibers. This new circuitry is expected to facilitate epileptiform discharge. Prolonged seizures induce the long-lasting neoexpression of neuropeptide Y (NPY) in mossy fibers. NPY is released spontaneously from recurrent mossy fiber terminals, reduces glutamate release from those terminals by activating presynaptic Y2 receptors, and depresses granule cell epileptiform activity dependent on the recurrent pathway. These effects are much greater in rats than in C57BL/6 mice, despite apparently equivalent mossy fiber sprouting and neoexpression of NPY. This species difference can be explained by contrasting changes in the expression of mossy fiber Y2 receptors; seizures upregulate Y2 receptors in rats but downregulate them in mice. The recurrent mossy fiber pathway may synchronize granule cell discharge more effectively in humans and mice than in rats, due to its lower expression of either NPY (humans) or Y2 receptors (mice).

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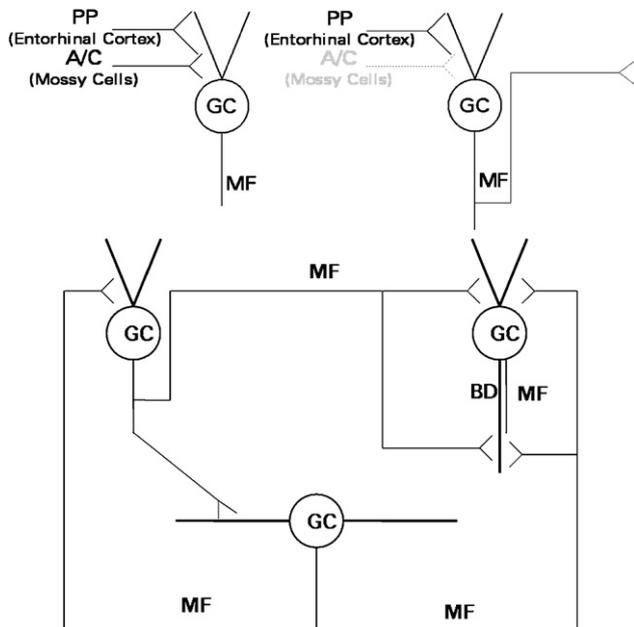
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## 1. Introduction

Temporal lobe epilepsy is the most common form of epilepsy in the adult population. This condition afflicts at least 800,000 Americans. Unfortunately, pharmacotherapy usually fails to achieve long-term remission [31]. A rational approach to improved pharmacotherapy of temporal lobe epilepsy requires investigation of its unique pathology and pathophysiology. One unique feature of temporal lobe epilepsy is the anatomical reorganization of the dentate gyrus (Fig. 1) [1,14,20,42,51]. This phenomenon is replicated in several animal models of epilepsy, including pilocarpine-treated rats and mice [35]. Dentate granule cells become interconnected through the growth of recurrent mossy fibers. These mossy fiber collaterals mediate recurrent excitation [13,33,36,57], a type of innervation that is hardly present on dentate granule cells in normal brain. In addition, seizures increase the rate of



**Fig. 1** – Schematic diagram of the excitatory innervation of dentate granule cells (GC). (Upper left) In normal brain, granule cells receive excitatory innervation from the perforant path (PP), which originates in the entorhinal cortex, and the associational-commissural (A-C) fibers, which originate from mossy cells of the dentate hilus. Their axons, the mossy fibers (MF), innervate pyramidal cells of area CA3 and interneurons of area CA3 and the dentate gyrus, but only minimally innervate granule cells. (Upper right) Seizures kill the hilar mossy cells, triggering the development of mossy fiber collaterals that grow into the synaptic territory abandoned by the degenerated associational-commissural fibers. (Bottom) The granule cell network develops with time after brain-damaging seizures. Components of this network include normally located granule cells of normal cellular morphology (upper left), normally located granule cells with a basal dendrite (BD; upper right), and newly generated hilar ectopic granule cells (bottom). These components are synaptically interconnected by mossy fibers. In animal models, these fibers express NPY *de novo*.

