



ROLE OF INHIBITION IN EMOTIONAL LEARNING

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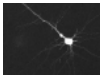
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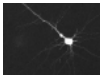
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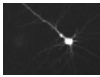


Abbreviations

AC	adenylate cyclase
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BLA	basal nucleus of the amygdala
CACA	cis-aminocrotonic acid (selective GABA _C R agonist)
cAMP	cyclic adenosine monophosphate
CGP52432	GABA _B receptor antagonist
ChR2	channelrhodopsin-2
CNS	central nervous system
CREB	cAMP response element-binding
CS	conditioned stimulus
DAG	diacyl-glycerol
DNA	deoxyribonucleic acid
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAT	GABA transporter
5-HT3	serotonin (5-hydroxytryptamine) receptor
IP3	inositol-1,4,5-triphosphate
KCC2	K ⁺ /Cl ⁻ co-transporter
LA	lateral amygdala
LC	locus coeruleus
LTM	long term memory



mRNA	messenger Ribonucleic acid
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate receptor
NpHR	Natronomonas pharaonis - anion pump - halorhodopsin
PKA	protein kinase A
PKC	protein kinase C
PLP	pyridoxal phosphate
SCG	superior cervical ganglia
STM	short-term memory
TPMPA	(1,2,5,6-tetrahydropyridine-4-yl) and methyl-phosphinic acid (GABA _C R antagonist)
US	unconditioned stimulus
VGAT	vesicular neurotransmitter transporter
VGCC	voltage-gated calcium channels
YFP	yellow fluorescent protein

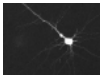


Resumo

No cérebro os neurónios trocam bilhões de impulsos sinápticos a cada segundo. Existem dois tipos de sinapses que asseguram esse fluxo de dados: sinapses excitatórias e sinapses inibidoras. Na minha tese de doutoramento concentro-me na inibição, o mecanismo menos explorado.

No meu primeiro projecto, estava interessada no papel do circuito inibitório na memória do medo. O neurotransmissor inibitório ácido γ -aminobutírico (GABA) actua através de três tipos diferentes de receptores, denominados: GABA_A, GABA_B, e GABA_C. Enquanto os receptores GABA_A e GABA_B são ubiquamente distribuídos no SNC de mamíferos, os receptores GABA_C só têm sido descritos apenas num número limitado de estruturas do sistema nervoso central. Dentro da amígdala foi encontrada a mRNA das subunidades dos receptores GABA_C. Tendo como suporte essa informação efectuei gravações patch clamp com o fim de examinar a presença de receptores GABA_C funcionais na amígdala lateral (LA). Usando vários estímulos e protocolos de gravação verificou-se que neurónios no rato expressam receptores GABA_C funcionais na LA. Os resultados de estimulação eléctrica de aferentes das células piramidais indicam que os receptores GABA_C estão envolvidos na comunicação intra neuronal na LA em que modulam uma fracção considerável de correntes pós-sinápticos. Interpretamos que os receptores GABA_C estão localizados no lado pré-sináptico nos axónios dos interneurónios, e agem como auto inibidores para reduzir libertação do GABA sináptica. A infusão de agonistas e antagonistas de receptores GABA_C na LA durante medo condicionado auditivo pavloviano, indicou que receptores GABA_A e receptores GABA_C desempenharam papéis opostos na modulação da aquisição de medo e sua consolidação: receptores GABA_A prejudicaram, e receptores GABA_C melhoraram, a aprendizagem da memória do medo. O presente estudo expande a nossa compreensão do papel dos receptores de GABA na aprendizagem medo, e poderá conduzir a melhorias no tratamento de perturbações relacionadas com ansiedade.

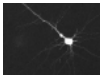
Neste projecto examinei modulações de inibição através de neuromoduladores, que são conhecidos por contribuir na plasticidade sináptica, aprendizagem e memória. Melhorias de memória para situações emocionalmente carregadas, estão associadas com a liberação de norepinefrina (NE) na amígdala, que é uma estrutura do cérebro fundamental na aprendizagem emocional. Estudos no nosso laboratório demonstram que NE pode ter efeitos



diferenciais modulatórios devido a interneurónios inibidores GABAérgicos. O papel de receptores alfa1-adrenérgicos no condicionamento do medo, um modelo importante de aprendizagem emocional, é pouco compreendido. Foi examinado o efeito de terazosina, um antagonista do receptor alfa 1-adrenérgico, em medo condicionado. Aplicou-se terazosina sistemicamente ou intra-lateral da amígdala antes do treino do condicionamento da memória a curto e a longo prazo. Terazosina aplicada após o condicionamento, não afectou a consolidação. *In vitro*, a terazosina prejudicou correntes inibitórias pós-sinápticas levando à facilitação de correntes pós-sinápticas excitatórias e potenciação dessas mesmas a longo prazo.

Tendo como base este estudo, é importante considerar os resultados obtidos visto que os mesmos podem ter importantes implicações clínicas, uma vez que estão a ser aplicados bloqueadores de receptores alfa1 para tratamentos de stress pós-traumático.

No terceiro projecto trabalhei no desenvolvimento de um novo método para silenciamento neuronal *in vitro* e *in vivo*. A capacidade de controlar e manipular a actividade neuronal dentro de um cérebro mamífero intacto é de importância fundamental para a elaboração de um mapa das ligações funcionais e para distinguir a função de circuitos neurais subjacentes. Nesta parte da minha tese descrevo ratinhos transgénicos que expressam halorodopsina (NpHR), uma bomba de cloreto, que pode ser usada para silenciar a actividade neuronal, através de aplicação de luz. Usando o promotor Thy-1 para expressão de NpHR nos neurónios, verificou-se que os neurónios nestes ratinhos transgénicos revelam altos níveis de NpHR-YFP. Por sua vez, a iluminação de neurónios piramidais corticais com NpHR-YFP resultou na fotoinibição, rápida e reversível..

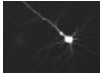


Summary

In the brain, neurons exchange many billions of synaptic impulses every second. There are two kinds of synapses that ensure that this flow of data is regulated: excitatory and inhibitory synapses. In my doctoral thesis I concentrated on inhibition, the less well understood mechanism in the brain.

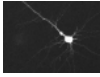
I was interested in the role of the inhibitory circuit in fear memory. The inhibitory neurotransmitter γ -aminobutyric acid (GABA) acts through three different types of receptors, termed GABA_A, GABA_B, and GABA_C receptors. While GABA_A and GABA_B receptors are ubiquitously distributed in the mammalian CNS, GABA_C receptors have been described as appearing in only a restricted number of CNS structures. mRNA for GABA_C receptor specific subunits have been found within the amygdala. We performed patch clamp recordings in order to examine the presence of functional GABA_C receptors in the lateral amygdala (LA). Using various stimulations and recording protocols, we were able to demonstrate that neurons in the rat LA express functional GABA_C receptors. The results from electric stimulation of Pyramidal cell afferents indicate that GABA_C receptors are involved in intra LA neuronal communication where they modulate a considerable fraction of postsynaptic currents. We interpret this to suggest that GABA_C receptors are located on the presynaptic side on the axons of the interneurons and act as autoinhibitors to reduce synaptic GABA release. Infusion GABA_CR agonists and antagonists in LA In auditory Pavlovian fear conditioning indicate that GABA_ARs and GABA_C receptors play opposite roles in the modulation of fear acquisition and consolidation: GABA_A receptors impair and GABA_C receptors enhance fear learning and memory. The present study furthers the understanding of the role of GABA receptors in fear learning, and could lead to improvements in the treatment of anxiety-related disorders.

In my next project I intend to examine modulations of inhibition through neuromodulators, which are known to contribute to synaptic plasticity, learning and memory. Memory enhancements for emotionally charged events are associated with the release of norepinephrine (NE) in the amygdala, which is a key brain structure in emotional learning. Our lab has shown that NE can have differential modulatory effects due to differences in local GABAergic inhibitory interneurons. The role of α 1-adrenergic receptors in fear conditioning, a major model of emotional learning, is poorly understood. We examined the effect of terazosin, an α 1-adrenergic receptor antagonist, on cued fear conditioning.



Systemic or intra-lateral amygdala terazosin delivered before conditioning enhanced short- and long-term memory. Terazosin delivered after conditioning did not affect consolidation. *In vitro*, terazosin impaired lateral amygdala inhibitory postsynaptic currents, leading to facilitation of excitatory postsynaptic currents and long-term potentiation. Since alpha1 blockers are prescribed for hypertension and post-traumatic stress disorder, these results may have important clinical implications.

In the third project I worked on we developed a new tool for neuronal silencing *in vitro* and *in vivo*. The ability to control and manipulate neuronal activity within an intact mammalian brain is of key importance for mapping functional connectivity and for dissecting the neural circuitry underlying behaviors. In this part of my thesis I describe transgenic mice that express halorhodopsin (NpHR), a light-driven chloride pump that can be used to silence neuronal activity via light. Using the Thy-1 promoter to target NpHR expression to neurons, we found that neurons in these mice expressed high levels of NpHR-YFP and that illumination of cortical pyramidal neurons expressing NpHR-YFP led to rapid, reversible photoinhibition of action potential firing in these cells.



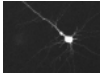
Chapter 1

I. Introduction

In the neuroscience literature, the term 'inhibition' has been in use since the 19th century (Smith, R., 1992). There are three concepts of inhibition:

1. The neural concept: It is accepted that neurons can inhibit the activity of other neurons.
2. The physical-response concept: It is accepted that actions can be initiated and then cancelled (Logan, G. D., et al., 1984), although the extent to which these two domains of inhibition relate to each other remains to be established.
3. The cognitive concept: This is the idea that mental processes or representations can be inhibited.

In the present thesis, I will concentrate on the first concept, the neural concept of inhibition, because this is the area that directly relates to the central questions addressed in my studies. In addition, cognitive inhibition will be introduced very briefly as efforts were made to analyze drug effects not just on the neural level, but also *in vivo* on behavior.

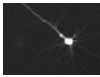


I.1. Neural inhibition

Inhibition in the brain seems simple, but there is an underlying complexity that makes it one of the most challenging aspects of brain function to understand. The main inhibitory neurotransmitter at synapses in the cerebral cortex is gamma-aminobutyric acid (GABA). This molecule plays a crucial role in how the brain functions. The inhibitory synapses are also the targets of various modulators, such as alcohol, barbiturates, and benzodiazepines such as valium.

Until recently, not very much was known about GABAergic synapses in the human brain. All organisms, from bacteria to humans, can synthesize GABA, and multiple functions of GABA had evolved (Barker, J. L., et al., 1998; Morse, D. E., et al., 1980). However, GABA-mediated signaling has also been implicated in the regulation of nearly all the key developmental steps, from cell proliferation to circuit refinement. A recent study has shown how GABA synapses in the human brain change across the lifespan from infants to older adults (Pinto, J. G., et al., 2010).

GABAergic synapses are complex structures with many pre- and post-synaptic proteins that affect how these inhibitory synapses function. GABA signaling stops neural activity that could lead to a seizure and it also sets the balance between excitation and inhibition that is important for neural plasticity. Moreover, GABA is involved in a variety of biological functions such as locomotor activity, learning, reproduction, circadian rhythms as well as cognition, emotions and sleep, synapse formation and cell death in the developing CNS. Beside its function in the CNS, GABA has been also detected in many peripheral neuronal and non-neuronal tissues and suggested to be involved in the physiology of glia cells, the digestive tract, pancreas, liver, adrenal medulla, respiratory and kidney epithelium, osteoblasts, chondrocytes, and germ cells.



I.1.1. Neurotransmitter GABA

GABA was the first discovered example of an inhibitory neurotransmitter substance, which changed the prevailing view of synaptic transmission that had previously allowed for only excitatory signaling molecules (Cowan, W. M. and Kandel, E. R., 2001). More than half a century ago, GABA was first identified in the mammalian brain (Awapara, J., et al., 1950; Roberts, E. and Frankel, S., 1950). In the 1950s and 1960s, strong evidence accumulated that GABA acted as an inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems (Squires, R. F., 1988). One of the first experiments was conducted in crayfish. Extracts from the mammalian brain, named factor I, blocked impulse generations in crayfish stretch receptor neurons (Elliott, K. A. and Jasper, H. H., 1959). Factor I was shown to be GABA (Basemore, A. W., et al., 1957). GABA was found in crustaceans to be approximately 100 times more concentrated in inhibitory than in excitatory axons (Kravitz, E. A., et al., 1965; Kravitz, E. A. and Potter, D. D., 1965). Stimulation of inhibitory nerve terminals leads to the release of GABA (Otsuka, M., et al., 1966). Similar results were observed in the vertebrate brain, i.e. GABA was concentrated and released from inhibitory neurons (Obata, K., 1972). The biosynthetic and metabolic pathways for GABA were identified and showed that the production, release, reuptake and metabolism of this neurotransmitter occurred in the nervous system (Roberts, E. and Frankel, S., 1950). Further, it was shown that when GABA was applied to nerve and muscle cells of both vertebrates and invertebrates, it was generally found to have inhibitory effects (Basemore, A. W., et al., 1957; Kuffler, S. W., 1958; Kuffler, S. W. and Edwards, C., 1958; Purpura, D. P., et al., 1957) and to produce conductance changes with ion sensitivities similar to those observed after the activation of inhibitory nerves (Boistel, J. and Fatt, P., 1958; Dreifuss, J. J., et al., 1969; Krnjevic, K. and Schwartz, S., 1967; Kuffler, S. W., 1958; Takeuchi, A. and Takeuchi, N., 1965; Takeuchi, A. and Takeuchi, N., 1966). In the 1970s, lastly, GABA was localized to mammalian nerve terminals (Bloom, F. E. and Iversen, L. L., 1971), and antibodies raised against GABA-biosynthesizing enzymes were shown to be localized preferentially to known inhibitory neurons (Roberts, E. and Frankel, S., 1950).

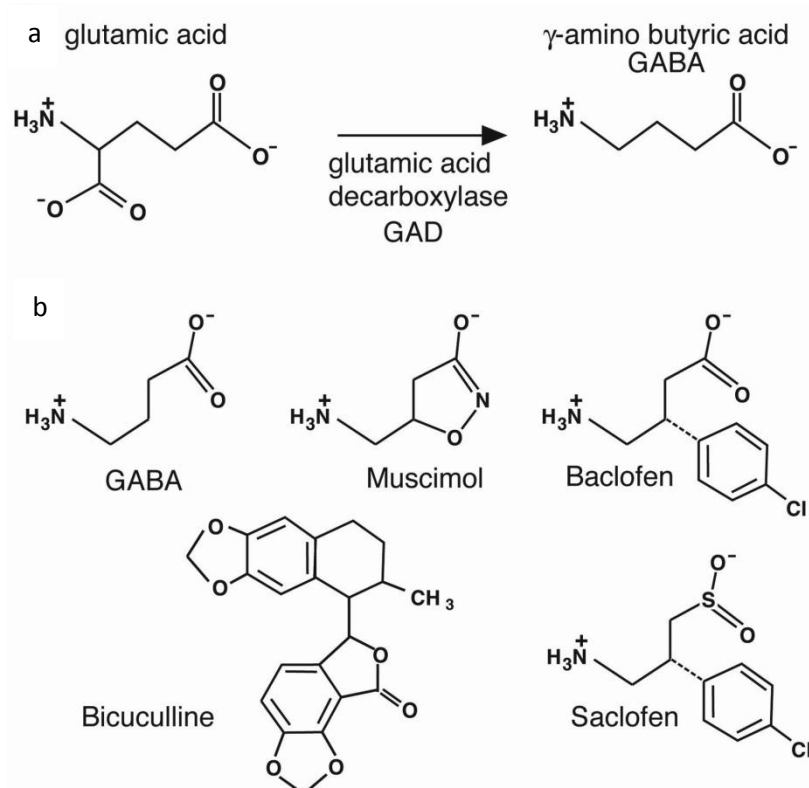
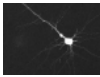
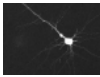
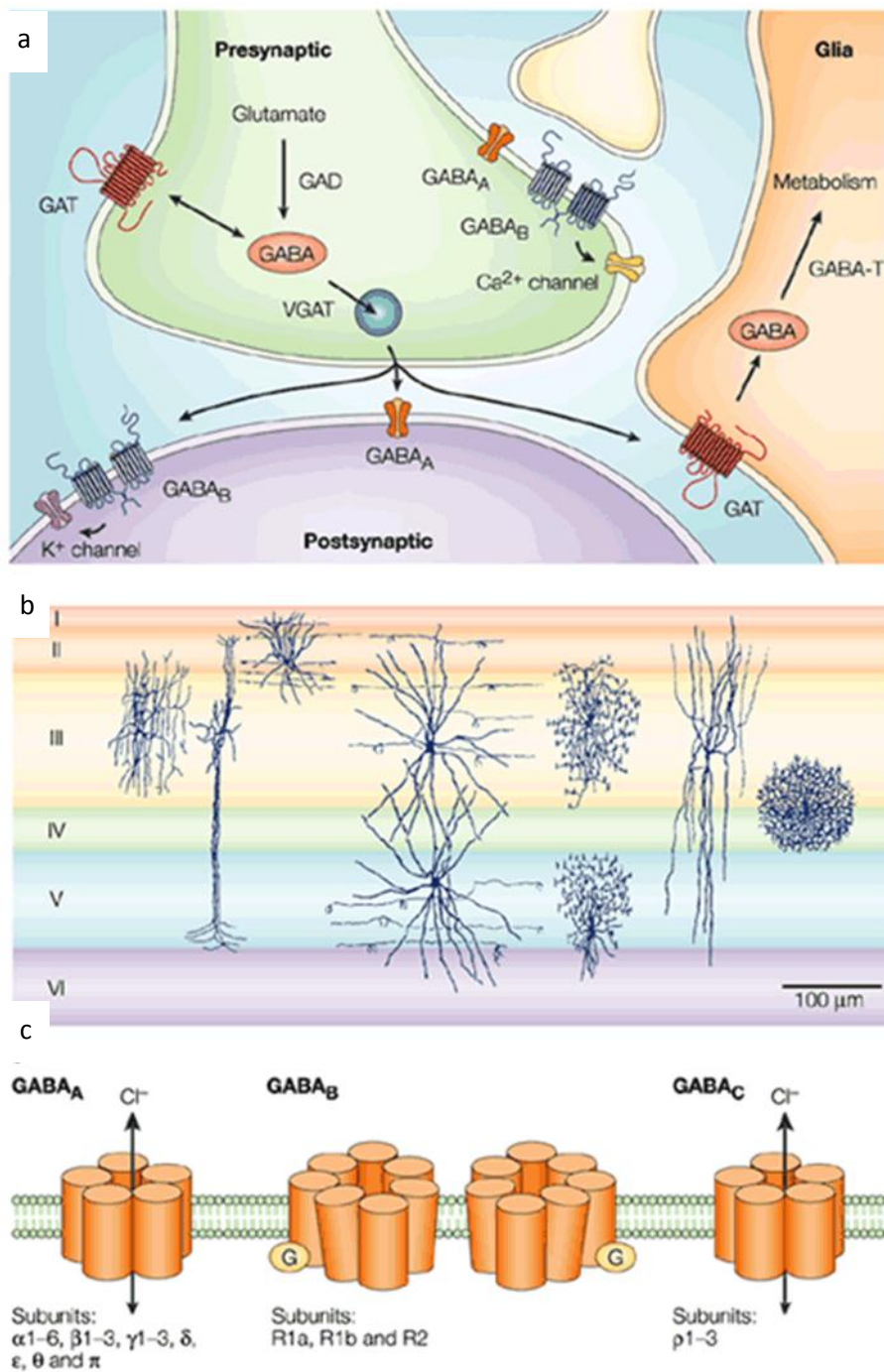
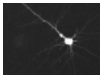


Figure 1: a) Metabolism of the neurotransmitter GABA. b) Chemical structure of GABA and GABA receptor agonists and antagonists (Jorgensen, E. M., 2005).