granule cell replication, and some of these newly born neurons migrate to ectopic locations, most notably the dentate hilus [39,44]. Finally, many granule cells in epileptic brain are found to have a basal dendrite [7,43,47], which provides a novel surface for innervation by recurrent mossy fibers [43]. Normal granule cells, granule cells with a basal dendrite, and hilar ectopic granule cells are synaptically interconnected by recurrent mossy fibers, forming a reverberating network unique to the epileptic brain. Formation of recurrent excitatory circuitry in the dentate gyrus is associated with a reduced threshold for granule cell synchronization in both humans [16,27] and animal models [11,19,37,40,52]. It may thus contribute to progressively enhanced excitability [18,58], because in non-epileptic animals dentate granule cells have been shown to resist the propagation of seizures from the entorhinal cortex to the hippocampus [9,25,48]. Development of monosynaptic recurrent excitation is not the only mechanism that can synchronize granule cells. However, recurrent excitatory circuitry serves as the major substrate for synchronization of CA3 pyramidal cells [32], and it would be expected to play a similar role in the dentate gyrus. Thus we suggested that granule cell synaptic reorganization plays a significant role in epileptogenesis [34,35,52].

## 2. Functions of NPY in the normal and reorganized dentate gyrus

The anticonvulsant/antiepileptogenic properties of NPY have drawn considerable interest [10,55]. Exogenous NPY attenuates epileptiform activity *in vitro* [3,23] and blocks seizures when infused into the CSF [56]. NPY<sup>-/-</sup> mice sometimes develop spontaneous seizures and exhibit severe, and often fatal, seizures upon the administration of kainic acid [2]. Conversely, overexpression of NPY raises the seizure threshold [54]. NPY is particularly abundant in the dentate gyrus. In this region, it is normally expressed mainly by a subset of hilar GABA neurons (HIPP cells) that also express somatostatin [24]. Somatostatin/NPY-immunoreactive neurons provide feedback (mossy fiber-evoked) inhibition to the granule cell dendrites. These neurons are killed readily by seizures [5,8,28], with a corresponding reduction in feedback inhibition to granule cells [36]. HIPP cells innervate granule cell dendrites in the outer part of the molecular layer (perforant path terminal zone). Although NPY is generally viewed as an endogenous anticonvulsant, microelectrode recordings detected no effect of applied NPY on granule cell membrane properties (except for depressed function of N-type calcium channels [30]), and NPY does not inhibit excitatory transmission to granule cells [22]. In fact, the only significant action of NPY found to date in the normal dentate gyrus is disinhibitory. NPY hyperpolarizes about half the “non-mossy” cells (presumed GABA interneurons) of the dentate hilus, probably the HIPP cells, by Y1 receptor-mediated activation of a G<sub>i</sub>-regulated inwardly-rectifying K<sup>+</sup> (GIRK) current [38]. Thus NPY acts on somatic/dendritic autoreceptors to reduce HIPP cell activity. It is not known whether or under what circumstances endogenous NPY might reach those receptors. Released NPY might also feed back on the terminal from

which it is released to reduce the probability of subsequent GABA release [49]. Thus endogenous NPY might facilitate seizures in the normal dentate gyrus. Indeed NPY can have proconvulsant actions; intracerebroventricular administration of NPY reportedly increases the duration of pentylene-tetrazole seizures [41].