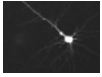
I.1.2. Metabolism and system of the neurotransmitter GABA

A summary of the GABA neurotransmitter system and metabolism is shown in Figures 1 and 2. GABA is synthesized from its precursor, glutamate, and this reaction is catalyzed by two isoforms of glutamic acid decarboxylase (GAD), GAD65 and GAD67 (Erlander, M. G., et al., 1991). The two GAD isoforms are highly homologous at the protein level, but show different affinity to the co-factor pyridoxal phosphate (PLP) and distinct sub-cellular localization: GAD65 is predominantly localized to nerve endings and is thought to synthesize GABA for vesicular release, whereas GAD67 is enriched in the cytoplasm and is probably involved in the synthesis of the cytoplasmic GABA pool. During mouse and rat embryonic development two additional, alternatively-spliced forms of GAD67 gene (I80 and I86) are synthesized coding for the truncated GAD25 and enzymatically active embryonic GAD44 that are transiently expressed in a strict temporal order during neuronal differentiation, and the latter of which is thought to synthesize a GABA pool used for paracrine signaling. The vesicular neurotransmitter transporter VGAT (Fon, E. A. and Edwards, R. H., 2001) transfers GABA into synaptic vesicles and GABA is released from nerve terminals by calcium-dependent exocytosis. In addition, non-vesicular secretion of GABA has also been described (by reversed transporter action), which could be important during development (Attwell, D., et al., 1993; Taylor, J. and Gordon-Weeks, P. R., 1991). Synaptic transmission through GABA is mediated by ionotropic and metabotropic receptors, which are located both pre- and postsynaptically. GABA mediated signals are terminated by re-uptake of the neurotransmitter into nerve terminals and into surrounding glial cells by a class of plasma-membrane GABA transporters (GATs) (Cherubini, E. and Conti, F., 2001). After removal from the extracellular space, GABA is metabolized by transamination reactions that are catalyzed by GABA-T, a GABA transaminase.



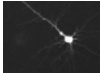
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Figure 2: a) Metabolism of the inhibitory neurotransmitter GABA. b) GABAergic neurons of the mammalian neocortex. c) Schematic image of the three GABA receptor types (Owens, D. F. and Kriegstein, A. R., 2002).



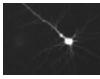
I.1.3. Inhibitory neurons

Most of the inhibitory neurons in the brain are GABAergic, which have a wide variety of dendritic morphologies (Figure 2b). For example, in the neocortex, most GABA-containing neurons are local interneurons with few dendritic spines, classified as sparsely spiny, aspiny or smooth cells (Hendry, S. H., et al., 1987; Shepherd, G. M., 1998). Further classifications of these GABAergic neurons are for example basket cells, chandelier cells, double bouquet cells, local plexus neurons or neurogliaform cells (Fig. 2b). These subtypes are differentiated by their morphology, neurochemical composition, somatic location and terminal arborization (Gutnick, M. J. and Mody, I., 1995; Houser, C. R., et al., 1984; Jones, E. G., 1995). In addition, GABAergic interneurons can be classified by intrinsic membrane properties and synaptic connectivity (Connors, B. W. and Gutnick, M. J., 1990; Gupta, A., et al., 2000).



I.1.4. GABA synapses

Electron-microscopic analyses of neocortical synapses, based on differences in ultrastructure, showed that there are two general synaptic types, termed type 1 and type 2 synapses (Gray, E. G., 1959). In general, type 1 synapses have an asymmetrical membrane density at the synaptic cleft and are considered to be excitatory. In contrast, type 2 synapses have a symmetrical appearance and are generally thought to be inhibitory, and most of them contain GABA (Colonnier, M., 1968). GABA synapses are observed most frequently on cell somata, proximal dendrites and axon initial segments (Douglas & Martin, 1998; (De Felipe, J., et al., 1997; Houser, C. R., et al., 1984; Micheva, K. D. and Beaulieu, C., 1996). However, like asymmetrical synapses, GABA synapses can also be found on distal dendrites and dendritic spines (Douglas & Martin, 1998). Two general types of GABA-mediated postsynaptic potential properties have been described on the basis of distinctive pharmacological sensitivity, ionic selectivity and kinetic properties. Ionotropic GABA_A receptors mediate fast responses and slow responses are mediated by metabotropic GABA_B receptors (Bormann, J., 1988; Connors, B. W., et al., 1988; Kaila, K., 1994).

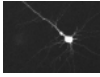


I.1.5. GABA receptors

GABA_A receptors are part of the ligand-gated ion channels class, which include nicotinic acetylcholine receptors (nAChRs), glycine receptors and the serotonin (5-hydroxytryptamine) 5-HT₃ receptor (Figure 2c). After the binding of a ligand the receptors go through a conformational change in the channel protein, which allows a net inward or outward flow of ions through the membrane-spanning pore of the channel, depending on the electrochemical gradient of the particular permeant ion. GABA_A receptors mediate primarily chloride currents; however, other anions, such as bicarbonate (HCO₃⁻), can also permeate the channel pore, although less efficiently (Bormann, J., et al., 1987; Kaila, K., 1994). Chloride-dependent GABA_A receptor-mediated synaptic inhibition can occur either pre- or postsynaptically. GABA_A receptors are heteropentameric proteins that are constructed from subunits derived from several related genes or gene families (Macdonald, R. L. and Olsen, R. W., 1994). So far, six α -subunits, three β -subunits, three γ -subunits, one δ -subunit, one ϵ -subunits, one π -subunit and one θ -subunit have been identified (Macdonald, R. L. and Olsen, R. W., 1994; McKernan, R. M. and Whiting, P. J., 1996; Mehta, A. K. and Ticku, M. K., 1999; Schofield, P. R., et al., 1987). Although there are many combinations of subunit types to build a receptor, data shows that certain combinations seem to be favored (McKernan, R. M. and Whiting, P. J., 1996). GABA_A receptors that are native contain at least one α -, β -, and one γ - subunit, with the δ -, ϵ -, π -, and θ -subunits able to substitute for the γ -subunit (McKernan, R. M. and Whiting, P. J., 1996). Cellular locations seem to define different subunit compositions, depending on whether the receptors are positioned to mediate primarily synaptic or extra synaptic signaling (Mody, I., 2001).

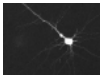
GABA_C receptors, which are closely related ionotropic GABA receptors, have also been identified (Figure 2c). This receptor is also chloride-selective ion channel, but insensitive to the GABA_A receptor antagonist bicuculline (Bormann, J. and Feigenspan, A., 1995).

During the late 1970s and early 1980s, evidence was found for a bicuculline-insensitive, chloride-independent GABA response in the brain, mediated by a metabotropic receptor that was termed the GABA_B receptor, as shown in Figure 2c (Bowery, N. G., et al., 1980; Hill, D. R. and Bowery, N. G., 1981; Nicoll, R. A., 1988). Signaling in metabotropic receptors is mediated by the activation of heterotrimeric G proteins (LeVine, H., 3rd, 1999). Subsequently, G proteins modify signals through the positive and negative regulation of primary effectors, second messengers and their associated enzymes, which can modulate



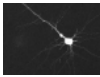
channel and receptor function (Nicoll, R. A., 1988). How GABA_B receptors regulate cell excitability depends on whether the receptors are localized pre- or postsynaptically. Presynaptic inhibition results from a GABA_B receptor-mediated reduction in calcium current at the nerve terminal and a subsequent reduction in transmitter release, whereas postsynaptic inhibition occurs by GABA_B-receptor-mediated activation of outward potassium currents that hyperpolarize the neuron (Bormann, J., 1988).

Cloning of GABA_B receptors showed that it is a putative seven-transmembrane G-protein-coupled receptor, which exists as two isoforms, R1a and R1b (Kaupmann, K., et al., 1997). The amino acid sequence of GABA_B receptors shows homology with metabotropic glutamate receptors (mGluRs) (Kaupmann, K., et al., 1997). Native receptors that are functional seem to be heterodimers that are composed of the R1a or R1b subunit, and the more recently identified R2 subunit (Kaupmann, K., et al., 1998). Data suggest that the R1a and R1b isoforms localize preferentially to presynaptic and postsynaptic membranes (Billinton, A., et al., 1999).



I.1.6. Excitation and inhibition

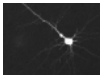
In the adult mammalian brain, GABA has been associated primarily with the mediation of synaptic inhibition. However, there are several ways in which synaptic inhibition can be produced, and this has resulted in some confusion in the classification of GABA receptor actions in immature neurons. The first intracellular recordings in the vertebrate CNS showed that activation of inhibitory neurons resulted in membrane hyperpolarization in the postsynaptic neuron, leading to the suggestion that inhibition resulted from driving the membrane potential further away from the action potential threshold (Brock, L. G., et al., 1952; Kandel, E. R. and Spencer, W. A., 1961). On the other hand, experiments in the crustacean nervous system showed that inhibition could be produced by an increase in membrane conductance that was associated with either no change in membrane potential or even depolarization (Fatt, P. and Katz, B., 1953; Kravitz, E. A., et al., 1963; Takeuchi, A. and Takeuchi, N., 1966). Recordings from adult cortical neurons have shown that activation of GABA_A receptors, either synaptically or by the application of exogenous GABA_A receptor agonists, can result in hyperpolarization, depolarization or no change in membrane potential, depending on the experimental conditions (Connors, B. W., et al., 1988; Dreifuss, J. J., et al., 1969; Krnjevic, K. and Schwartz, S., 1967; McCormick, D. A., 1989; Scharfman, H. E. and Sarvey, J. M., 1987). However, in nearly all cases GABA-mediated signaling in adult neurons produces a decrease of firing in the postsynaptic neuron. These results show that the direction of membrane potential change might not be the most important factor in the inhibitory process. Stephen Kuffler stated in 1960: "Since the inhibitory potentials may be repolarizing [hyperpolarizing] or depolarizing or may be absent if the cell is at the inhibitory equilibrium level, it follows that the electrical potential changes themselves are secondary and are not essential part of inhibition" (Edwards, C. and Kuffler, S. W., 1959). The key element in synaptic inhibition is the increase in membrane conductance, as this will act to shunt the ability of excitatory potentials to depolarize the membrane to spike threshold, provided that the inhibitory equilibrium potential is below this value. This formulation generally applies to fast chloride-dependent GABA_A-mediated synaptic inhibition. In adult cells, the equilibrium potential (or reversal potential) for chloride ions (E_{Cl}) is -60 to -70mV, which is usually well below the threshold for action potential generation (-40 to -50 mV). By contrast, GABA_B receptor-mediated postsynaptic potentials are potassium dependent, and they generally hyperpolarize the membrane towards the equilibrium potential for potassium ions (below –



70mV). These potentials typically produce less change in membrane conductance than GABA_A potentials, but are strongly inhibitory because they keep the membrane potential further away from the spike threshold (McCormick, D. A., 1989).

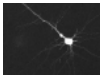
It should therefore be stressed that, in adult neurons, GABA_A-receptor-mediated synaptic inhibition can be produced effectively by membrane depolarization that can in some cases directly evoke action potential discharge (Ben-Ari, Y., et al., 1989; Brickley, S. G., et al., 1996; Chen, G., et al., 1996; Dammerman, R. S., et al., 2000; Gao, X. B. and van den Pol, A. N., 2001; Owens, D. F., et al., 1996; Owens, D. F., et al., 1999; Wang, Y. F., et al., 2001). The more intense depolarizing actions of early GABA_A receptor activation are due to relatively high intracellular chloride concentrations ($[Cl]_i$) of immature neurons and resting membrane potentials that are significantly more negative than E_{Cl} (Ben-Ari, Y., et al., 1989; Brickley, S. G., et al., 1996; Chen, G., et al., 1996; Owens, D. F., et al., 1996; Rohrbough, J. and Spitzer, N. C., 1996).

As development proceeds neuronal $[Cl]_i$ decreases and the GABA_A reversal potential (E_{GABA_A}) becomes more negative (Owens, D. F., et al., 1996), allowing the effect of GABA to become progressively inhibitory. This is reflected in the ability of the GABA_A receptor antagonist bicuculline to induce epileptiform activity by blocking inhibition, an effect that develops during the later part of the first postnatal week (Kriegstein, A. R., et al., 1987; Wells, J. E., et al., 2000). Among neurons, E_{GABA_A} was found to shift negatively by more than 20 mV over a three-week developmental period. This shift corresponds to an approximately 20-mM drop in $[Cl]_i$ (Owens, D. F., et al., 1996). These data indicate that, during development, cells might go from a stage of chloride accumulation to one of chloride extrusion. Consistent with this idea, cation-chloride co-transporters are expressed differentially in the cortex at different stages of development (Clayton, G. H., et al., 1998; Rivera, C., et al., 1999). In addition, as early GABA_A-receptor-mediated synaptic potentials invariably depolarize postsynaptic cells, it has been suggested that, in the immature brain, fast excitatory synaptic transmission is mediated by GABA_A receptors (Ben-Ari, Y., et al., 1997; Cherubini, E., et al., 1991). However, as stated above, depolarization and excitation are not necessarily equivalent. Even when GABA_A receptor activation can depolarize the membrane potential above spike threshold and excite a cell, it is still able to shunt and inhibit other inputs (Gao, X. B., et al., 1998; Lu, T. and Trussell, L. O., 2001). Therefore, in the embryonic and early postnatal brain, when GABA_A receptor activation can, by itself, be excitatory, the resulting change in conductance can modulate other excitatory inputs as well, either inhibiting or facilitating them depending on their timing (Gao, X. B., et al., 1998).



I.1.7. GABA as a developmental signal

One of the first indications that GABA might act as a trophic substance during nervous system development came from studies by Wolff et al. (Wolff, J. R., et al., 1978) in the rat superior cervical ganglia (SCG). Here, it was shown that the continuous application of GABA could promote dendritic growth *in vivo*, influence ganglion cell sensitivity to acetylcholine and alter the development of presynaptic specializations (Joo, F., et al., 1987; Wolff, J. R., et al., 1978). Furthermore, only in the presence of GABA could an ectopic nerve innervate the SCG. From these studies, it was concluded that GABA acts to promote synaptogenesis or the synaptogenic capacity of the SCG (Joo, F., et al., 1987; Wolff, J. R., et al., 1978). During the 1980s, several studies showed that the application of GABA could influence aspects of neuronal differentiation *in vitro* (Madtes, P., Jr. and Redburn, D. A., 1983; Meier, E. and Jorgensen, O. S., 1986). For example, in cultured cerebellar granule cells, GABA treatment increased the number of neurite-extending cells and the density of cytoplasmic organelles (Hansen, G. H., et al., 1987). In addition, GABA application was found to result in a change in expression of the GABA receptor itself (Meier, E., et al., 1987). Similar results were found in chick cortical and retinal neurons (Spoerri, P. E., 1988) and in neuroblastoma cells (Spoerri, P. E. and Wolff, J. R., 1981), leading to the conclusion that GABA could act as a general neuro-developmental factor (Meier, E., et al., 1987). Although the mechanisms of GABA's trophic actions were not elucidated, they seemed to be mediated by the activation of GABA receptors, as GABA_A receptor antagonists could block the effects. It was further suggested that GABA-mediated membrane hyperpolarization was an important step (Meier et al., 1991). This contrasts with more recent studies, which indicate that the trophic actions of GABA probably result from the depolarizing effects of GABA_A receptor activation in immature cells.

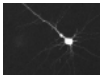


I.1.8. Functional consequences of early depolarizing actions

GABA_A-receptor-mediated membrane depolarization has been observed in developing cells from many brain regions, indicating a general role for GABA-mediated depolarization during development (Ben-Ari, Y., 2002). This depolarization is sufficient to increase $[Ca^{2+}]_i$ by the activation of voltage-gated calcium channels (VGCCs) (Leinekugel, X., et al., 1995; Lin, M. H., et al., 1994; LoTurco, J. J., et al., 1995; Owens, D. F., et al., 1996; Yuste, R. and Katz, L. C., 1991). These results indicate one potential downstream consequence of early GABA_A receptor second-messenger pathways (Cherubini, E., et al., 1991). These findings, coupled with evidence that endogenous GABA receptor activation occurs early in development (in some cases before synapse formation) have provided a signaling framework in which GABA-mediated cell communication can influence many processes in brain development, from cell proliferations to synaptogenesis and circuit formation.

In precursor cells in the neocortical proliferative zone, activation of GABA_A receptors has been shown to influence DNA synthesis (Haydar, T. F., et al., 2000; LoTurco, J. J., et al., 1991). Activating GABA_A receptors in intact rat neocortical explants led to significant decrease in DNA synthesis, as assessed by tritiated-thymidine incorporation, and a reduction in the number of 5-bromodeoxyuridine (BrdU-)labelled cells (LoTurco, J. J., et al., 1995). Depolarizing cells by exposing cortical explants to elevated potassium was sufficient to inhibit DNA synthesis, and the effect of GABA was abolished when the chloride gradient was altered so that GABA was no longer depolarizing.

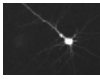
More compelling evidence that GABA_A receptor activation regulates DNA synthesis during cortical development came from experiments in which explants were exposed to only the GABA_A receptor antagonist bicuculline. In the presence of bicuculline, there was a significant increase in DNA synthesis in cortical precursor cells, indicating that GABA is released endogenously and influences the rate of DNA synthesis (LoTurco, J. J., et al., 1995). The bicuculline-induced increase in DNA synthesis was seen readily at E19, but was absent in younger (E14-E16) cortical explants (LoTurco, J. J., et al., 1995). This implies that endogenous GABA_A receptor activation affects only late-stage neurogenesis and/or gliogenesis. However, GABA_A-receptor-mediated reduction of cell proliferation might occur indirectly by interactions with other factors. Experiments in dissociated cell culture have shown that activation of GABA_A receptors can inhibit the proliferative effect that basic fibroblast growth factor (bFGF) exerts on neocortical precursor cells, but has no effect when



applied alone (Antonopoulos, J., et al., 1997). Thus, it is possible that the observed effects in intact tissue represent an indirect action of GABA_A receptor activation shown to influence the movement and migration of immature cortical neurons (Behar, T. N., et al., 1996; Behar, T. N., et al., 2000; Behar, T. N., et al., 2001). In cultured slices of rat brain, it was determined that GABA_C and GABA_B receptor activation provided a stop signal once cells reached the CP (Behar, T. N., et al., 2000). Selectively blocking GABA_C and GABA_B receptors for two days could retard migration, but this effect could be overcome with longer culture periods. These results indicate that, although GABA-mediated signaling might influence neuronal migration, it is not essential for this process, or can be compensated for if absent. Interestingly, in similar studies with cultured slices of mouse brain, GABA seemed to have little, if any, role in neuronal migration (Behar, K. L., et al., 1999). Rather, NMDA-type glutamate receptors appeared to promote migrations in this system. The reason for the differences between these two rodent species is unclear, but these studies indicate, once again, that the results and conclusions of a study might depend on the experimental conditions.

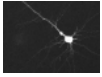
In addition to proliferation and migration, aspects of neuronal differentiation might be regulated by early GABA-mediated signaling. In cultured embryonic hippocampal and neocortical neurons, GABA_A receptor activation has been shown to promote neurite outgrowth and maturation of GABA interneurons (Barbin, G., et al., 1993; Maric, D., et al., 2001; Marty, S., et al., 1996). These effects depend on GABA_A-receptor-mediated membrane depolarization and increases in $[Ca^{2+}]_i$ (Maric et al., 2001; Berninger, B., et al., 1995), as do GABA effects on the survival of rat embryonic striatal neurons *in vitro* (Ikeda, Y., et al., 1997). In the case of interneuron development, GABA might exert its trophic effects by stimulating an increase in brain-derived neurotrophic factor (BDNF) expression and release from target neurons, an effect that diminishes with development (Marty et al., 1996; Vicario-Abejon, C., et al., 1998). The observation that the enhancement by GABA of interneuron growth is diminished when using cells derived from BDNF-knockout mice supports the link between GABA-mediated growth effects and BDNF (Marty et al., 1996; Berninger et al., 1995).