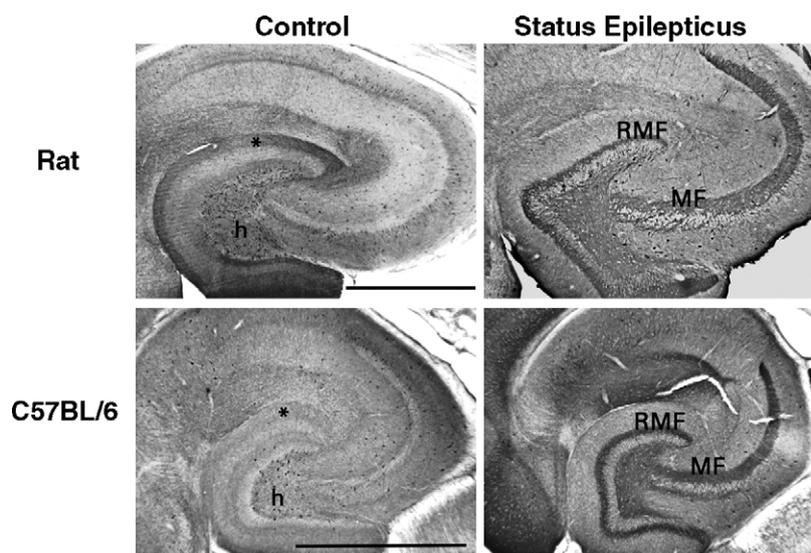
Seizures dramatically alter NPY localization and function in the dentate gyrus. In the pilocarpine and/or kainic acid model of temporal lobe epilepsy, HIPP cells degenerate and granule cells and their mossy fibers express NPY *de novo* [26,46]. NPY is transported through the mossy fibers to their terminals, from which it can be released [29]. This is the only known instance in which NPY is released from a glutamate pathway. Applied NPY inhibits synaptic transmission at mossy fiber synapses on CA3 pyramidal cells [22]. Thus release of NPY at recurrent mossy fiber synapses might feed back onto synaptic terminals and inhibit the release of glutamate. Corelease of NPY may explain, in part, why activation of the mossy fibers usually does not evoke reverberating excitation in the dentate gyrus of epileptic brain, despite the presence of robust recurrent mossy fiber growth [6,11,19,40,52]. Moreover, studies in other systems indicated that NPY is released preferentially by stimulus trains, compared with stimuli presented at low frequency [21,50]. Thus release of NPY could have explained the “reversal of facilitation” we noted when the recurrent mossy fiber pathway is stimulated at a frequency of 10 Hz with pulses of increasing magnitude [13].

Our studies investigated the actions of mossy fiber NPY on synaptic transmission in the recurrent circuit and on granule cell epileptiform activity. They utilized the pilocarpine model of temporal lobe epilepsy. Pilocarpine administered to rats or mice induces 6-8 h of status epilepticus, followed by a

hippocampal lesion similar to those found in persons with temporal lobe epilepsy, formation of recurrent excitatory circuitry in the dentate gyrus, and spontaneous recurrent seizures.

### 3. Long-lasting expression of NPY in mossy fibers of rats and mice after pilocarpine-induced status epilepticus

NPY immunostaining was performed simultaneously on brain sections from rats and mice that had and had not experienced pilocarpine-induced status epilepticus (Fig. 2). In the dentate gyrus of control rats and mice, somata immunoreactive for NPY were mainly confined to the hilus. These neurons were presumably HIPP cells. Immunostained fibers were present in the outer part of the molecular layer, where axons of the HIPP cells terminate. NPY immunoreactivity was significantly more intense in the outer molecular layer of rats ( $P < 0.001$  by densitometry, Student's *t*-test;  $n = 6$  rats and 4 mice), in accordance with the apparently smaller number of NPY-immunoreactive HIPP cells. Three changes were evident in sections from pilocarpine-treated epileptic animals. First, NPY immunoreactivity appeared *de novo* in the mossy fiber pathway, particularly in the recurrent mossy fibers. Second, there were markedly fewer NPY-immunoreactive somata in the dentate hilus. Third, immunostaining in the outer part of the molecular layer was less distinct, consistent with a loss of HIPP cell axons and terminals. No species difference in the response to status epilepticus was evident when hippocampal sections from rats and mice were processed simultaneously. These findings replicate previous reports [4,44].