Early GABA signaling might also interact with NMDA receptor activation to regulate synapse maturation (Ben-Ari, Y., et al., 1997). NMDA receptors are often functionally silent at negative membrane potentials due to blockade of the channel by magnesium (Durand et al., 1996). Therefore, an endogenous depolarizing influence must be present at immature synapses to relieve the magnesium block of the NMDA receptor, a role that has been attributed to non-NMDA receptor activation in the more mature brain. In the developing



hippocampus, GABA_A-receptor-mediated synaptic activity has been shown to depolarize cells and relieve the magnesium block from NMDA channels, allowing calcium influx (Leinekugel, X., et al., 1997). The resulting increase in intracellular Ca²⁺ might activate downstream signaling pathways that are crucial for neuronal maturation and synaptogenesis (Ben-Ari, Y., et al., 1997).

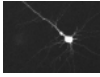
Finally, a recent study in cultured hippocampal neurons provides intriguing evidence that GABA_A-receptor-mediated signaling itself seems to produce the developmental [Cl]_i switch (Ganguly, K., et al., 2001). In this study, the authors found that application of GABA produced increases in [Ca²⁺]_i through GABA_A-receptor-mediated membrane depolarization and activation of VGCCs in young neurons. When GABA_A receptors were chronically blocked with specific receptor antagonists, the authors found that the GABA-induced [Ca²⁺]_i increases persisted and that EGABAGA remained at a relatively depolarized value in older neurons. Evidence was provided that GABA_A-receptor-mediated miniature synaptic potentials were the endogenous source of GABA_A receptor activation. Levels of the K⁺/Cl⁻ co-transporter KCC2 were reduced in cells that had been chronically treated with GABA_A receptor antagonists, indicating that the activation of GABA_A receptors is required to upregulate the expression of KCC2 and decrease [Cl]_i. Moreover, the GABA-mediated upregulation of KCC2 expression was dependent on the activation of VGCCs and calcium influx.



I.2. Cognitive inhibition

The concept of cognitive inhibition is the idea that mental processes or representations can be inhibited. The existence of such a inhibitory mechanisms in the structure of cognition seems logical and crucial, because the substrate on which that structure operates (i.e. the brain) uses both excitatory and inhibitory processes to perform neural computation, and because computational analyses show that inhibitory mechanisms are critical for maintaining stability in neuronal networks (Anderson, M. C. and Spellman, B. A., 1995).

Attempting to fit these two levels of analysis to each other such that they can share a common term will not help in the understanding of either (Breese, 1899). From a cognitive standpoint, inhibition is the blocking or overwriting of a mental process, in whole or in part, with or without intention (Hourihan, K. L. and MacLeod, C. M., 2007). The influenced mental process could be selective attention or memory retrieval or a host of other cognitive processes. This influence would not be to eradicate or entirely prevent some process from occurring, but to slow it down or reduce its probability of taking place. Inhibition could be automatic, applied as an act of will, or represent a by-product of other cognitive processes (Hourihan, K. L. and MacLeod, C. M., 2007).



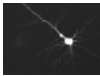
I.3. Lateral amygdala

The amygdala is a key structure underlying fear conditioning (for review, see (Blair, H. T., et al., 2001; Fanselow, M. S. and Poulos, A. M., 2005; LeDoux, J. E., 2000; Maren, S. and Quirk, G. J., 2004; Maren, S., et al., 2001; Pare, D., et al., 2004; Rodrigues, S. M., et al., 2004; Rosen, J. B., 2004; Walker, D. L., et al., 2002); for a different view, see (Cahill, L., 1998; Cahill, L., et al., 1999)). In Pavlovian fear conditioning, a neutral conditioned stimulus (CS) that is paired with a painful shock unconditioned stimulus (US) comes to elicit fear responses such as freezing behavior and related physiological changes.

Within the amygdala, the lateral nucleus (LA) receives auditory (CS) inputs and somatosensory (US) inputs from the thalamus and cortex and connects, directly and indirectly, with the central nucleus (CE) to control the expression of fear responses. Convergence of the CS and US in the LA is believed to play a key role in the initiation of amygdala plasticity, though it is possible that plasticity also occurs in the CE or basal amygdala.

Other possible sites of plasticity include areas of the auditory system that are afferent to the amygdala (Edeline, J. M. and Weinberger, N. M., 1992; Weinberger, N. M. and Bakin, J. S., 1998; Weinberger, N. M., et al., 1995). However, amygdala lesions disrupt conditioned changes in auditory areas (Armony, J. L., et al., 1998; Poremba, A. and Gabriel, M., 1997); Maren et al., 2001), suggesting that auditory system changes may in part be dependent on amygdala plasticity (Apergis-Schoute, A. M., et al., 2005). Together with molecular evidence described below, these various findings point to the LA as an important site of neural changes underlying the acquisition and storage of fear memories. While LA may not be the only site of learning and plasticity during fear conditioning, it is clearly a site of central functional significance and, therefore, an interesting target to examine.

Studies of the amygdala often gloss over the anatomical distinction between LA and the basal nucleus, focusing on the so-called BLA, which includes these and other nuclei (Amaral, D. G. and Insausti, R., 1992; Pitkanen, A., et al., 1997). Most drug infusion studies cannot distinguish between effects in these areas. However, lesion studies have shown that damage to LA (but not to other parts of the BLA) disrupts fear conditioning (Amorapanth, P., et al., 2000; Bauer, E. P., et al., 2001; Nader, K., et al., 2001). It is possible that the basal nucleus also plays a role, since lesions to a small region of anterior basal nucleus can disrupt fear conditioning (Goossens, K. A. and Maren, S., 2001), and post-training basal nucleus



lesions prevent the expression of a previously established fear memory (Anglada-Figueroa, D. and Quirk, G. J., 2005), but this needs further confirmation. Recent studies using viral approaches show that disruption of plasticity in networks within LA is sufficient to disrupt fear conditioning (Rumpel, S., et al., 2005). The amygdala also contributes to the regulation or modulation of memories stored in other brain areas, such as the hippocampus or striatum (Cahill, L. and McGaugh, J. L., 1998; Gold, J. M., et al., 1995; McGaugh, J. L., 2000; Roozendaal, B., et al., 1999). The evidence that the amygdala is also involved in the acquisition and storage of memories about fear conditioning is complementary rather than contradictory to the memory modulation view. Indeed, one way that the amygdala may participate in the regulation of memory in other areas is by detecting emotionally relevant stimuli and, via efferent connections, activating brainstem arousal systems, including norepinephrine (NE) systems, and initiating the release of peripheral hormones, both of which can then affect the amygdala itself and other forebrain regions.

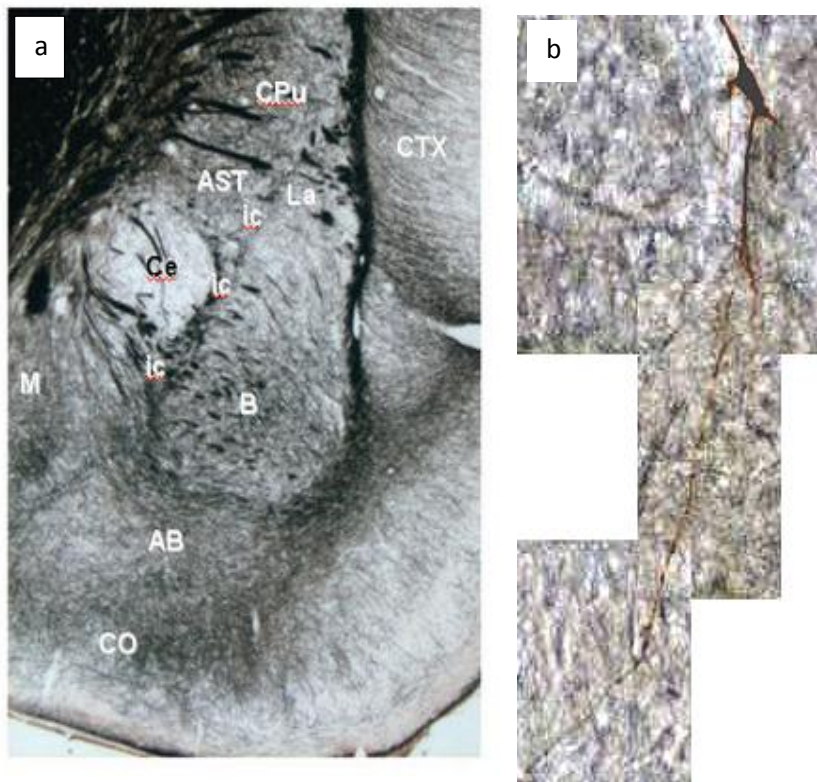
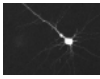


Figure 3: a) Image of amygdala with LA from rat brain slice. B) Recorded and labeled projection neuron of the LA.



I.4. Role of GABA_C receptors in fear memory

As stated earlier, in the mammalian brain GABA is the most frequent inhibitory neurotransmitter (Nicoll, R. A., et al., 1990). The various GABA receptor types not only differ in their responses to GABA but, more important from an experimental point of view, can be selectively activated and blocked by specific agonists and antagonists (Bormann, J., 2000; Bowery, N. G., et al., 2002; Chebib, M. and Johnston, G. A., 1999; Johnston, G. A., 1996). GABAergic neurotransmission is mediated by GABA_A receptors, so one could assume that GABA_A receptors are expressed by every mammalian CNS neuron. In a large number of neurons GABA_B receptors are co-expressed with GABA_A receptors. Compared to the ubiquitous expression of these two GABA receptor types, however, the appearance of the third identified GABA receptor type, the GABA_C receptor, seems to be topographically more restrained. GABA_C receptors have been characterized more recently than GABA_A and GABA_B receptors, and their functional significance is not understood as well.

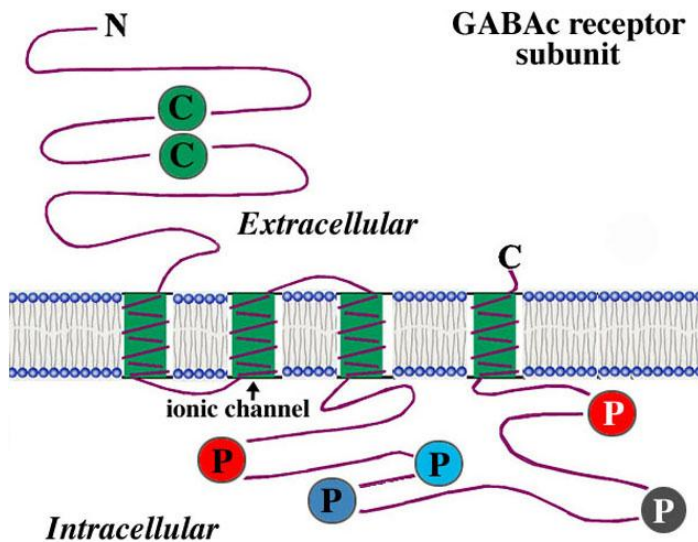
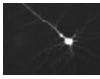


Figure 4: Schematic diagram of GABA_C receptor subunits (Qian, H. and Ripps, H., 2009).

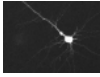
GABA_BR are coupled to second messenger systems by G proteins and to potassium and calcium channels (Kerr, D. I. and Ong, J., 1995). GABA_BR are sensitive for the agonist baclofen and for the antagonist CGP52432. In contrast to GABA_BR, the ionotropic GABA receptors conduct Cl⁻ currents. However, GABA_AR and GABA_CR differ in their composition of subunits. While GABA_AR are formed by a mixture of various α , β , γ , δ and ϵ subunits, GABA_CR



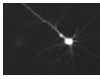
contain only ρ subunits. The subunit composition also causes a number of biophysical differences. Thus, GABA_CR are about 10 times more sensitive to GABA, they activate and inactivate more slowly, the mediated currents are smaller, they do not show desensitization, and their mean open time is longer than that of the GABA_AR. GABA_AR are sensitive to the antagonists bicuculline and picrotoxine, while muscimol is known as an agonist. In contrast, GABA_CR are by definition insensitive to bicuculline but sensitive to picrotoxin. The GABA_AR agonist muscimol is also an effective GABA_CR agonist, but it activates at lower concentrations (Bormann, J., 2000). Cis-aminocrotonic acid (CACA) is regarded as a selective GABA_CR agonist, (1,2,5,6-tetrahydropyridine-4-yl) and methylphosphinic acid (TPMPA) is a selective GABA_CR antagonist (Bormann, J., 2000; Bormann, J. and Feigenspan, A., 1995; Ragozzino, D., et al., 1996).

Because it is the most abundant GABA receptor in the mammalian CNS, GABA_ARs are present on every cerebral cell type. Their cellular distribution includes synaptic as well as extrasynaptic sites (Brickley, S. G., et al., 1996; Wall, M. J. and Usowicz, M. M., 1997). Functional GABA_C receptors are known from the retina (Feigenspan, A. and Bormann, J., 1998; Lukasiewicz, P. D., 1996), the hippocampus (Hartmann, K., et al., 2004), the superior colliculus (Boller, M. and Schmidt, M., 2003; Kirischuk, S., et al., 2003; Schmidt, M., et al., 2001), cerebellum (Delaney, A. J. and Sah, P., 1999) and from the spinal cord (Johnston, G. A., et al., 1975). In the lateral amygdala (LA), functional GABA_C receptors have not yet been reported.

We wanted to analyze the role of GABA_C receptors in the amygdala, which is a key brain structure in emotional learning (LeDoux, J. E., 1995; Longman, J. and Valente, M. I., 1957; Ramsay, J., 1939). The lateral nucleus of the amygdala (LA) is a key site of plasticity underlying fear learning (Blair, H. T., et al., 2001; LeDoux, J. E., 2000; Maren, S. and Quirk, G. J., 2004). It is known that the inhibitory circuit plays an important role in fear memory and its extinction (Akirav, I. and Richter-Levin, G., 2006; Wilensky, A. E., et al., 1999; Zhang, S. and Cranney, J., 2008). The majority of studies concentrate on GABA_A and GABA_B receptors. The study of (Delaney, A. J. and Sah, P., 1999) described the existence of GABA_C receptors in the lateral part of the central nucleus of the amygdala. However, using in situ-hybridization of GABA_C receptor-specific $\rho 1$ and $\rho 2$ subunits in the amygdala has been demonstrated (Allen Brain Atlas [Internet]). The functional significance of GABA_CRs is generally less well understood than that of the other GABARs, and few studies have explored the contribution of GABA_CRs to learning, memory and plasticity. Research performed in the forebrain of chicks

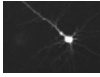


found GABA_ARs and GABA_CRs to play opposite roles in short-term memory (STM) in passive and discriminated avoidance tasks (Gibbs, M. E. and Johnston, G. A., 2005). Also, a novel selective GABA_CR antagonist facilitates learning and memory in the Morris Water Maze task in mice (Chebib, M., et al., 2009). Furthermore, GABA_CRs could be an important target in the effort to develop pharmacological treatments for anxiety-related disorders since, unlike GABA_ARs, GABA_CRs do not desensitize (Bormann, J., 2000). Patients with generalized anxiety disorder or panic disorder show lower benzodiazepine binding, which targets GABA_ARs (Kaschka, W., et al., 1995; Malizia, A. L., et al., 1998; Tiihonen, J., et al., 1997). Because GABA_CRs have different pharmacological properties, they provide an alternative avenue to treatment.



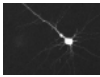
I.5. Norepinephrine

Neuromodulators are known to contribute to synaptic plasticity, learning and memory (Summers, R. J. and McMartin, L. R., 1993). Memory enhancements for emotionally charged events are associated with the release of norepinephrine (NE) in the amygdala (McGaugh, J. L. and Izquierdo, I., 2000), which is a crucial structure in emotional learning (LeDoux, J. E., 2000; Phelps, E. A. and LeDoux, J. E., 2005). The release of NE in forebrain areas occurs via axons of neurons located in the pons and medulla, especially in the locus coeruleus (LC) (Aghajanian, G. K., 1978; Amaral, D. G. and Sinnamon, H. M., 1977; Aston-Jones, G. and Harris, G. C., 2004; Aston-Jones, G., et al., 1996; Dahlstrom, A. and Fuxe, K., 1964; Foote, S. L., et al., 1983; Lindvall, O. and Stenevi, U., 1978; Nestler, E. J., et al., 1999). During alert, non-stressed states the LC exhibits low tonic and high phasic firing, while threatening or stressful conditions elicit high tonic firing rates (Aston-Jones, G. and Bloom, F. E., 1981; Aston-Jones, G., et al., 1999; Aston-Jones, G., et al., 2000; Grant, S. J., et al., 1988; Grant, S. J. and Redmond, D. E., Jr., 1984) and high levels of NE release (McGaugh, J. L. and Roozendaal, B., 2002; McIntyre, C. K., et al., 2003). NE released from terminals and varicosities binds to α_1 , α_2 and β G-protein coupled receptors (Berridge, C. W. and Waterhouse, B. D., 2003; Bylund, D. B., et al., 1994; Minneman, K. P. and Esbenshade, T. A., 1994; Philipp, M. and Hein, L., 2004; Ruffolo, R. R., Jr. and Hieble, J. P., 1994). The regional distribution, synaptic organization (presynaptic vs. postsynaptic localization), and intracellular coupling to second messengers dictates the effects that NE has when binding to specific receptor subtypes in a given circuit. α_1 , α_2 and β receptors are all found at both pre- and postsynaptic sites (Braga, M. F., et al., 2004; Nicholas, A. P., et al., 1996; Timmermans, P. B. and van Zwieten, P. A., 1981). α_1 receptors are positively coupled to PLC through Gq proteins leading to the generation of DAG and IP₃, ultimately activating PKC (Drouva, S. V., et al., 1991). α_2 receptors are negatively coupled to adenylate cyclase (AC) through Gi proteins, suppressing cAMP production and PKA activation (MacMillan, L. B., et al., 1998). When located presynaptically, or on cell bodies or dendrites of NE neurons, α_2 receptors mediate a powerful auto-inhibitory mechanism resulting in NE-triggered suppression of subsequent NE release (Collis, M. G. and Shepherd, J. T., 1980). β receptors are positively coupled to AC through G-proteins leading to production of intracellular cAMP and PKA activation (DeBlasi, A., et al., 1986). When located presynaptically, β receptor stimulation enhances transmitter release (Majewski, H., 1983).



I.5.1. Role of norepinephrine in the amygdala

One role of NE is the transient regulation of amino acid transmission. NE can facilitate processing of specific stimuli both by suppressing background activity relative to stimulus-evoked activity and by enhancing evoked activity (Berridge, C. W. and Waterhouse, B. D., 2003; Foote, S. L., et al., 1983). These effects of NE are believed to be mediated by altering the AHP and accommodation that follow spike discharges (Berridge, C. W. and Waterhouse, B. D., 2003; Madison, D. V. and Nicoll, R. A., 1986; Moises, H. C., et al., 1981; Oades, R. D., 1985). A second important role of NE is to trigger intracellular processes that lead to persistent consequences for synaptic transmission and plasticity, and thus for memory. Little effort has been directed towards understanding how the transient effects on cellular function and synaptic transmission relate to the more persistent effects that occur when plasticity is induced. The effects of NE on target neurons are dose related. Spike discharges elicited by glutamate are enhanced by intermediate levels of NE, with higher or lower levels of NE being less effective (Waterhouse, B. D., et al., 1998). This 'inverted-U' shaped pattern for NE-induced responses involves dose-related interactions between the different adrenoceptor subtypes and their affinity to bind NE (Arnsten, A. F., 2000). This is consistent with reported inverted-U shaped effects of NE on memory and attention (Aston-Jones, G. and Cohen, J. D., 2005; Aston-Jones, G., et al., 2000; Introini-Collison, I. B., et al., 1994), and suggests that NE functions within a narrow range to tune neural activity in specific circuits to cognitive, emotional, and behavioral demands (Berridge, C. W. and Waterhouse, B. D., 2003).

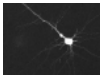


I.5.2. Contribution of NE to memory and synaptic plasticity.

NE has long been thought to serve as a signal that facilitates synaptic transmission in response to biologically significant events (Harley, C. W., 2004; Kety, S. S., 1972; Livingston, P., 1967), and thus contribute to learning and memory (Aantaa, R., et al., 1995; Levitzki, A., et al., 1993; MacDonald, E. and Scheinin, M., 1995; Molinoff, P. B., 1984; Sirvio, J. and MacDonald, E., 1999; Summers, R. J. and McMartin, L. R., 1993). Consistent with this view, LC neurons are activated by novel, arousing and rewarding stimuli (Aston-Jones, G. and Bloom, F. E., 1981; Aston-Jones, G., et al., 1994; Sara, S. J., 2009; Sara, S. J. and Segal, M., 1991).

The specific contribution of NE to learning and memory has been studied extensively by manipulation of NE receptor subtypes systemically or within specific brain structures using a variety of different learning tasks (Izquierdo, I. and Dias, R. D., 1985; Lalumiere, R. T., et al., 2004; Marti Barros, D., et al., 2004; McGaugh, J. L., 2002; Roozendaal, B., et al., 2004). Most studies have examined the effects of β -adrenergic receptor manipulation, but there is also evidence of a role of α receptors (Coull, J. T., et al., 2004; McGaugh, J. L., 2004).

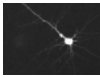
NE modulates synaptic plasticity and memory by G-protein coupled activation of second messengers (Bailey, C. H., et al., 2000; Gelinass, J. N. and Nguyen, P. V., 2005; Swope, S. L., et al., 1999; Watabe, A. M., et al., 2000; Winder, D. G., et al., 1999). Particularly well understood is the role of β receptor stimulation, which interacts with cAMP and PKA. PKA translocates to the nucleus and activates transcription and translation (Sassone-Corsi, P., 1998) and thereby contributes to the persistence of plasticity and memory (Bailey, C. H., et al., 2000). Importantly, calcium influx through NMDA receptors also activates the cAMP/PKA/CREB pathway (Collingridge, G. L. and Singer, W., 1990). α 2 receptor stimulation inhibits PKA signaling and may suppress CREB activation, gene expression and protein synthesis (Aghajanian, G. K. and Wang, Y. Y., 1987; Davies, M. F., et al., 2004; Lu, L. and Ordway, G. A., 1997). Thus, an important function of α 2 and β receptors may be to suppress or amplify PKA signaling, thereby gating the transformation of short-term memory (STM) into long-term memory (LTM) (Bailey, C. H., et al., 2000). PKC, activated by α 1 receptor stimulation, may be involved in memory acquisition and consolidation (Goosens et al., 2000; Selcher et al., 2002; Weeber et al., 2000). However, NE stimulation of α 1 receptors facilitates amygdala GABA signaling (Braga, M. F., et al., 2004), suggesting a role in gating excitatory plasticity or even facilitating inhibitory plasticity.



I.6. Optogenetic control of neural activity

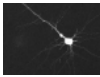
The term 'optogenetics' was created in 2006 (Deisseroth, K., et al., 2006) to define "the branch of biotechnology which combines genetic engineering with optics to observe and control the function of genetically targeted groups of cells with light, often in the intact animal" (Miesenbock, G., 2009). Identification and visualization of neurons has always been a key aspect in studying not only neuronal morphology but also function, as is it clearly illustrated by the still widespread use of the Golgi staining technique.

At present, there is a vast variety of techniques available to facilitate the visualization of single neurons, such as the Golgi staining, diffusible dyes (Gan, 2000) or fluorescent proteins (Feng, G., et al., 2000). Neuronal pathways can also be traced using viral mediated retrograde labeling of synaptic partners (Marshall, J. H., et al., 2010) or even by labeling synaptic contacts between neurons using pre- and postsynaptic fluorophore complementation (Feinberg, E. H., et al., 2008). Additionally, specific populations of neurons can be labeled based on common genetic markers using bacterial artificial chromosome (BAC) transgenic mice to drive expression of green fluorescent protein (GFP) (Gong, S., et al., 2003). Also increasingly common are tools that optically report on neuronal activity and other neuronspecific variables, such as membrane depolarization, calcium ion concentration changes or presynaptic vesicle fusion events (Miesenbock, G. and Morris, R. G., 2005). Functional characterization requires tools to perform manipulations, such as a stimulating electrode. Optical based actuators are the object of intense interest in neuroscience research as they offer a counterpart to optical sensor technology (Miesenbock, G., 2009; Miesenbock, G. and Kevrekidis, I. G., 2005). Recently, there have been several improvements in this area, mainly directed toward circumventing the shortcomings of the electrode as the sole provider of control over neuronal activity. Even though the electrode is still the standard for many electrophysiological techniques, some of the most difficult challenges presented by this method are readily visible when targeting multiple cells or performing *in vivo* experiments. This is mainly because intracellular electrodes are impractical in targeting more than a small number of cells, while at the same time extracellular electrodes exhibit a very poor spatial resolution in a tissue as heterogeneous as the brain. This is further complicated when moving from an *in vitro*, or *ex vivo* setting, to controlling neuronal activity in an awake behaving animal, where the necessity to keep electrodes immobile while both stimulating and recording is critical (Lee, A. K., et al., 2009; Lee, E., et al., 2009).



In order to be able to link cellular inhibitory mechanisms with behavior, one would like to elucidate the role of inhibition onto neurons within intact tissue and neural circuitries. Several chemical genetic approaches have been developed to silence neuronal activity in mammalian neurons (Tervo, D. and Karpova, A. Y., 2007). These methods allow perturbation of neuronal activity in specific subpopulations of neurons by genetic targeting and administration of chemical inducers, enabling neuroscientists to probe how individual groups of neurons regulate circuitry activity and behavior (Armbruster, B. N., et al., 2007; Karpova, A. Y., et al., 2005; Lerchner, W., et al., 2007). The major advantages of these chemical genetic approaches are their on-and-off inducibility, their ability to silence large numbers of neurons and their ability to reach deep brain regions through systemic administration of chemical inducers. The major disadvantages are the slow time course of onset/offset (minutes in cultured neurons and hours to days *in vivo*) and the potential toxicity or interference with physiological processes by chemical inducers (Tervo, D. and Karpova, A. Y., 2007). In neuroscience, noninvasive, genetically targeted, and temporally precise manipulation of neuronal activity would enable exploration of the functional roles of individual neuron types in intact circuits. Moreover, precise control over specific molecularly distinct neuronal cell types would be likely to drive new clinical understanding and novel treatment development in neuropsychiatric disease. Photostimulation presents a path to deconvolute several of these problems, particularly if allied to the potential for genetic encoding. These two powerful strategies can combine the rapidity and ease of light directed stimulation with the cellular specificity conferred by promoter driven expression. Recent additions to the growing neuro-engineering toolbox have been the algae derived cation permeable channel channelrhodopsin-2 (ChR2) (Boyden, E. S., et al., 2005) and the bacterial *Natronomonas pharaonis* - anion pump - halorhodopsin (NphR).

To enable precise perturbation of living circuits, in previous work, George Augustine's and Guoping Feng's lab have generated transgenic mice that express ChR2 in subsets of neurons and demonstrated their utility for *in vivo* light-induced activation and mapping of neural circuits (Aravanis, A. M., et al., 2007; Arenkiel, B. R., et al., 2007) for genetically targeted, millisecond-timescale optical excitation of neurons without the need for exogenous co-factor or agonists. ChR2 can be used to test sufficiency of particular spike patterns in circuit responses, and many neuroscience laboratories are now interested in applying ChR2 to their experimental questions. However, ChR2 cannot be used as a diagnostic circuit-breaker to inhibit native spike trains, which precludes testing the necessity, or physiological



function, of targeted cell types. The ideal solution to this problem would involve a complementary optical hyperpolarizer, to permit excitation or inhibition using two well-separated wavelengths of light in the same experiment, while maintaining high temporal precision and genetic targeting. As a possible solution, the high-speed light-activated chloride pump NpHR has been described (Boyden, E. S., et al., 2005; Nagel, G., et al., 2003). NpHR is a seven-transmembrane domain halorhodopsin isolated from the halophilic bacterium *Natronobacterium pharaonis* (Kolbe, M., et al., 2000; Lanyi, J. K., et al., 1990; Lanyi, J. K., et al., 1990). This microorganism can be isolated from soda lakes where it has to cope with two extreme conditions, high salt concentrations and an alkaline pH of 11. It grows optimally in 3.5 M NaCl and at pH 8.5.



Figure 5: Lake Zug from Wadi Natrum in the Sahara Desert Egypt (photograph from Antiquity 77, No 296, June 2003), from which *Natronomonas* has been isolated (Soliman and Truper, 1982).

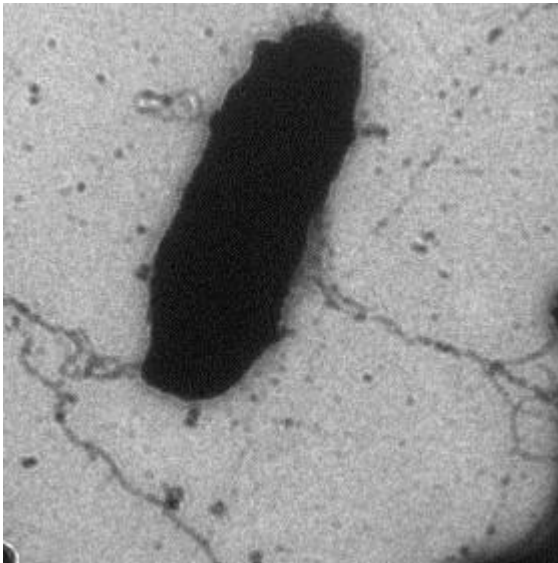
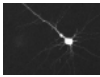
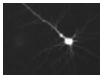


Figure 6: *Natronomonas pharaonis* electron microscopical image with about 10 000-fold magnification (photo by Christian Klein).

Recent studies have demonstrated that expression of NpHR in mammalian neurons by transfection or viral infection allows rapid, light-induced reversible inhibition of neuronal activity (Han, X. and Boyden, E. S., 2007; Zhang, F., et al., 2007). The activating wavelength spectrum of NpHR is strongly red-shifted relative to ChR2, and NpHR functions in mammalian neurons without exogenous cofactors. To expand this tool into a mammalian model system, we generated transgenic mice that express NpHR-YFP using the neuron-specific Thy1 promoter. We found that high levels of NpHR-YFP were expressed in subsets of neurons in these mice and that illumination of NpHR-expressing neurons led to rapid, reversible photoinhibition of action potential firing in these cells. Together, NpHR and ChR2 form a complementary system for multimodal, high-speed, genetically-targeted, all-optical interrogation of intact neural circuits.

In a set of studies relevant to human disorders, introduction and activation of ChR2 was successful in ameliorating several dysfunctions. Viral transduction of ChR2 in the retina of a mouse model of retinal degeneration restored the ability of the surviving retinal neurons to encode light signals to the visual cortex (Bi, A., et al., 2006). Also, in ovo electroporation of ChR2 followed by neuron activation demonstrated the potential for a noninvasive



manipulation of intact chick spinal cord, while in a rat lesion model for diaphragm paralysis, light activation of ChR2 in the spinal cord motor neurons was sufficient to induce recovery of respiratory diaphragmatic motor activity (Alilain, W. J., et al., 2008; Arenkiel, B. R. and Peca, J., 2009).

It is therefore clear that the use of an optogenetic tool such as ChR2 and NpHR can facilitate inquiries into neuronal circuits, in the manipulation of behavior or in the correction of dysfunctional or diseased neural pathways. The main advantages of using ChR2 and NpHR as an actuator over neuronal activity arise from the high temporal and spatial control overexcitation, and the possibility for genetic encoding, allowing for expression both in large populations of cells or in a cell-type specific manner. Furthermore, establishing a qualitative and quantitative link between neuronal activity and perception driven behavioral output represents a major step toward the comprehension of the interplay between several circuits in the brain.

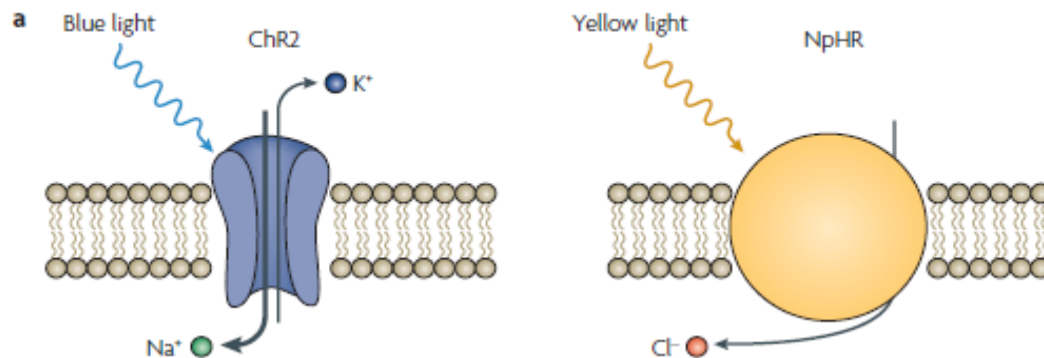
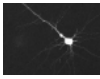


Figure 7: a) Model of ChR2 and NpHR activation with light (Zhang, F., et al., 2007).



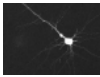
II. Aim and outline of this study

In this study, I intended to contribute to knowledge of the subject of inhibition in the mammalian brain. To get a better insight into the mechanisms of inhibition I used a variety of approaches.

1. First I studied transmission of inhibition, concentrating on different receptors and their modulations by different drugs.
2. The next step was to use the obtained results on the neural level and analyze whether these effects had any relevance for the behaving animal *in vivo*.
3. Finally, I participated in a project developing new tools for analyzing neural inhibition.

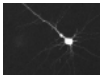
The involvement of inhibitory circuits in fear conditioning (the behavioral model upon which current understanding of the neurobiology of fear is largely grounded) in the LA is poorly understood. Previous studies have often focused on the basolateral complex (BLA), which includes several regions besides the LA. Most studies concentrate only on the GABA_A and GABA_B receptor, although it is known that all three GABA_{A,B,C} receptors are expressed in the amygdala. The aim of this study was to verify the expression of functional GABA_C receptor in the LA, using whole-cell patch clamp recordings in acute slices *in vitro*. To analyze the role of GABA_C receptors in the LA *in vivo* we used auditory Pavlovian fear conditioning (Fanselow, M. S. and LeDoux, J. E., 1999; LeDoux, J. E., 2000), a learning paradigm in which an emotionally neutral auditory conditioned stimulus (CS) comes to elicit fear after it is paired with an aversive unconditioned stimulus (US), which is a useful tool for studying the neural basis of fear learning and memory (Rodrigues, S. M., et al., 2004). Electrical stimulation of Pyramidal cell afferents allowed for analysis of a possible participation of GABA_C receptors in local GABAergic neurotransmission. The results indicate that GABA_C receptors are involved in intra LA neuronal communication where they modulate a considerable fraction of postsynaptic currents. We suggest that GABA_C receptors are located on the presynaptic side on the axons of the interneurons and act as autoinhibitors. Auditory Pavlovian fear conditioning under the influence of GABA_C receptor agonists and antagonists showed that GABA_A and GABA_C receptors play an opposing role on modulations of fear acquisition, and consolidation: GABA_A receptors impair and GABA_C receptors enhance fear learning and memory.

In a variety of neural systems in wide ranging species, neuromodulators are known to



contribute to synaptic plasticity and learning and memory (Bailey, C. H., et al., 2000). We chose to focus on NE because of the mismatch between its long established relation to fear and anxiety (Gray, J. A., 1978; Redmond, D. E., Jr. and Huang, Y. H., 1979; Sullivan, G. M., et al., 1999), and the paucity of information about its role in fear conditioning. By building on progress that has been achieved in understanding fear conditioning mechanisms, it should be possible to elucidate the role of NE in fear, and to gain new insights into the function of NE, and possibly other modulators, in neural circuits. LA principal neurons receive converging information about CS inputs from thalamic and cortical auditory areas (Doron, N. N. and Ledoux, J. E., 2000; Li, X. F., et al., 1996; Romanski, L. M., et al., 1993). NE can have differential modulatory effects at these two pathways, showing a stronger inhibitory effect on synaptic transmission at the cortical pathway than the thalamic pathway (Johnson, L. R., et al., 2011). This pathway asymmetry is due to differences in local GABAergic inhibitory interneurons regulating the two pathways. Additionally, bath application of a β -adrenergic receptor (β -AR) agonist, isoproterenol (ISO), can induce a synaptic potentiation similar to LTP induction (Huang, Y. Y. and Kandel, E. R., 1996). In this potentiation, the modulatory effect of NE is again greater at the cortical pathway than at the thalamic, and this difference is mediated by GABAergic interneurons. In addition, LTP induction by electrical stimulation at either pathway depends on β -AR activation (Johnson, L. R., et al., 2011). However, the role of α 1-adrenergic receptors in fear conditioning, a major model of emotional learning, is poorly understood. In chapter 3 we examined the effect of terazosin, an α 1-adrenergic receptor antagonist, *in vitro* and *in vivo*.

A better understanding of the functional organization of neuronal circuits is of key importance for neurobiological research. However, the heterogeneity of neural tissues complicates our ability to differentiate between specific cell types and circuits imbedded in areas that are not highly laminated. One objective of this study was to develop and test actuating optogenetic tools as a means of mapping neuronal circuitry in the mouse brain. In chapter 4 I describe a strategy that was designed to use the Thy1 promoter to drive expression of NpHR-YFP in random subsets of cells. However, even though Thy1 will drive transgenic expression in random populations of cells across different transgenic lines, characterization of founder mice and their progeny usually reveals a stable and predictable transgene expression in subsequent generations. Therefore, this work entailed the detailed characterization of multiple lines of transgenic NpHR-YFP mice across the central and peripheral nervous system. These animals were then used for the dual purpose of proving the



feasibility of using NpHR in the intact mammalian brain, and assisting in the mapping of neuronal circuits. Using brain slices from these transgenic animals, the endogenous properties of NpHR-positive and NpHR-negative neurons were compared to assess maintenance of naturalistic neuronal properties. The evoked photocurrents elicited by illumination of NpHR-positive neurons were also characterized, as well as the effect of light intensity and stimulus frequency on the inhibition of the neural activity. For my studies in the amygdala concentrating on the role of the inhibitory circuitry in the lateral amygdala in fear learning and memory, these tools will be very useful. In Figure 8 I show that I found expressions of NpHR in the amygdala of the Thy1-NpHR-YFP transgenic mice (unpublished data). In these mice I could switch off specific neurons in the amygdala and analyze the effect of the precise inhibition *in vitro* and *in vivo*.