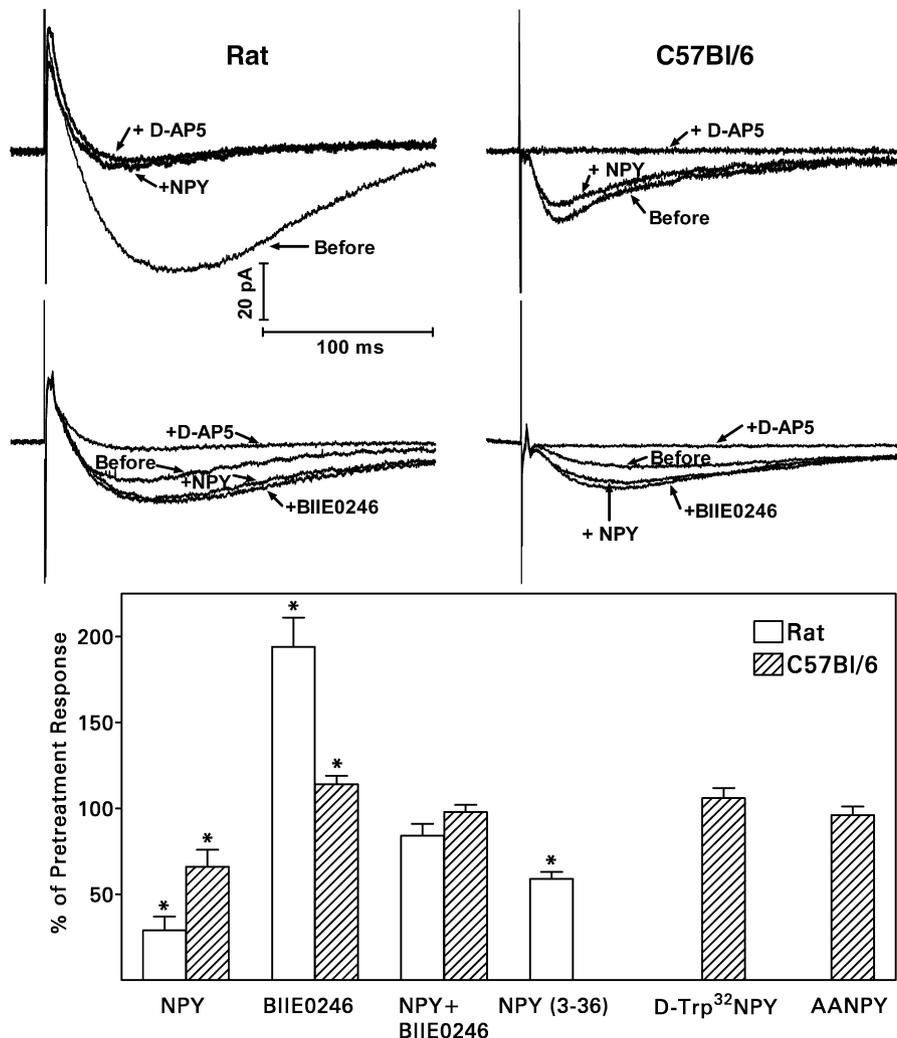


**Fig. 2 – Similar expression of NPY in mossy fibers of rats and C57BL/6 mice after pilocarpine-induced status epilepticus. Mossy fibers in area CA3 (MF) of control animals lack NPY immunoreactivity. NPY-immunoreactive HIPP cells appear less numerous in the dentate hilus (h) of mice than of rats and their terminal zone in the outer part of the dentate molecular layer (\*) is less intensely immunostained. Mossy fibers in area CA3 and the dentate hilus and recurrent mossy fibers (RMF) in the dentate molecular layer develop NPY immunoreactivity after status epilepticus. There are many fewer NPY-immunoreactive HIPP cells, and their terminal zone in the dentate molecular layer is hardly discernable. Scale bars, 0.5 mm.**

#### 4. Spontaneous release of NPY tonically inhibits recurrent mossy fiber transmission in rats

To study the actions of applied and endogenous NPY at recurrent mossy fiber synapses, whole cell patch clamp recordings were made in dentate granule cells of hippocampal slices prepared from pilocarpine-treated epileptic rats [53]. Some of these experiments, to be described later, were also performed with hippocampal slices prepared from C57BL/6 mice. First, we tested the effect of bath-applied NPY (1  $\mu$ M) on synaptic responses evoked by stimulating each

of the three excitatory projections to granule cells: mossy fibers, the perforant path, and the associational–commissural fibers. NPY reversibly inhibited synaptic transmission at recurrent mossy fiber synapses, but not at perforant path or associational–commissural synapses. It reduced the pharmacologically isolated NMDA receptor-mediated component of the compound recurrent mossy fiber EPSC (recorded instead of the full EPSC to eliminate polysynaptic components) by 71% (Fig. 3) and increased the failure rate of minimal electrically evoked EPSCs by 23%. Applied NPY also reduced the frequency (but did not affect the amplitude or kinetics) of