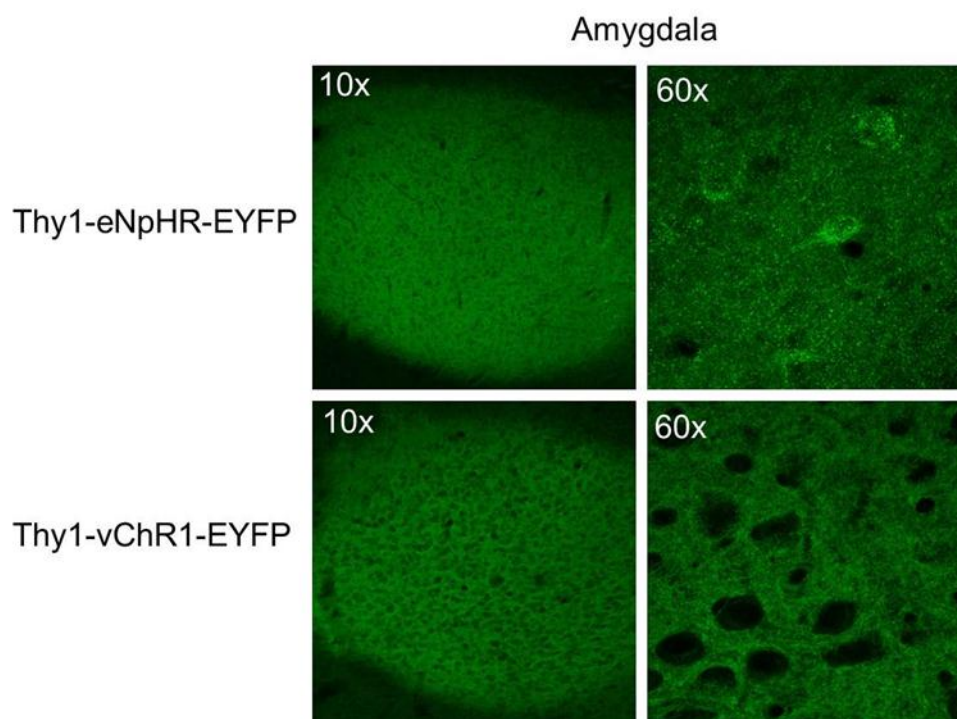
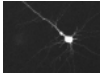


Figure 8: Thy1-NpHR-YFP transgenic mice confocal images showing expression of NpHR-YFP in amygdala.

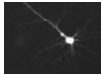


Chapter 2

GABA_C receptors in the lateral amygdala: a possible novel target for the treatment of fear and anxiety disorders?

In the subsequent work, Catarina Cunha made the following contributions:

- Planned the research and designed the experiments
- Tested drug concentrations for electrophysiological and behavioral experiments
- Performed the surgery
- Collected, analyzed and interpreted:
 - Electrophysiology recordings, behavioral experiments, histology
- Wrote the paper



GABA_C receptors in the lateral amygdala: a possible novel target for the treatment of fear and anxiety disorders?

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Activation of GABA_ARs in the lateral nucleus of the amygdala (LA), a key site of plasticity underlying fear learning, impairs fear learning. The role of GABA_CRs in the LA and other brain areas is poorly understood. GABA_CRs could be an important novel target for pharmacological treatments of anxiety-related disorders since, unlike GABA_ARs, GABA_CRs do not desensitize. To detect functional GABA_CRs in the LA we performed whole cell patch clamp recordings *in vitro*. We found that GABA_ARs and GABA_BRs blockade lead to a reduction of evoked inhibition and an increase increment of excitation, but activation of GABA_CRs caused elevations of evoked excitation, while blocking GABA_CRs reduced evoked excitation. Based on this evidence we tested whether GABA_CRs in LA contribute to fear learning *in vivo*. It is established that activation of GABA_ARs leads to blockage of fear learning. Application of GABA_C drugs had a very different effect; fear learning was enhanced by activating and attenuated by blocking GABA_CRs in the LA. Our results suggest that GABA_C and GABA_ARs play opposing roles in modulation of associative plasticity in LA neurons of rats. This novel role of GABA_CRs furthers our understanding of GABA receptors in fear memory acquisition and storage and suggests a possible novel target for the treatment of fear and anxiety disorders.

Keywords: GABA, GABA_C receptors, muscimol, TPMPA, lateral amygdala, PPD, fear learning memory

INTRODUCTION

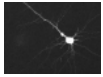
The lateral nucleus of the amygdala (LA) is a key site of plasticity underlying fear learning (LeDoux, 2000; Blair et al., 2001; Maren and Quirk, 2004), and inhibitory circuits in that structure play an important role in the regulation of fear memory and its extinction (Wilensky et al., 1999; Akirav and Richter-Levin, 2006; Zhang and Cranney, 2008). In the mammalian brain, γ -aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter (Nicoll et al., 1990). It acts through different receptor types, including the ionotropic GABA_A (GABA_AR) and GABA_C (GABA_CR) receptors (both of which activate Cl⁻ currents), and the metabotropic GABA_B receptor (GABA_BR). Most studies that have examined the role of GABA receptors in the LA have focused on GABA_ARs. GABA_ARs and GABA_CRs are activated by the same ligands (GABA or GABA_A agonists; Lukasiewicz et al., 1994), but GABA_CRs are many times more sensitive than GABA_ARs to these. If GABA_CRs are present in the LA, they should thus contribute to amygdala functions under different conditions than GABA_ARs. To test this, we used different concentrations of the GABA agonist muscimol, since low concentrations should activate GABA_CRs and high concentrations GABA_ARs.

The functional significance of GABA_CRs is generally less well understood than that of the other GABARs, and few studies have

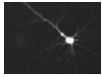
explored the contribution of GABA_CRs to learning, memory and plasticity. Research performed in chicks found GABA_ARs and GABA_CRs in the forebrain play opposite roles in short-term memory (STM) of avoidance learning (Gibbs and Johnston, 2005). Also, the selective GABA_CR antagonist (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid (TPMPA), facilitates learning and memory in the Morris Water Maze task in mice (Chebib et al., 2009).

GABA_CR-specific $\rho 1$ and $\rho 2$ subunits were detected in the amygdala of mice using *in situ*-hybridization [Allen Brain Atlas (Internet)]. Functional GABA_CR have been identified in several regions of the nervous system (Johnston et al., 1975; Lukasiewicz et al., 1994; Pan and Lipton, 1995; Delaney and Sah, 1999; Boller and Schmidt, 2001, 2003; Kirischuk et al., 2003; Hartmann et al., 2004), including the lateral part of the central nucleus of the amygdala (CE; Delaney and Sah, 1999), but have not been reported in the LA. Demonstration of the existence of GABA_CRs in the LA and determining their role in amygdala circuitry could lead to a better understanding of inhibitory plasticity in this brain region. Furthermore, GABA_CRs could be an important target in the effort to develop pharmacological treatments for anxiety-related disorders since, unlike GABA_ARs, GABA_CRs do not desensitize (Bormann, 2000).

The goals of the current study were to verify the expression of



functional GABA_CR in the LA, and to analyze their role in circuitry using whole-cell patch clamp recordings in acute slices *in vitro*. To examine the relevance of the role of GABA_CRs on fear learning and memory, we performed *in vivo* auditory Pavlovian fear conditioning a learning paradigm in which an emotionally neutral auditory conditioned stimulus (CS) comes to elicit fear responses after it is paired with an aversive unconditioned stimulus (US). Because fear conditioning is rapidly acquired and long lasting and the neural circuits underlying the learning are well understood and known to critically involve the LA, fear conditioning has been a popular technique for exploring the cellular and molecular mechanisms that contribute to learning, memory and plasticity (Rodrigues et al., 2004).



MATERIALS AND METHODS

SUBJECTS

All animal experiments were performed in accordance with our institutional guidelines after obtaining the approval of the Institutional Animal Care and Use Committee (IACUC). For the electrophysiological experiments we received 37 Sprague Dawley 21-day-old rats. For the behavioral experiments we received 28 naïve male Sprague Dawley rats, weighing 250–300 g. These rats were housed individually and placed on a 12-h light/dark cycle with *ad libitum* food and water. The rats were acclimatized to laboratory conditions for 3 days before undergoing surgery.

ELECTROPHYSIOLOGICAL EXPERIMENTS

Slice preparations

The amygdala slice preparation has been described previously (Weisskopf et al., 1999). Rats were anesthetized with subcutaneous injection of ketamine (100 mg/kg body weight) and thiazine hydrochloride (1 mg/kg). To obtain acute slices of the LA for recording, the rats were deeply anesthetized with a subcutaneous injection of ketamine (100 mg/kg body weight) and thiazine hydrochloride (1 mg/kg). After transcardial perfusion with ice-cold artificial cerebro-spinal fluid (ACSF) containing (in mM), NaCl 124, KCl 5, NaH₂PO₄ 1.25, NaHCO₃ 26, MgSO₄ 2, CaCl₂ 2, glucose 10, that was continuously gassed with 5% CO₂/95% O₂, the brain was removed and cut into 300 µm thick coronal slices on a vibratome in ice cold ACSF. To allow recovery, slices were incubated for 1 h in ACSF at a temperature of 36°C. For recording, the slices were transferred into a submerged type recording chamber where they were continuously superfused at 3 ml/min with ACSF at room temperature.

Electrophysiology

For whole-cell recordings, slices were transferred to a submersion-type recording chamber where they were continuously perfused with oxygenated ACSF at a rate of 4 ml/min. Whole-cell recordings were obtained from the pyramidal cells in the LA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 5.0–8.0 MΩ. The pipettes were filled with internal solution composed of (in mM): potassium gluconate, 130; sodium gluconate, 2; HEPES, 20; MgCl₂, 4; Na₂ATP, 4; NaGTP, 0.4; EGTA, 0.5. In order to block sodium spikes, 5 mM QX 314 (Sigma-Aldrich) was added, as was 0.5% biocytin for morphological single cell reconstruction. Neurons were visualized with an upright microscope (Nikon Eclipse E600fn) using the Nomarski-type differential interference optics through a 60× water immersion objective. Neurons with a pyramidal appearance were selected for recordings. Neurons were voltage clamped using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Excitatory (EPSCs) and inhibitory postsynaptic currents (IPSCs) were recorded at a holding potential of –35 mV. Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (2 mm diameter, World Precision Instruments, Sarasota, FL, USA) placed in the cortical and thalamic pathway, 50–100 mm from the recording electrode. Stimulation was applied, at 0.1 Hz, using a photoelectric stimulus isolation unit having a constant current output (PSIU6, Grass Instrument Co., West Warwick, RI, USA). Access resistance

(8–26 MΩ) was regularly monitored during recordings, and cells were rejected if it changed by more than 15% during the experiment. The signals were filtered at 2 kHz, digitized (Digidata 1440A, Axon Instruments, Inc.), and stored on a computer using the pCLAMP10.2 software (Axon Instruments, Inc.). The peak amplitude, 10–90% rise time, and the decay time constant of IPSCs were analyzed off-line using pCLAMP10.2 software (Axon Instruments).

Drugs

All pharmacologically active substances were bath applied for 10 min to achieve stable responses before their effects were tested. We used 0.1 µM muscimol (C5-aminomethyl acid; Sigma) as GABA_AR agonist. To make sure that the evoked responses were not GABA_AR-mediated, 20 µM bicuculline (GABA_AR antagonist; Sigma) was co-applied. GABA_CRs were blocked with 30 µM TPMPA (Sigma). As GABA_BR agonist we applied 50 µM CGP52432 (Tocris).

Paired pulse depression

Neurons were stimulated by using an interstimulus interval of 100 ms. EPSCs and IPSCs were recorded.

Histochemistry

After each recording session, slices were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 24 h. The slices were processed using standard histochemical techniques for visualization of biocytin with 3,3-diaminobenzidine (Sigma-Aldrich). For documentation, stained cells were photographed using a digital camera attached to a standard laboratory microscope.

Analysis

Data were analyzed by paired Student's *t*-tests, because one group, the neurons, of units that has been tested twice (control and drug).

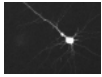
BEHAVIORAL STUDIES

Surgery

Rats were anesthetized with subcutaneous injection of ketamine (100 mg/kg body weight) and thiazine hydrochloride (1 mg/kg) and treated with atropine sulfate (0.4 mg/kg). Using a stereotaxic frame, guide cannulae (22 gauge; Plastics One, Roanoke, VA, USA) fitted with internal cannulae that extended out by 1.5 mm were positioned just above the lateral and basal amygdala (LBA) using coordinates 3 mm posterior to bregma, 7.2 mm ventral to skull surface, and 5.5 mm lateral to midline. The guide cannulae were fixed to screws in the skull using cranioplastic cement (Plastics One). After the cement hardened, internal cannulae were replaced with dummy cannulae, cut 0.5 mm longer than the guides, to prevent clogging. Rats were tested the following week after recovery.

Intracranial injections

Rats were held in the experimenter's lap while dummy cannulae were replaced with 28-gauge injector cannulae attached to 1.0 ml Hamilton syringes via polyurethane tubing. The tubing was back-filled with distilled water, and a small air bubble separated the water from the drug solution. The drug volume was 0.0003 ml, and was



infused bilaterally by an infusion pump at a rate of 0.05 $\mu\text{l}/\text{min}$. After drug infusion, cannulae were left in place for additional 3 min to allow diffusion of the drug away from the cannula tip, after which the dummy cannulae were replaced.

Apparatus

Fear conditioning took place in a Plexiglas rodent conditioning chamber with a metal grid floor (model E10-10; Coulbourn Instruments, Lehigh Valley, PA, USA), dimly illuminated by a single house light and enclosed within a sound-attenuating chamber (model E10-20). Testing for conditioned fear responses occurred in a brightly lit Plexiglas chamber with three house lights (ENV-001; Med Associates Inc., Georgia, VT, USA), fitted with a flat black Formica floor that had been washed with a peppermint-scented soap. Previous studies have shown that this distinct testing environment minimizes generalization from the training environment (Nader and LeDoux, 1999; Schafe et al., 1999). A video camera mounted at the top of the chamber recorded behavior for later scoring.

Habituation, conditioning, and testing

Figure 2 shows the behavior procedure. On day 1, rats received either muscimol (0.03 nmol/side in 0.0003 ml), TPMPA (30 nmol/side in 0.0003 ml) or ACSF vehicle (0.0003 ml) 60 min before training. All rats were habituated to the training and testing chambers for 10 min right before conditioning.

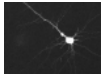
For training, rats were allowed 2–3 min to acclimate to the conditioning chamber and were then presented with three pairings of a 20-s tone CS (5 kHz, 75 dB) that co-terminated with a foot shock US (0.5 s, 0.7 mA). The intertrial interval varied randomly between 90 and 120 s. After drug infusion and conditioning, rats were returned to their home cages and to the colony.

Testing took place for STM and long-term memory (LTM). The STM test consisted of two CSs presentations 3 h after conditioning and the LTM test 18 CS presentations 24 h after conditioning. Rats were videotaped during testing for later scoring. After a 3-min acclimation period to the test chamber, rats were presented with 20 s tones (5 kHz, 75 dB). After tone testing, rats were returned to their home cages and to the colony. Fear memory was evaluated from the videotape by measuring the number of seconds during each tone presentation where rats engaged in freezing behavior, defined as a lack of all movement with the exception of respiration.

Data were analyzed with the unpaired Student's *t*-test, because two separate independent and identically distributed samples were obtained, where one from each of the two populations were compared. Measures were compared by ANOVA with *post hoc* testing where appropriate ($p < 0.05$).

Histology

To verify injector tip location, rats were anesthetized with an overdose of Nembutal (100 mg/kg, i.p.) and perfused transcardially with 0.9% NaCl followed by 10% buffered Formalin. Brains were postfixed in 10% buffered Formalin and subsequently blocked, sectioned on a cryostat at 50 μm . Sections were cover slipped with Permount and examined under light microscopy for injector tip penetration into the amygdala.



RESULTS

We recorded from 47 pyramidal cells across the LA. Input resistances of recorded neurons in patch clamp experiments ranged from 104.7 to 198.0 M Ω (mean 160.2 M Ω , SD 64.5), resting membrane potentials varied between -55.7 and -68.3 mV (mean -61.3 mV, SD 5.0).

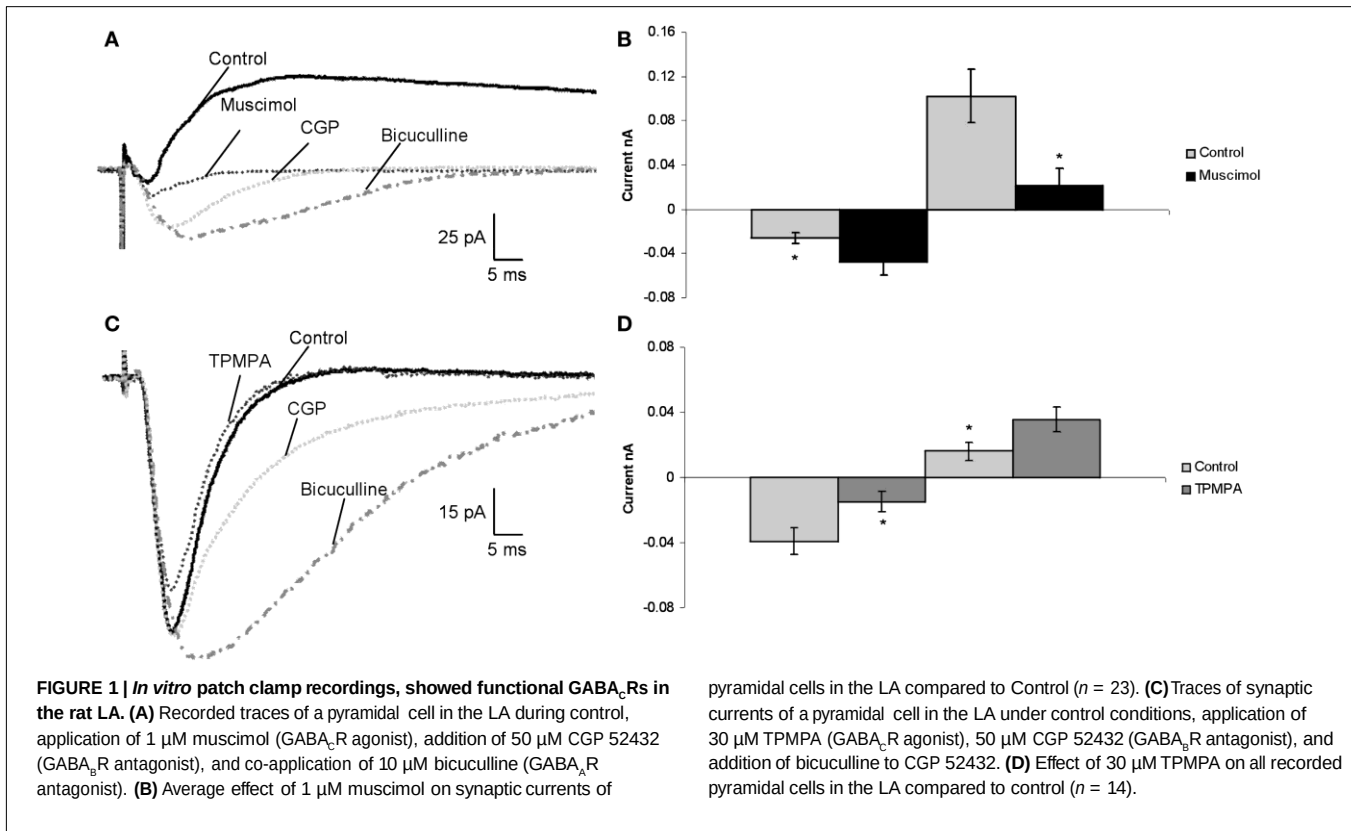
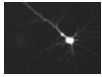
EFFECTS OF MUSCIMOL ON POSTSYNAPTIC CURRENTS

In the first experiments we used whole-cell patch clamp recordings in acute slices *in vitro* to test whether GABA_CRs are present and participate in synaptic transmission within the LA. To elicit post-synaptic currents, electric stimulation was applied to the cortical or thalamic pathway to mimic, *in vitro*, testing the effects of stimulation of sensory inputs known to occur during fear conditioning *in vivo* (Romanski et al., 1993; Repa et al., 2001). The effects of the 1 μ M muscimol, the GABA agonist, on the electrically evoked responses were assessed since this concentration has been reported to selectively activate GABA_CRs as opposed to GABA_ARs (Pasternack et al., 1999; Boller and Schmidt, 2001; Schmidt et al., 2001). To determine whether this 1 μ M muscimol primarily activated GABA_CRs we also examined the effects of blockade of GABA_ARs and GABA_BRs on the electrically evoked responses by adding the GABA_AR antagonist bicuculline and the GABA_BR antagonist CGP 52432 separately to the bath during recordings.