**Fig. 3** – NPY (1  $\mu$ M) and BIIE0246 (100 nM) have qualitatively similar, but quantitatively very different, effects on recurrent mossy fiber synaptic transmission in rats and C57BL/6 mice. (Top) Whole cell patch clamp recordings from representative experiments on hippocampal slices are averages of 10 traces. NMDA receptor-mediated recurrent mossy fiber EPSCs were isolated pharmacologically with use of 10  $\mu$ M NBQX (to block AMPA/kainate receptors) and 30  $\mu$ M bicuculline (to block GABA<sub>A</sub> receptors) and recorded at a holding potential of  $-20$  or  $-30$  mV. “Before” refers to responses recorded before exposure of the tissue to NPY or BIIE0246. (Upper traces) NPY was added to the superfusion medium and then 50  $\mu$ M D-AP5 was added in the continued presence of NPY. In slices from both rats and mice, NPY inhibited synaptic transmission and the subsequent addition of D-AP5 abolished the response. The latter result confirms that the response was mediated entirely by the synaptic activation of NMDA receptors. (Lower traces) BIIE0246 was added to the superfusion medium and then NPY and NPY + D-AP5 were added in the continued presence of BIIE0246. BIIE0246 enhanced synaptic transmission in both species when it was applied by itself, and the subsequent addition of NPY to the medium had no effect. (Bottom) The effects of NPY and BIIE0246 on the peak amplitude of the NMDA receptor-mediated EPSC were much greater in rats than in mice.  $P < 0.01$ , paired *t*-test.

miniature EPSCs (mEPSCs) in granule cells from pilocarpine-treated epileptic rats, but not from control rats. These actions of NPY were mediated by activation of presynaptic Y2 receptors. The Y2 receptor agonist NPY (3–36) (1  $\mu$ M) replicated the actions of NPY and the selective Y2 receptor antagonist BIIE0246 (100 nM) blocked them. Y1 receptor ligands had no effect. Importantly, BIIE0246 produced effects opposite to those of NPY, when it was applied by itself. It increased the amplitude of compound NMDA receptor-mediated recurrent mossy fiber EPSC by 94%, reduced the failure rate of minimally evoked EPSCs by 20%, and increased the frequency (but did not affect the amplitude or kinetics) of mEPSCs in granule cells from pilocarpine-treated epileptic rats. These effects were obtained in the absence of a prepulse. Several observations supported the selectivity of BIIE0246, including no effect on perforant path and associational–commissural synaptic transmission or on membrane properties of granule cells. An action of an antagonist when applied by itself can be interpreted to indicate that the endogenous agonist acts in the opposite way. Because BIIE0246 enhanced recurrent mossy fiber synaptic transmission without requiring any prior stimulation, we concluded from these results that NPY (or possibly an active metabolite, such as methionine sulfoxide NPY [29]) is released spontaneously from recurrent mossy fiber terminals and that the quantity released is sufficient to depress glutamate release from those terminals. This is in contrast to the need for high-frequency activity to release NPY from most terminals that normally express this peptide. Recurrent mossy fiber synapses are rather weak; 60–70% of minimal stimuli fail to evoke glutamate release in hippocampal slices maintained at room temperature [13,33]. Tonic release of NPY accounts for about one-third of these response failures.

We observed no effect of NPY or BIIE0246 that appeared to be postsynaptically mediated. However, our experimental conditions precluded the detection of a GIRK current, such as applied NPY has been shown to produce in HIPP cells [38]. If present in granule cells, an NPY-mediated GIRK current would add an inhibitory postsynaptic action to the tonic inhibition of glutamate release we demonstrated.

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## 5. Spontaneous release of NPY depresses granule cell epileptiform activity

Effects of NPY receptor ligands on granule cell epileptiform activity were studied during superfusion of hippocampal slices at 35 °C with 30  $\mu$ M bicuculline and 6 mM  $[K^+]_o$  [53]. Under these conditions, mossy fiber stimulation evokes epileptiform activity in the granule cell body layer that depends entirely on the formation of recurrent mossy fiber connections; no epileptiform activity is observed in slices from control rats [19,37,52]. The effects of NPY receptor ligands on mossy fiber-evoked granule cell epileptiform activity were entirely predictable from their effects on recurrent mossy fiber synaptic transmission. NPY substantially reduced the magnitude of short-latency epileptiform activity and markedly attenuated or abolished the delayed bursts evoked by mossy fiber stimulation. Delayed bursts are presumably generated by polysynaptic activation of recurrent mossy fibers. BIIE0246

blocked these actions and enhanced epileptiform activity when applied by itself. Stimulation of the perforant path under the same conditions evoked multiple granule cell population spikes superimposed on either a positively directed or negatively directed wave. The negative wave indicated the presence of a current sink close to the granule cell body layer, consistent with disinaptic activation of recurrent mossy fibers. Delayed bursts were often evoked as well. In these experiments, NPY reduced the magnitude of the short-latency activity and abolished the delayed bursts. NPY had no effect when the waveform was positively directed. Perforant path-evoked epileptiform activity of the latter type can be evoked in disinhibited slices from control rats, which lack a significant recurrent mossy fiber pathway. Thus NPY receptor ligands only affected granule cell epileptiform activity dependent on the recurrent mossy fiber pathway. We concluded that tonic release of NPY impedes the ability of recurrent mossy fibers to synchronize granule cell discharge and may thus protect the hippocampus from seizures that involve the entorhinal cortex.

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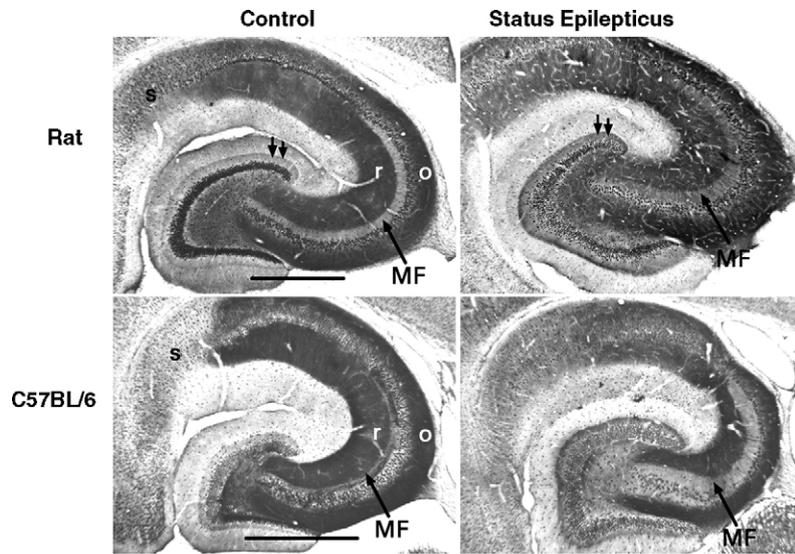
## 6. Lesser effects of endogenous and applied NPY on recurrent mossy fiber transmission in mice

We aimed to gain a more detailed understanding of the role of NPY in the normal and reorganized dentate gyrus with use of mutant mice genetically engineered to lack expression of NPY or Y2 receptors. In preparation for these studies, we repeated the experiments on the NMDA receptor-mediated component of the compound recurrent mossy fiber EPSC with pilocarpine-treated C57BL/6 mice. Experiments on mice were interleaved with some of those on rats, so that the results could be compared across species. The qualitative effects of NPY and BIIE0246 on recurrent mossy fiber synaptic transmission were the same in mice as in rats: applied NPY was inhibitory, BIIE0246 blocked its action, and BIIE0246 enhanced transmission when applied by itself (Fig. 3). The Y5 receptor agonists D-trp<sup>32</sup>NPY and [ala<sup>31</sup>,aib<sup>32</sup>]-NPY did not replicate the action of NPY. These results argue that Y2 and not Y5 receptors mediate the NPY-induced reduction in glutamate release from mossy fiber terminals in C57BL/6 mice. Our most important finding, however, was that both NPY and BIIE0246 were much less effective in mice than in rats. NPY reduced the size of the NMDA receptor-mediated component of the compound EPSC by an average of only 34% (compared with 71% in rats) and BIIE0246 increased the size of this response by an average of only 14% (compared with 94% in rats). This finding implies that both endogenous and applied NPY are less efficacious in mice, despite apparently equivalent expression of NPY in the recurrent mossy fibers of both species.

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## 7. Species difference explained by plasticity of Y2 receptor expression

The lesser effect of both endogenous and applied NPY on the recurrent mossy fiber pathway of mice suggested a paucity of



**Fig. 4 – Opposite changes in mossy fiber Y2 receptor expression in rats and C57BL/6 mice after pilocarpine-induced status epilepticus.** In control animals, stratum radiatum (r) and stratum oriens (o) of areas CA1 and CA3 (loci of the Schaffer collateral-commissural projections) are strongly immunoreactive. The mossy fiber terminal zone of area CA3 (MF) is far more immunoreactive in mice than in rats, whereas the subiculum (s) is more immunoreactive in rats. Status epilepticus reduces Y2 receptor immunostaining in the mossy fiber terminal zone of mice, but increases it in rats. In rats, but not in mice, growth of recurrent mossy fibers into the inner third of the dentate molecular layer was associated consistently with increased Y2 receptor immunoreactivity (double arrows). Scale bars, 0.5 mm.