EPSCs and IPSCs, evoked through external and internal capsule stimulation, were recorded from 23 pyramidal cells. Application of 1 μ M muscimol, the concentration selective for activation of GABA_CRs, led to a decrease of IPSCs by $84 \pm 13.7\%$ ($p < 0.05$) and an increase in EPSCs of $90 \pm 21.5\%$ ($p < 0.05$) in all cells (**Figures 1A,B**). CGP 52432 the GABA_BR antagonist decreased inhibition by $82 \pm 9.9\%$ ($p < 0.05$) and increased excitation $75 \pm 7.1\%$ ($p < 0.05$) in all pyramidal cells. Co-application of bicuculline to 1 μ M muscimol and CGP blocked all the IPSCs (100%, $p < 0.05$) and the excitatory current increased by additional $330 \pm 82\%$ ($p < 0.05$; **Figure 1A**). These results suggest that muscimol, in a concentration that selectively activates GABA_CRs, reduced inhibition in the LA and increased excitation. This is the opposite of the effects of activating GABA_ARs and GABA_BRs, which, upon ligand activation, hyperpolarize pyramidal neurons and reduce excitation. Consistent with this, we found that GABA_ARs and GABA_BRs blockade led to reduction of evoked inhibition and increment of excitation.

EFFECTS OF GABA_CR ANTAGONISTS ON POSTSYNAPTIC CURRENTS

In the next set of experiments the effects of a blockade of GABA_CRs on the electrically evoked responses in pyramidal cells was examined ($n = 14$). The selective GABA_CR antagonist TPMPA was bath-applied and led to enhancement of inhibition: IPSC amplitudes were increased ($41 \pm 18.2\%$, $p < 0.05$), and EPSC amplitudes were reduced by $60 \pm 26.7\%$ ($p < 0.05$; **Figures 1C,D**). GABA_CRs are resistant to GABA_AR antagonist bicuculline and GABA_BRs antagonist CGP 52432. We added bicuculline and CGP 52432 to TPMPA to verify that the recorded impact of TPMPA applications were a GABA_CR related effect. The IPSCs were completely blocked ($100 \pm 0\%$) in all 14 pyramidal cells, leading to increased EPSC amplitudes ($350 \pm 75\%$, $p < 0.05$; **Figure 1C**), so all inhibition was extinguished. Blocking GABA_ARs and GABA_BRs had the opposite effect of blocking GABA_CRs.

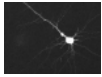


Next we verified that TPMPA application blocks the effect of muscimol. We applied just 1 μ M muscimol to the bath for 15 min, and after this we added TPMPA ($n = 10$). One micromolar muscimol led to a decrease of IPSCs by $86 \pm 11.5\%$ ($p < 0.05$) and an increase in EPSCs of $89 \pm 11.9\%$ ($p < 0.05$) in all cells. After the addition of TPMPA the effect of muscimol was completely blocked and IPSC amplitudes were increased ($71 \pm 10.6\%$, $p < 0.05$), and EPSC amplitudes were reduced by $84 \pm 23.9\%$ ($p < 0.05$).

In summary, our physiological studies show that blockade of GABA_cRs with TPMPA increases inhibition and their activation with 1 μ M muscimol leads to the opposite. If the GABA_cRs were located on the postsynaptic side, we would have expected for muscimol to increase inhibition and TPMPA enlarge inhibition. The fact that muscimol led to a decrease in inhibition and TPMPA to an increase in excitation suggests the possibility that the GABA_cRs are located on the presynaptic side. We next tested this hypothesis directly.

PAIRED PULSE DEPRESSION AND FACILITATION WAS AFFECTED BY MUSCIMOL AND TPMPA

Paired pulse depression (PPD) and facilitation (PPF) are tests of presynaptic effects. To examine whether GABA_cRs act on presynaptic sites, we used PPD and PPF of IPSCs and EPSCs by using an interstimulus interval of 50 ms. PPD of IPSCs is associated with decreased GABA release and a PPD of EPSCs with increased GABA release. In all 12 neurons recorded, TPMPA increased the PPD of EPSCs by $35.6 \pm 7.3\%$ ($p < 0.05$). Muscimol significantly increased the PPD ratio and of IPSCs by $42.5 \pm 5.2\%$ ($p < 0.05$).



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and increased the PPF ratio of EPSCs by $39.2 \pm 3.9\%$ ($p < 0.05$) (**Figure 2**). These data support our hypothesis that GABA_CRs could be located on the presynaptic sites of GABAergic interneurons in the LA. Therefore, the function of GABA_CRs seems to be opposite from GABA_ARs and GABA_BRs *in vitro*. Based on these results we next asked the question, if this function of GABA_CRs would affect learning and memory abilities in the living animal.

BEHAVIORAL EFFECTS OF GABA_CR ACTIVATION

We tested whether GABA_CRs participate in fear learning and memory using auditory Pavlovian fear conditioning. Because our electrophysiological results showed that direct activation of GABA_CRs reduced evoked inhibition and enhanced excitation in LA pyramidal neurons, we hypothesized that stimulation of GABA_CRs in the LA prior to fear conditioning should enhance the acquisition of fear memory formation. To test this hypothesis, rats were chronically implanted with bilateral cannulae in the LA and muscimol or vehicle (ACSF) were injected in the LA in separate groups of animals prior to fear conditioning. Based on a previous study (Wilensky et al., 1999), where 4.4 nm muscimol was used to activate GABA_ARs, we used a very weak concentration (0.03 nM) of muscimol 1 h before FC (**Figure 3**). As noted, GABA_CRs are at least 10-fold more sensitive to muscimol than GABA_ARs (Bormann, 2000), and our concentration was more than 100 times less than that used in previous studies to block fear learning. We tested STM 3 h after conditioning and LTM 24 h later. We analyzed pre-tone freezing; the average was $5 \pm 0.3\%$, and there was no significant difference between groups ($p > 0.05$).

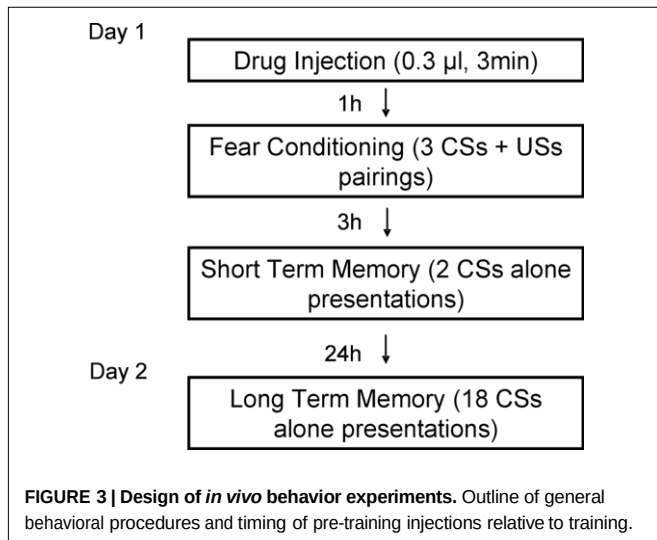
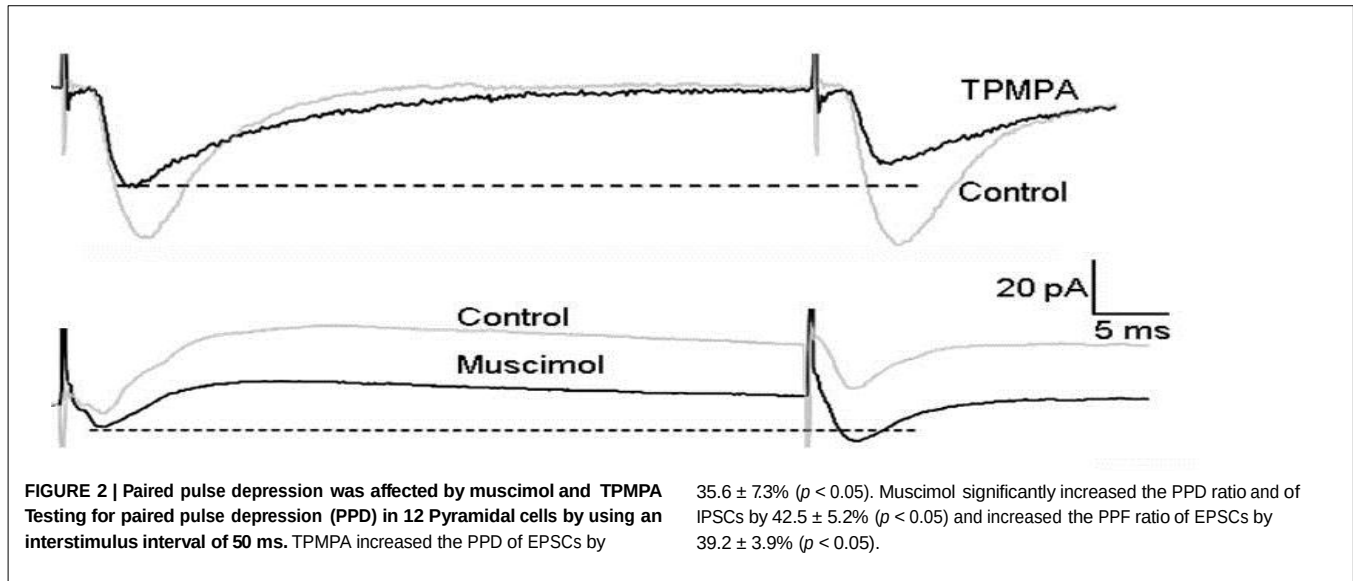
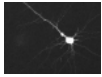


Figure 4A shows the mean $\pm$ SE percent freezing during the test tone presentations for rats injected before conditioning with 0.03 nM muscimol ($n = 6$) and saline ($n = 7$). The results show a significant difference between the saline and muscimol groups for FC ($p < 0.05$), STM ($p < 0.05$), and LTM ($p < 0.05$) (**Figures 4A–C**). Administration of 0.03 nM muscimol enhanced fear acquisition and consolidation.

BEHAVIORAL EFFECTS OF GABA_CR BLOCKADE

Because the electrophysiological data indicate that blocking GABA_CRs with TPMPA enhances evoked inhibition, we hypothesized that blocking GABA_CRs with TPMPA would impair fear learning and memory in rats. As before, bilateral cannulae injections of 30 nM TPMPA ($n = 7$) into the LA were performed 1 h before FC whereas the control group received intra-LA administration of ACSF ($n = 6$) and both STM and LTM were assessed. In **Figure 5A** we show mean $\pm$ SE percent freezing levels for TPMPA and control

treated animals, demonstrating that intra-LA microinjections of TPMPA significantly impaired fear learning and memory at STM ($p < 0.05$) and LTM ($p < 0.05$) time points (**Figures 5B–D**).

HISTOLOGY

Histological reconstruction of cannulae placements revealed that all injector tips were located in the LA in 26 out of 47 animals and were thus included in the analysis.

DISCUSSION

Our results indicate that GABA_CRs are involved in intra-LA neuronal communication by modulating a considerable fraction of postsynaptic currents. As justified below, we interpret this to suggest that GABA_CRs could be located on the presynaptic side on the axons of the interneurons and act as autoinhibitors to reduce synaptic GABA release. Infusion of GABA_CR agonists and antagonists in LA in conjunction with auditory Pavlovian fear conditioning showed that GABA_ARs and GABA_CRs play opposing roles in fear acquisition and consolidation: GABA_ARs impair and GABA_CRs enhance fear learning and memory. Our results demonstrate a novel role of GABA_CRs, which advances our understanding of the function of GABA_CRs in the brain, our knowledge of the circuitry of the LA, and the mechanisms by which fear memories are formed and stored.

IN VITRO PATCH CLAMP RECORDINGS

Previous studies noted the existence of GABA_CRs in the lateral part of the CE (Delaney and Sah, 1999). If GABA_CRs are also present in the LA, their activation would be expected to influence the amplitudes of postsynaptic currents that are evoked by electric stimulation. To test this we used coronal slices that included the LA since inhibitory responses of neurons are known to be stronger than in horizontal slices (Samson et al., 2003). Pyramidal cell afferents from the cortex and thalamus were stimulated electrically. This simultaneously elicits monosynaptic EPSCs, through direct activation of excitatory thalamic and cortical afferents onto LA pyramidal cells, and heterosynaptic through a direct stimulation, and IPSCs,

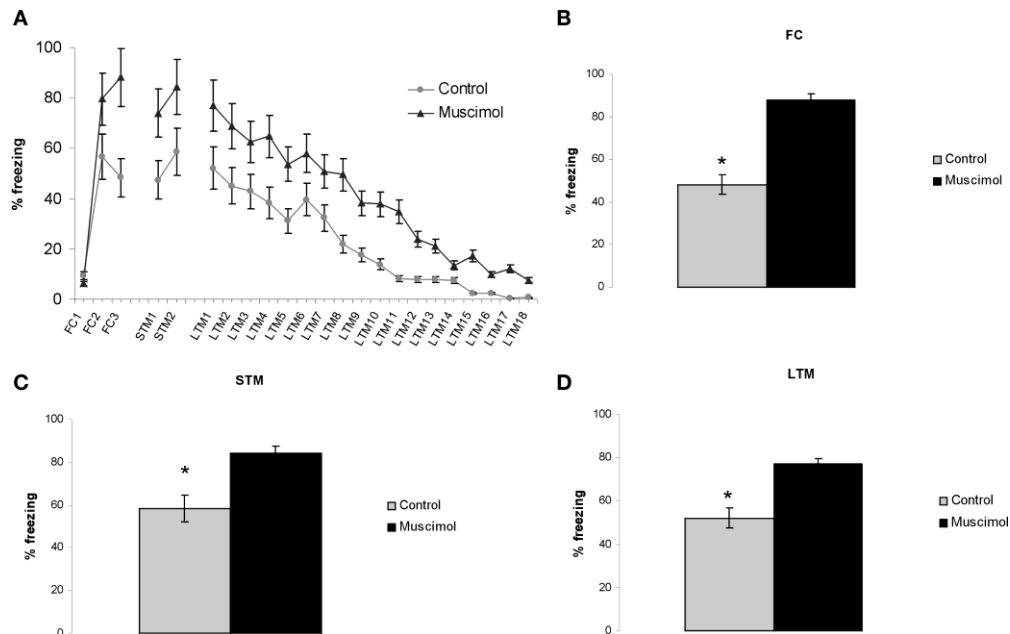
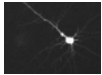


FIGURE 4 | Intracranial injections of 0.03 nM muscimol into the LA enhanced fear learning and memory. (A) Image shows the mean $\pm$ SE percent freezing during the test tone presentations for rats injected before conditioning with 0.03 nM muscimol ($n = 6$) and saline ($n = 7$). **(B–D)**

Diagrams show a significant difference between the control (vehicle injection) and muscimol groups for FC, fear conditioning; STM, short-term memory test; and LTM, long-term memory test ($p < 0.05$).

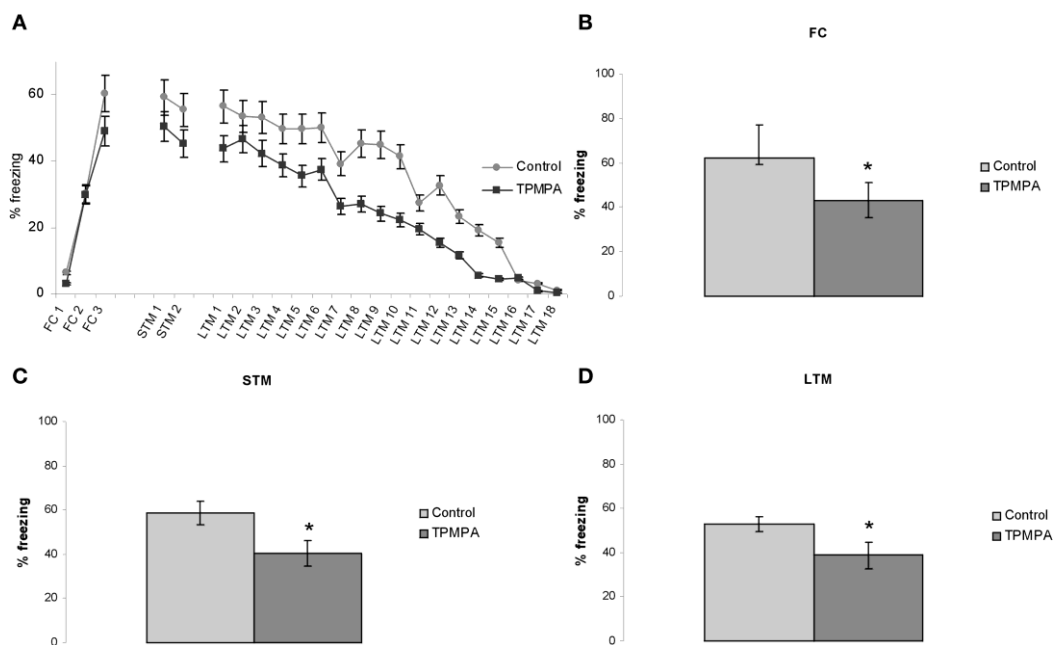
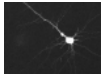


FIGURE 5 | Intracranial injections of 30 nM TPMPA into the LA impaired fear learning and memory. (A) Whole experiment with mean $\pm$ SE percent freezing levels for injections of 30 nM TPMPA ($n = 7$) and control ($n = 6$). **(B–D)** Diagrams show TPMPA impaired fear learning for FC, fear conditioning; STM, short-term memory test; and LTM, long-term memory test ($p < 0.05$).

through indirect afferent activation of GABAergic interneurons. An increase of the excitatory amplitudes of the postsynaptic currents in the presence of muscimol at low concentrations (see above) was regarded as an indication of the expression of GABA_CRs by the presynaptic interneurons to the pyramidal cells. In addition, these results demonstrate that GABA_CRs activation reduces feed forward



or feedback inhibition and enhances excitatory transmission. Such an effect of muscimol was observed in all of the recorded pyramidal cells.

To answer the question of whether endogenous activation of GABA_CRs contributes to information processing within the LA, the specific GABA_CR blocker TPMPA (Ragozzino et al., 1996; Bormann, 2000) was applied and the effects on evoked postsynaptic responses of pyramidal cells were investigated. If the GABA_CRs are located on the presynaptic interneurons their blockade should increase inhibition of the pyramidal cells due to higher GABA release. This effect was registered in all 14 pyramidal cells we recorded from.

Next we examined PPD and PPF of IPSCs and EPSCs in the presence of TPMPA, and separately muscimol (**Figure 2**). TPMPA blocked the GABA_CRs on the presynaptic side so that during the second pulse GABA was still being released. The second pulse showed a stronger reduction of excitation, which was recorded as smaller EPSCs (PPD). On the other hand, muscimol application activated the presynaptic GABA_CRs. During the second pulse the IPSC amplitudes were decreased (PPD) and EPSCs increased (PPF). This was a result of suppressed GABA release.

These results lead us to the conclusion that GABA_CRs could be located on the presynaptic side. We propose in our model that GABA_CRs would act as autoinhibitors on interneurons (**Figure 6**). A similar function for GABA_CRs was described in the retina, where GABA_CRs are expressed predominantly at the bipolar cell terminals wherefrom they mediate feedback inhibition from amacrine cells (Lukasiewicz and Werblin, 1994; Vaquero and de la Villa, 1999; Euler and Masland, 2000; Shields et al., 2000). In general inhibition mediated by GABA_CRs is slower compared to the kinetics of GABA_ARs, which induce much faster responses to GABA, suppress

glutamate release more rapidly and transiently (Pan and Lipton, 1995). The presence of the three receptor types on the synapse leads to a much larger dynamic range in the overall response to GABA than each subtype alone.

In our model muscimol or GABA would activate the GABA_CRs on the axons of the interneurons, which would lead to less or no GABA release that would inhibit the Pyramidal cells. Application of TPMPA would block the presynaptic GABA_CRs, the axon would release GABA which activates the GABA_ARs and GABA_BRs that inhibit the Pyramidal cells.

IN VIVO AUDITORY PAVLOVIAN FEAR CONDITIONING

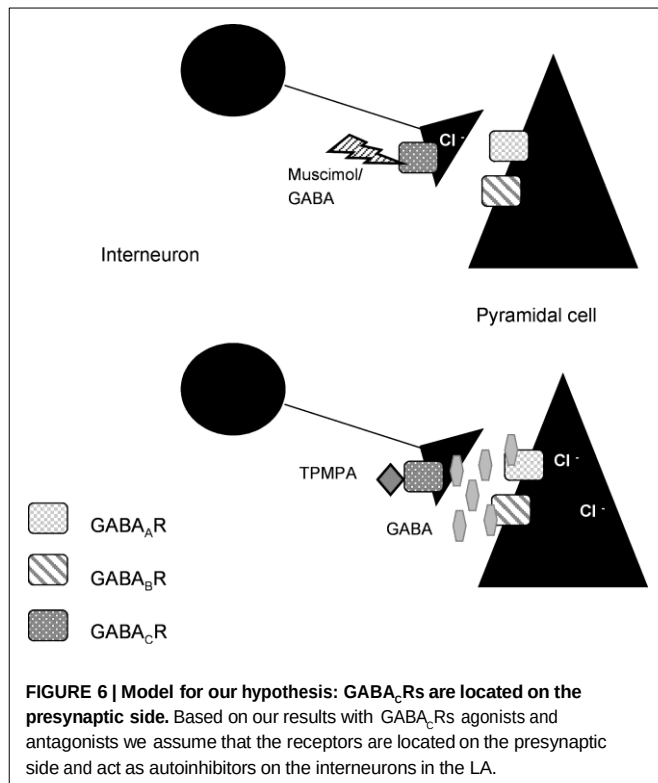
LA is a key site of plasticity underlying fear learning (LeDoux, 2000; Blair et al., 2001; Maren and Quirk, 2004). It is known that inhibitory circuits in LA and related amygdala areas play an important role in fear memory and its extinction (Wilensky et al., 1999; Akirav and Richter-Levin, 2006; Zhang and Cranney, 2008). Most studies have focused on GABA_ARs, and to a lesser degree and GABA_BRs. GABA_ARs have a very large chloride channel (Farrant and Nusser, 2005), and drugs targeting this receptor produce strong cellular hyper polarization and have a profound impact on processing in neural circuits.

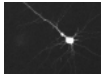
Our physiological results in coronal slices suggest that GABA_CRs could be present in the LA on the presynaptic terminals of inhibitory inputs onto pyramidal cells, and act as autoinhibitors on the interneurons. If our interpretation is accurate, their activation should also influence fear learning and memory.

To test this we examined the effects of intra-LA infusion of a GABA_CR agonist and antagonist on auditory Pavlovian fear conditioning. Previously 4.4 nM muscimol was used as a GABA_AR agonist (Wilensky et al., 1999). It is known that GABA_CR are at least 10-times more sensitive to GABA and muscimol than GABA_ARs (Bormann, 2000). We used 0.03 nM muscimol as a GABA_CR agonist, and found a significant difference to the control (**Figure 4**) for FC, STM, and LTM. We found that activation of GABA_CRs with low concentrations of muscimol enhanced fear learning and memory and that blocking GABA_CRs with TPMPA produced the opposite effect (**Figure 5**). These results showed two things: (1) GABA_CRs modulates fear acquisition and consolidation; and (2) the role of GABA_CRs is opposite to GABA_ARs: their activation enhances fear learning and memory.

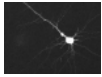
IMPLICATIONS FOR ANXIETY DISORDERS

GABAergic agonists (e.g., benzodiazepines), are commonly used to treat anxiety-related disorders, especially by targeting GABA_ARs. Yet, beyond their potent anxiolytic properties, these drugs also lead to side effects that include sedation, motor and memory impairments. These side effects are, in part, due to the fact that GABA_ARs are ubiquitously distributed throughout the mammalian brain. Another problem is that these drugs often lead to dependency. A third problem is that GABA_ARs desensitize, possibly explaining why patients with generalized anxiety disorder or panic disorder show lower benzodiazepine binding in some forebrain areas (Kaschka et al., 1995; Tiitonen et al., 1997; Malizia et al., 1998). Due to the non-specific effects of benzodiazepines, their potential for dependence, and their tendency to





desensitize, GABA_ARs are not optimal as a target for long-term treatment of patients with anxiety disorders. As a result, there has been a continued search for new, more specific, anxiolytic agents, either by indirect modulation of GABA_ARs via targeting norepinephrine (NE), serotonin, and dopamine, or by research aimed at altering specific GABA_ARs subunits. Given that amygdala processing is altered in anxiety disorders (LeDoux, 2007; Monk, 2008), our results suggest that GABA_ARs in the amygdala might be a useful alternative target for the development of anti-anxiety drugs.

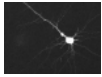


CONCLUSION

The present results expand our understanding of the role of GABA receptors in fear learning, and suggest possible ways to improve the treatment of anxiety-related disorders. However further studies are needed to fully understand the role of GABA_CRs in the amygdala, where neuromodulators like NE play crucial roles in synaptic plasticity and learning and memory (Cahill et al., 1994; McGaugh, 2000; Debiec and LeDoux, 2004). The GABA_ARs antagonist, picro-toxin, and high dosages of muscimol, which target GABA_ARs, are known to modulate the NE levels in the amygdala (Hatfield et al., 1999). It would be very important to know if and how GABA_CRs agonists and antagonists influence NE and other neuromodulator concentrations in the LA.

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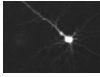
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Chapter 3

Antagonism of lateral alpha1- adrenergic receptors facilitates fear conditioning and long-term potentiation

In the subsequent work, Catarina Cunha made the following contributions:
Designed the electrophysiological experiments
Tested drug concentrations for electrophysiological experiments
Collected, analyzed and interpreted electrophysiology recordings
Assisted in writing the paper



Antagonism of lateral amygdala alpha1-adrenergic receptors facilitates fear conditioning and long-term potentiation

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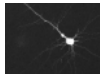
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Brief Communication

Antagonism of lateral amygdala alpha1-adrenergic receptors facilitates fear conditioning and long-term potentiation

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Norepinephrine receptors have been studied in emotion, memory, and attention. However, the role of alpha1-adrenergic receptors in fear conditioning, a major model of emotional learning, is poorly understood. We examined the effect of terazosin, an alpha1-adrenergic receptor antagonist, on cued fear conditioning. Systemic or intra-lateral amygdala terazosin delivered before conditioning enhanced short- and long-term memory. Terazosin delivered after conditioning did not affect consolidation. *In vitro*, terazosin impaired lateral amygdala inhibitory postsynaptic currents leading to facilitation of excitatory postsynaptic currents and long-term potentiation. Since alpha1 blockers are prescribed for hypertension and post-traumatic stress disorder, these results may have important clinical implications.

Although norepinephrine (NE) has been widely studied as an important modulator of memory and emotion, comparatively little is known about the role of NE in amygdala-dependent Pavlovian fear conditioning, a major model for understanding the neural basis of fear learning and memory. In fear conditioning, an emotionally neutral conditioned stimulus (CS; i.e., tone) is temporally paired with an aversive unconditioned stimulus (US; i.e., footshock). After very few pairings, a lasting, robust CS–US association is acquired, and the CS elicits stereotypical defensive responses, including behavioral freezing (Blanchard and Blanchard 1969; Bolles and Fanselow 1980).

The lateral nucleus of the amygdala (LA) is a key structure underlying fear conditioning (LeDoux 2000). Convergence of CS and US information in LA is believed to play an important role in initiating synaptic plasticity. Long-term potentiation (LTP)-like changes in LA CS processing are critical for fear memory storage (LeDoux 2000; Blair et al. 2001; Maren 2001; Walker and Davis 2002). LA receives auditory CS inputs from the thalamus and cortex and connects directly and indirectly with the central nucleus of the amygdala to control expression of Pavlovian fear responses.

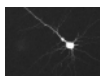
Of the noradrenergic receptor subtypes, alpha1 receptors have received the least attention in fear conditioning. LA receives NE-containing inputs from the locus coeruleus that fire tonically and phasically in response to aversive stimuli like footshock (Pitkänen 2000; Tanaka et al. 2000; Aston-Jones and Cohen 2005). Alpha1-adrenergic receptors are expressed in LA, most likely on both excitatory and inhibitory neurons (Jones et al. 1985; Domyancic and Morilak 1997). Alpha1 receptor activation stimulates GABA-mediated miniature inhibitory postsynaptic currents in LA (Braga et al. 2004), suggesting that alpha1 receptors contribute to inhibition in fear conditioning. elegantly demonstrated that LA inhibition gates synaptic plasticity necessary for fear conditioning, and this inhibitory gate can be influenced by

neuromodulators including NE (Stutzmann and LeDoux 1999; Shumyatsky et al. 2002; Bissière et al. 2003; Shaban et al. 2006; Shin et al. 2006; Tully et al. 2007). However, the role of alpha1 receptor activity in gating amygdala LTP and fear learning has never been examined.

We hypothesized that alpha1 blockers would facilitate fear learning, possibly by impairing LA inhibition and facilitating LA LTP. To test this hypothesis, we injected rats with terazosin, a selective alpha1-adrenergic receptor antagonist, systemically or directly into LA before or after fear conditioning. We examined *in vitro* the effect of terazosin on LA pyramidal cell inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) in response to fiber stimulation of the thalamic CS input pathway to LA, as well as the effect of terazosin on LA LTP in this same pathway. We found that intra-LA terazosin facilitated fear conditioning when injected before but not after training. We also found that terazosin impaired IPSCs in LA pyramidal cells, leading to facilitated EPSCs and LTP.

Behavioral experiments were conducted on adult, male Sprague–Dawley rats (Hilltop Laboratory Animals) weighing approximately 300 g upon arrival. Rats were individually housed, maintained on a 12/12 h light/dark schedule, and allowed free access to food and water. Testing was conducted during the light phase. All procedures and experiments were approved by NYU's Animal Care and Use Committee.

For systemic injections, terazosin (20 mg/kg; Sigma) was dissolved in saline and injected intraperitoneally (i.p.) 30 min prior to conditioning in 1 mL/kg volume. For bilateral infusions, terazosin (125 ng/0.25 mL) was dissolved in aCSF and infused into the LA at 0.1 mL/min 30 min prior to or immediately after fear conditioning. Bilateral guide cannulae (22 gauge; Plastics One) aimed at LA (23.3 mm anterior, 5.2 mm lateral, 2.7 mm dorsal to bregma) were surgically implanted as previously described (Sotres-Bayon et al. 2009). Rats were given at least 7 d to recover from surgery before testing. For infusions, dummy cannulae were



Role of inhibition in emotional learning

removed, and infusion cannulae (28 gauge, +1 mm beyond guides) were inserted into guides. Infusion cannulae were connected to a 1.0 mL Hamilton syringe via polyethylene tubing. Infusion rate was controlled by a pump (PHD22/2000; Harvard Apparatus), and infusion cannulae were left in place for an additional 60 sec to allow diffusion of the solution away from the cannula tip, then dummy cannulae were replaced. Upon completion of the experiment, rats were euthanized, brains removed, and cannulae placements verified histologically as previously described (Sotres-Bayon et al. 2009).

Two contexts (A and B) were used for all testing as previously described (Schiller et al. 2008). The grid floors in Context B were covered with black Plexiglas inserts to reduce generalization. No odors were used and chambers were cleaned between sessions. CSs were 30 sec, 5 kHz, 80 dB tones, and USs were 1 sec, 0.8 mA scrambled electric footshocks. Experiments consisted of two phases separated by 48 h: (1) fear conditioning in Context A and (2) long-term memory (LTM) test in Context B. On Day 1, rats were placed in Context A, allowed 5 min to acclimate, and then received three CS-US pairings separated by variable 5 min ITIs. On Day 3, rats were placed in Context B and allowed 5 min to acclimate before receiving one CS-alone presentation.

The index of fear in behavioral experiments was "freezing," the absence of all non-respiratory movement (Blanchard and Blanchard 1971; Fanselow 1980). Following testing, freezing was manually scored from DVDs by a scorer blind to group specification. Graphs represent group means $\pm$ SEM. Statistical analysis was conducted with GraphPad Prism.

Whole-cell electrophysiological recordings were obtained from LA pyramidal cells using in vitro coronal slices from rats aged P21–P30 d as described in Cunha et al. (2010). Terazosin was bath-applied for 10 min to achieve stable responses before testing. The cells were voltage-clamped using an Axopatch 200B amplifier at 235 mV for recording EPSCs and IPSCs. Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes. Internal capsule fibers containing thalamic afferents were stimulated for paired-pulse facilitation (PPF) (ISI $\frac{1}{4}$ 50 msec; 0.1 Hz) using a photoelectric stimulus isolation unit with a constant current output. Cells were rejected if access resistance (8–26 MV) changed more than 15%. Signals were filtered at 2 kHz and digitized (Digidata 1440 A; Axon Instruments), and peak amplitude, 10%–90% rise time, and IPSC decay time constants were analyzed offline using pCLAMP10.2 software (Axon Instruments).

Brain slices for LTP experiments were prepared from rats aged 3–5 wk as in Johnson et al. (2008) and maintained on an interface chamber at 31°C. Glass recording electrodes (filled with aCSF, 5 MV resistance) were guided to LA neurons. Bipolar stainless steel stimulating electrodes (75 kV) were positioned medial to LA in internal capsule fibers. Orthodromic synaptic potentials were evoked via an isolated current generator (Digitimer; 100 msec pulses of 0.3–0.7 mA). Evoked field potentials were recorded with an Axoclamp 2B amplifier and Axon WCP software (Axon Instruments). Data were analyzed offline using WCP PeakFit (Axon Instruments). LTP was measured as a change in evoked field potential amplitude.

Baseline responses were monitored at 0.05 Hz for 30 min with a stimulus intensity of 40%–50% of maximum fEPSP before LTP induction. Terazosin (10 mM) was superfused for 15 min, and then LTP was elicited by three tetanus trains (100 Hz $\times$ 1 sec, 3 min ITI) with the same intensity and pulse duration as the baseline stimuli. In one experiment, picrotoxin (PTX; 75 mM) was present in the perfusion solution to block fast GABAergic signaling.

Terazosin facilitates acquisition, but not consolidation, of fear conditioning

We first examined the effect of terazosin, injected systemically 30 min before training on fear learning and memory (Fig. 1A). Learning and short-term memory effects were assessed as differences in freezing during the second and third CSs of the three trial acquisition sessions. LTM effects were assessed as differences in freezing during the drug-free expression test CS 48 h after training. Although systemic terazosin caused sedation and reduced locomotion in some rats, it also increased CS-elicited freezing relative to controls, suggesting a learning facilitation. Freezing was distinguished from sedation by differences in posture and whisker tension. Two-way ANOVA of freezing scores during training revealed a significant effect of the group $\times$ trial interaction ($F_{(2,34)} = 3.06$, $P > 0.05$), and post-hoc Bonferroni tests showed a significant difference in freezing for the second ($P > 0.01$) and third ($P > 0.01$) CSs. Freezing was significantly elevated in terazosin-treated rats during the drug-free LTM test ($t_{(1,17)} = 3.09$, $P > 0.01$).

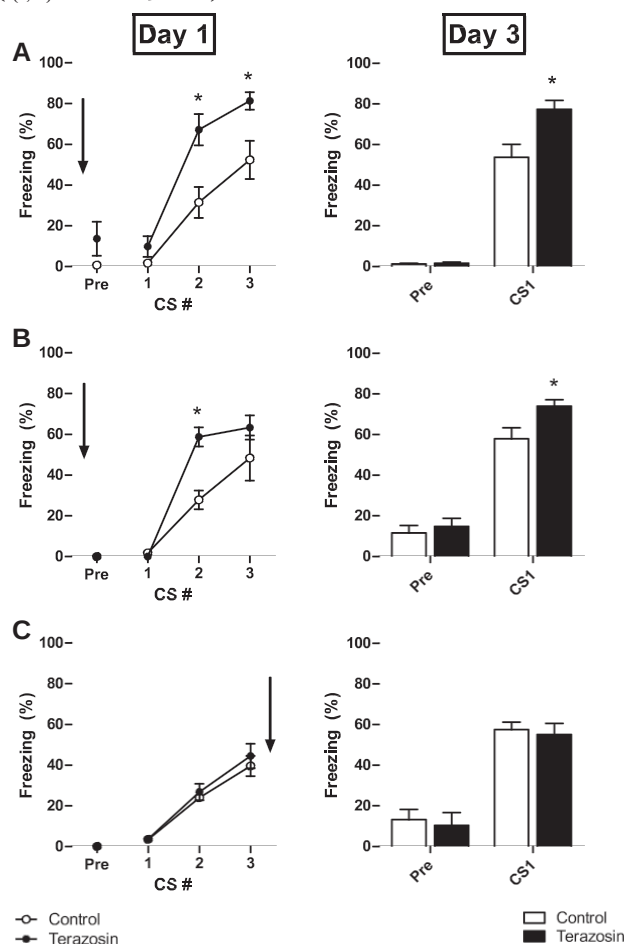
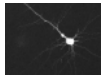


Figure 1. Pre-training terazosin facilitates conditioned fear learning when injected systemically or directly into LA. (A) Pre-CS- and CS-elicited freezing behavior during fear conditioning (left) and during a test CS 48 h after fear conditioning (right). Terazosin ($n = 10$) or saline ($n = 9$) was injected i.p. 30 min before conditioning. (B) Identical experiment except terazosin ($n = 8$) or aCSF ($n = 7$) was microinfused into LA 30 min prior to fear conditioning. (C) Identical experiment except terazosin ($n = 6$) or aCSF ($n = 6$) was microinfused into LA immediately after fear conditioning. Arrows indicate when drug treatment occurred. (*) $P > 0.05$ versus control.



We next infused terazosin directly into LA prior to fear conditioning to localize the drug effect and minimize concerns about nonspecific effects (sedation) associated with systemic injections. Intra-LA terazosin facilitated fear learning and LTM relative to control rats (Fig. 1B). Two-way ANOVA of freezing scores during training revealed a significant group $\times$ trial interaction effect ($F_{(2,26)} = 4.73$, $P < 0.05$), and post-hoc Bonferroni tests showed a significant difference in freezing for the second CS ($P < 0.01$). For the LTM test, intra-LA terazosin-treated rats froze more than control rats ($t_{(1,9)} = 2.556$, $P < 0.05$).

To differentiate between potential learning versus memory consolidation effects, we infused terazosin into LA immediately after fear conditioning in separate rats (Fig. 1C). Freezing was similar for terazosin- or vehicle-treated rats at the LTM test ($t_{(1,8)} = 0.37$), suggesting that terazosin facilitates learning, but not consolidation, of fear conditioning.