functional Y2 receptors. We tested this hypothesis by performing immunohistochemistry that utilized an antibody to the Y2 receptor (Neuromics, Edina, MN). This polyclonal antibody was raised in rabbits by immunization with a 10 amino acid polypeptide that corresponded to a portion of the Y2 receptor identical in rat and mouse. Thus equivalent immunoreactivity was expected in hippocampal sections from the two species. Indeed densitometry confirmed that the mean intensity of immunostaining differed by only about 10%.

Hippocampal sections from control mice and rats and from pilocarpine-treated epileptic mice and rats, five animals per group, were processed simultaneously. In control mice and rats, the terminal zones of Schaffer collateral-commissural-ipsilateral associational fibers in areas CA1 and CA3 and the mossy fiber terminal zones in area CA3 and the dentate hilus were immunoreactive (Fig. 4). In contrast, terminal zones of entorhinal cortical afferent fibers were not immunostained. These findings correlated well with the ability or inability of NPY to block synaptic transmission in those pathways. Notably, the immunostaining of the mossy fiber terminal zone in area CA3 (stratum lucidum) was considerably more intense in mice than in rats ( $P < 0.02$  by densitometry, Student's *t*-test). Immunoreactivity was also present in the inner third of the dentate molecular layer. There is no obvious explanation for this finding. The immunostaining was probably not associated with the associational-commissural pathway, the major excitatory pathway that projects to this part of the molecular layer, because NPY has no effect on transmission in the associational-commissural pathway and degeneration of this pathway after pilocarpine-induced status epilepticus did not reduce immunostaining. Further studies

are needed to determine which cellular element in the inner molecular layer expresses Y2 receptors. The lack of neuropil immunostaining in the hippocampus of  $Y2^{-/-}$  mice confirmed the specificity of the antibody.

Examination of animals that had survived pilocarpine-induced status epilepticus indicated a profound down regulation of Y2 receptors in the mossy fibers of pilocarpine-treated mice ( $P < 0.01$  in stratum lucidum by densitometry, paired *t*-test). Importantly, the growth of recurrent mossy fibers into the inner molecular layer was not accompanied by any significant increase in Y2 receptor immunostaining ( $P = 0.8$  by densitometry, paired *t*-test). This finding confirms that low expression of presynaptic Y2 receptors can explain the small effects of NPY and BIIE0246 on synaptic transmission in this pathway. In contrast, Y2 receptor immunoreactivity increased in stratum lucidum after pilocarpine-induced status epilepticus in rats ( $P < 0.02$  by densitometry, paired *t*-test). In addition, the growth of recurrent mossy fibers into the inner molecular layer was accompanied by a significant increase in the Y2 receptor immunoreactivity of that layer ( $P = 0.05$  by densitometry, paired *t*-test). Our findings in rats agree with the seizure-induced up regulation of mossy fiber Y2 receptor binding reported by previous investigators, although quantitation in those studies was limited to the dentate hilus [17,45]. These contrasting receptor changes can explain the quantitatively different effects of NPY and BIIE0246 on recurrent mossy fiber transmission in mice and rats. Other possible forms of seizure-induced plasticity, such as changes in receptor function caused by altered post-translational modifications, are not excluded by our results, however, and merit investigation.

## 8. Application to human epilepsy

Immunohistochemical studies have not detected in human material the robust expression of mossy fiber NPY reported in animal models. There is an increased density of NPY-immunoreactive fibers in the terminal lamina of the recurrent mossy fiber projection [12,15,45]. It has not been determined whether this finding signifies expression of NPY by some recurrent mossy fibers or the growth of NPY-immunoreactive interneuron processes. On this basis, one would expect a modest effect of endogenous NPY on recurrent mossy fiber synaptic transmission, similar to that observed in mice. We predict that this pathway synchronizes granule cell discharge more effectively in humans and mice than in rat, due to its lower expression of either NPY or Y2 receptors. Accordingly, mice may offer a better model than rats for studying this aspect of temporal lobe epilepsy.

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