Terazosin facilitates LTP in LA, most likely by impairing local inhibition

We first evaluated the effect of terazosin on thalamo-LA EPSCs and IPSCs. Synaptic current traces of an LA pyramidal cell ($n = 11$) under applied terazosin and control conditions are shown in Figure 2A. An application of 10 μ M terazosin significantly decreased IPSCs $80.7\% \pm 17.2\%$ ($p < 0.013$) and led to PPF of EPSCs $64.3\% \pm 14.5\%$ ($p < 0.006$).

We next evaluated the effect of terazosin on thalamo-LA LTP induction with and without PTX. Without PTX, terazosin

facilitated LTP (Fig. 2B). Two-way ANOVA of evoked responses post-LTP-induction showed a significant effect of the group $\times$ time interaction ($F_{(29,319)} = 5.97$, $P < 0.01$) and time ($F_{(29,319)} = 1.71$, $P < 0.05$). With PTX present (Fig. 2C), terazosin slightly impaired LTP, again showing a significant effect of interaction ($F_{(29,290)} = 1.63$, $P < 0.05$) and time ($F_{(29,290)} = 1.82$, $P < 0.01$). We also conducted identical LTP experiments in the cortex $\rightarrow$ LA pathway (stimulating external capsule), and terazosin failed to facilitate LTP without PTX and produced a modest impairment with PTX (data not shown). This null effect in the cortical pathway highlights the importance of the thalamic pathway in fear conditioning and suggests that α_1 receptors mainly constrain thalamo-LA plasticity underlying memory formation.

Noradrenergic modulation of memory and anxiety has been studied for decades (Gray 1978; Davis et al. 1979; Redmond and Huang 1979). However, less is known about the role of NE in Pavlovian fear conditioning, a major model for understanding the biological basis of memory and generation of normal and pathological fear. Of the major noradrenergic receptor classes, α_1 receptors have received less attention in studies of memory and fear than both the beta and α_2 receptors. Because α_1 receptors are strongly expressed in LA, a key region of plasticity in fear conditioning, we chose to examine the effects of terazosin on amygdala-dependent Pavlovian fear conditioning and its underlying synaptic processes. We found that systemic and intra-LA terazosin significantly enhanced fear learning without affecting memory consolidation. Terazosin also facilitated synaptic plasticity in an important LA fear conditioning pathway, most likely by impairing local GABAergic inhibition. These findings provide important insight into the neuromodulatory mechanisms affecting fear learning and may have important clinical implications.

We initially investigated the role of α_1 receptors in fear conditioning using the α_1 blocker prazosin injected systemically before training (Cain et al. 2006). Prazosin enhanced CS-elicited freezing within the training session and during the drug-free LTM test, suggesting that α_1 receptors contribute to a constraining process in fear conditioning. We switched to terazosin in these studies because it is approximately 25 times more soluble than prazosin and more selective for the α_1 receptors, although slightly less potent (Achari and Laddu 1992). Thus, terazosin was better for investigating α_1 contributions to brain processes important for fear conditioning. Like prazosin, we found that systemic terazosin facilitated CS-elicited freezing both within the training session and the drug-free LTM test (Fig. 1A). It is important to note that terazosin at the dose tested (20 mg/kg) did appear to induce sedation in some rats, possibly confounding within-session freezing scores.

To localize terazosin effects to a brain site and circumvent concerns about nonspecific systemic drug effects, we infused terazosin directly into LA before fear conditioning. Again, terazosin enhanced CS-elicited freezing during

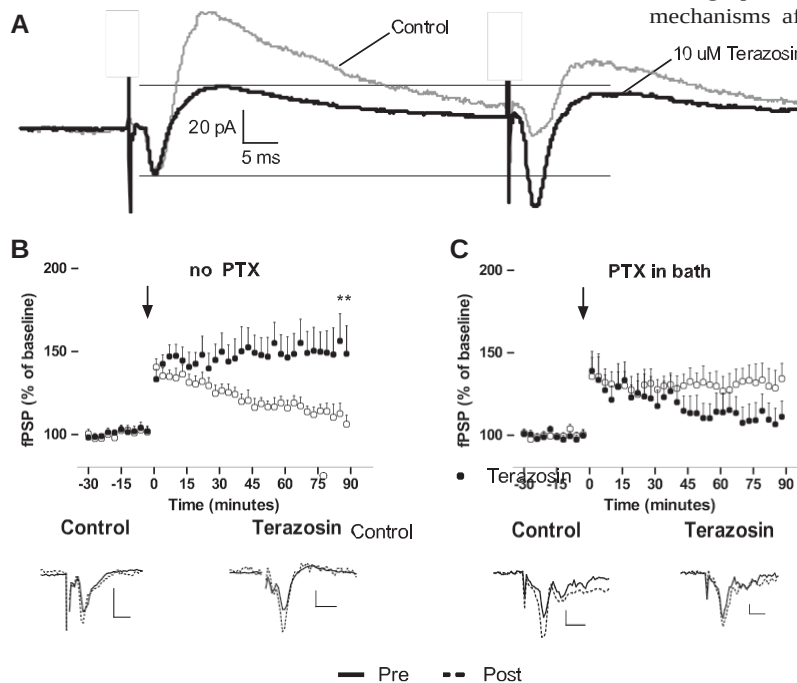
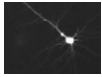


Figure 2. Terazosin impairs inhibition and facilitates LTP in LA. (A) Whole-cell recordings of LA pyramidal neurons in response to paired-pulse internal capsule stimulation. Eleven cells were examined, first in aCSF only, then after an application of terazosin (10 μ M). (B) LA field potential recordings in response to tetanic stimulation (three 100 Hz trains, 5 min ITI) of the internal capsule in control ($n = 6$) and terazosin-treated ($n = 7$) slices. (C) Identical experiment as in B with PTX (75 mM) added to the bath to remove GABAergic inhibition (control: $n = 6$; terazosin: $n = 6$). The arrow denotes terazosin application. Example traces show the mean response during the last 10 min of baseline (black line) versus the last 10 min of post-tetanic recording (dotted line). Scale bars: $x \times 5$ msec, $y \times 0.3$ mV. (*) $P < 0.05$ versus control.



training and during the drug-free LTM test (Fig. 1B). There were no signs of sedation with intra-amygdala infusions. The within-session enhancement of freezing suggests that LA alpha1 receptors normally act to constrain learning-related processes. NE, however, has been strongly implicated in consolidation processes with other fear-related behavioral paradigms such as inhibitory avoidance (Ferry and McGaugh 2008). Thus, we directly examined the contribution of LA alpha1 receptors to consolidation of fear conditioning by infusing terazosin immediately after training (Fig. 1C). We found no significant effect on CS-elicited freezing during the LTM test. Together, our findings implicate LA alpha1 receptors in a process that constrains acquisition, but not consolidation, of Pavlovian fear conditioning.

Although alpha1 receptors have never been directly examined in a cue fear conditioning procedure, amygdala-alpha1 receptor activity has been implicated in consolidation of inhibitory avoidance (McGaugh 2004). We do not believe that our findings contradict the avoidance findings. While the two procedures are related, they represent different forms of learning that depend on different brain regions. Fear conditioning is a Pavlovian procedure in which animals learn associations between environmental stimuli but have no control over their delivery. Inhibitory avoidance has an instrumental component—the animal withholds responses that lead to shock delivery. Additionally, whereas Pavlovian conditioning critically depends on LA for learning and memory, inhibitory avoidance appears to depend on LA mainly for modulation of consolidation processes taking place outside of the amygdala (McGaugh et al. 2002). It remains possible that LA alpha1 receptors can contribute both to acquisition of Pavlovian fear conditioning and consolidation of inhibitory avoidance. Future studies will be necessary to disentangle the exact contribution of LA alpha1 receptors to these two fear-related learning paradigms. One possibility is that alpha1 receptors on different neuron types (excitatory vs. inhibitory) or different populations of excitatory neurons explains the differential effects of alpha1 blockers in the two paradigms. This dissociation of noradrenergic contributions to fear conditioning and inhibitory avoidance is also seen with manipulations of beta-adrenergic receptors, where the antagonist propranolol blocks consolidation of inhibitory avoidance (McGaugh et al. 2002) but not fear conditioning (Lee et al. 2001; Debiec and LeDoux 2004; Bush et al. 2010).

Two ways NE may modulate fear acquisition have been identified. First, NE may amplify Hebbian mechanisms within pyramidal neurons that acquire plastic changes underlying conditioning (Bailey et al. 2000; Hu et al. 2007). Second, NE may suppress feed-forward inhibition, effectively lowering the depolarization threshold in postsynaptic pyramidal cells necessary for inducing learning-related plasticity (Tully et al. 2007; Ehrlich et al. 2009). LA alpha1-adrenergic receptors appear to be expressed on both excitatory pyramidal neurons and local GABAergic inhibitory neurons and thus may be positioned to affect fear conditioning in either manner. Although our behavioral findings seem more consistent with the second mechanism, we directly examined the effect of terazosin on LA synaptic currents and LTP *in vitro* to relate our behavioral findings to synaptic processes in a known fear conditioning pathway.

Electrophysiological recordings were made in LA in response to stimulation of the internal capsule, a fiber pathway known to carry thalamic sensory afferents relaying auditory CS information (Weiskopf and LeDoux 1999). NE-containing locus coeruleus fibers have been shown to reach the amygdala via the internal capsule (Jones and Moore 1977) and likely release NE during stimulation, especially high-frequency stimulation (Bailey et al. 2000). Whole-cell voltage clamp recordings indicated that terazosin impaired IPSCs leading to enhanced EPSCs with paired stimulation. Using

field potential recordings in the same pathway, we found that terazosin led to LTP with a tetanic stimulation protocol that produced only short-term potentiation in control slices. Finally, we conducted an identical LTP experiment, except that PTX was included in the bath to block GABAergic inhibition. With PTX present, terazosin failed to facilitate LA LTP and instead produced a modest impairment. Identical experiments in the cortex $\nrightarrow$ LA pathway (stimulating external capsule) failed to show LTP facilitation with terazosin (data not shown). Together, these findings are consistent with the hypothesis that the predominant role of LA alpha1-adrenergic receptor activity is to enhance feed-forward inhibition in the thalamic pathway and constrain plasticity related to fear conditioning. Consistent with our findings, alpha1 receptor activity has previously been reported to participate in feed-forward inhibition in LA (Braga et al. 2004). Our PTX experiment suggests that alpha1 receptors on pyramidal cells may actually enhance Hebbian LTP processes, but under normal conditions this contribution appears to be overshadowed by their contribution to inhibitory gating.

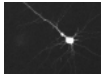
Our results suggest that alpha1-adrenergic receptor activity in LA contributes to feed-forward inhibition and constrains fear learning. Alpha1-adrenergic receptor antagonists, like prazosin and terazosin, appear to disinhibit plasticity in LA fear conditioning pathways even when given systemically. These results demonstrate another mechanism whereby NE neuromodulation can powerfully affect learning and fear. These same alpha1 blockers are commonly prescribed to combat both hypertension and nightmares associated with PTSD. Our findings suggest that clinicians should be especially aware of patients subjected to trauma while taking alpha1 blockers, as this may lead to stronger fear learning and possible exacerbation of existing fear-related disorders.

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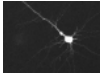
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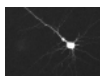


Chapter 4

Improved expression of halorhodopsin for light-induced silencing of neural activity

In the subsequent work, Catarina Cunha made the following contributions:

- Characterized expression patterns of the transgenic mice
- Designed the electrophysiological experiments
- Collected, analyzed and interpreted electrophysiology recordings
- Assisted in writing the paper



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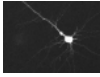
Brain Cell Biol. 2008 August; 36(1-4): 141–154. doi:10.1007/s11068-008-9034-7.

Improved expression of halorhodopsin for light-induced silencing of neuronal activity**Shengli Zhao¹, Catarina Cunha^{1,2}, Feng Zhang³, Qun Liu⁴, Bernd Gloss⁴, Karl Deisseroth³, George J. Augustine¹, and Guoping Feng^{1,4}**¹Department of Neurobiology, Box 3209, Duke University Medical Center, Durham, NC 27710, USA²Faculdade de Ciências da Universidade do Porto, Porto, Portugal³Department of Bioengineering, Stanford University, CA 94305, USA⁴Duke Neurotransgenic Laboratory, Box 3209, Duke University Medical Center, Durham, NC 27710, USA**Abstract**

The ability to control and manipulate neuronal activity within an intact mammalian brain is of key importance for mapping functional connectivity and for dissecting the neural circuitry underlying behaviors. We have previously generated transgenic mice that express channelrhodopsin-2 for light-induced activation of neurons and mapping of neural circuits. Here we describe transgenic mice that express halorhodopsin (NpHR), a light-driven chloride pump, that can be used to silence neuronal activity via light. Using the Thy-1 promoter to target NpHR expression to neurons, we found that neurons in these mice expressed high levels of NpHR-YFP and that illumination of cortical pyramidal neurons expressing NpHR-YFP led to rapid, reversible photoinhibition of action potential firing in these cells. However, NpHR-YFP expression led to the formation of numerous intracellular blebs, which may disrupt neuronal function. Labeling of various subcellular markers indicated that the blebs arise from retention of NpHR-YFP in the endoplasmic reticulum. By improving the signal peptide sequence and adding an ER export signal to NpHR-YFP, we eliminated the formation of blebs and dramatically increased the membrane expression of NpHR-YFP. Thus, the improved version of NpHR should serve as an excellent tool for neuronal silencing *in vitro* and *in vivo*.

Introduction

The complex and diverse functions of the brain depend on the unique properties of neural circuits formed by various subtypes of neurons with distinct molecular and electrical properties. Furthermore, many neurological disorders are often due to the dysfunction of specific subsets of neurons or neural circuits. Thus, elucidating the unique roles of each subtype of neuron in shaping circuitry function is critical to our understanding of both normal and abnormal brain function. This effort has been greatly facilitated by the recent development of optogenetic approaches for high-speed, light-induced activation or silencing of neurons through the use of light-sensitive, cation permeable channelrhodopsin-2 (ChR2) and the light-driven chloride pump halorhodopsin (NpHR) (Lanyi, 1990; Nagel et al., 2003; Boyden et al., 2005; Li et al., 2005; Bi et al., 2006; Zhang et al., 2006, 2007a,b; Han and Boyden, 2007; Petreanu et al., 2007; Zhang and Oertner, 2007; Gradinaru et



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al., 2007; Zhang et al., 2008; Ernst et al., 2008). We have previously generated transgenic mice that express ChR2 in subsets of neurons and demonstrated their utility for *in vivo* light-induced activation and mapping of neural circuits (Arenkiel et al., 2007; Wang et al., 2007). Consequently, genetic tools that permit photoinhibition of neuronal activity in mice using NpHR would complement the currently available ChR2 transgenic mice (JAX stock number 007615 and 007612), and significantly enhance our capability to dissect the cellular basis of circuitry function and dysfunction.

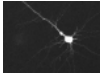
NpHR is a halorhodopsin isolated from the halophilic bacterium *Natronobacterium pharaonis* (Lanyi, 1990). It is a seven-transmembrane protein and functions as a light-driven chloride pump (Lanyi et al., 1990; Kolbe et al., 2000). Recent studies have demonstrated that expression of NpHR in mammalian neurons by transfection or viral infection allows rapid, light-induced reversible inhibition of neuronal activity (Zhang et al., 2007a; Han and Boyden, 2007; Gradinaru et al., 2007;). Furthermore, transgenic expression of NpHR in *C. elegans* permits rapid control of motor behavior by light, illustrating the potential in using NpHR as a genetic tool to determine cellular and circuitry bases of behavior (Zhang et al., 2007a). To expand this tool into a mammalian model system, we generated transgenic mice that express NpHR-YFP using the neuron-specific Thy1 promoter. We found that high levels of NpHR-YFP were expressed in subsets of neurons in these mice and that illumination of NpHR-expressing neurons led to rapid, reversible photoinhibition of action potential firing in these cells. However, we found that NpHR-YFP was not efficiently targeted to plasma membrane and that high levels of NpHR-YFP expression in transgenic mice led to the formation of numerous intracellular blebs in neurons. Similar blebs have also been found in transfected or viral infected neurons (Gradinaru et al., 2008). Using markers of various subcellular compartments we determined that the blebs arose due to retention of NpHR-YFP in the ER. To improve the expression of NpHR we introduced an improved signal peptide sequence and added an ER export signal to NpHR-YFP. The modified NpHR-YFP showed dramatically increased membrane expression and no bleb formation in transfected neurons. The improved version of NpHR-YFP should serve as an excellent tool for neuronal silencing *in vitro* and *in vivo*.

Results

Thy1-NpHR-YFP transgenic mice

We used the well-characterized mouse Thy1 promoter to drive codon-humanized NpHR-YFP expression specifically in neurons in transgenic mice. Our previous studies have shown that the modified Thy1 promoter predominantly drives transgene expression in subsets of projection neurons, and that due to transgenic position-effect variegation, transgene expression is often restricted to different subsets of neurons in different transgenic lines (Feng et al., 2000; Arenkiel et al., 2007; Wang et al., 2007). We generated 7 founder lines, 5 of which showed NpHR-YFP expression in the brain. Expression of NpHR in lines 1, 3 and 7 was widespread, including layer V pyramidal neurons of the cortex, CA1 and CA3 pyramidal neurons and dentate granule cells of the hippocampus, and various neurons in the superior and inferior colliculus, thalamus and brain stem (Fig. 1a–c and data not shown). In lines 6 and 9, NpHR-YFP expression was detected in isolated single neurons throughout various regions of the brain (Fig. 1d, e).

All Thy1-NpHR-YFP mice are viable and breed normally. However, we noticed two problems at the cellular level. First, unlike ChR2-YFP, which is mostly targeted to the plasma membrane in neurons of Thy1-ChR2-YFP mice (Arenkiel et al., 2007; Wang et al., 2007), a large fraction of the NpHR appeared to be cytoplasmic (Fig. 2a, b). In Thy1-ChR2-YFP mice, the intensity of the YFP fluorescence signal was highest in neuronal compartments with a high surface/volume ratio, such as in dendrites and axons (Arenkiel et al., 2007; Wang et al., 2007), whereas in Thy1-NpHR-YFP



mice, the highest YFP fluorescence signal was in cell body layers (Fig. 2a, b). These observations suggest that NpHR-YFP is not efficiently targeted to the plasma membrane. The second problem is that NpHR-YFP formed numerous bright intracellular blebs, evident as brightly fluorescent, spherical structures often found in neurons expressing NpHR (Fig. 2c, d). This was particularly apparent in transgenic lines that express high levels of NpHR-YFP, such as lines 3 and 7 (Fig. 2e, f). These blebs varied in size and could form in cell bodies or in dendrites (Fig. 2c, d). Large blebs often caused dramatic, local swelling of dendrites (Fig. 2d), raising the concern that these blebs could affect neuronal functional properties.

Photoinhibition of NpHR-YFP expressing cells

To determine whether transgenic expression of NpHR-YFP in mice allows light-induced inhibition of neuronal activity, we characterized the electrophysiological properties of neurons in a line of Thy1-NpHR-YFP mice with minimal blebbing (line 6; Fig. 1d, e).

We first asked whether NpHR expression altered the functional characteristics of neurons. For this purpose, we used whole-cell patch clamp recordings to measure the electrophysiological properties of NpHR-expressing pyramidal neurons in slices from the hippocampus and cortex of line 6 mice. No significant differences were observed between the resting electrical properties of NpHR-positive and NpHR-negative neurons, or in the ability of these neurons to generate action potentials (Table 1). We therefore conclude that, in the absence of illumination, NpHR expression does not affect the electrical properties of these neurons despite the presence of some blebbing.

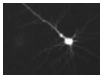
We next asked whether illumination of NpHR could photoinhibit neurons. For this purpose, large light spots ($\approx 0.4 \text{ mm}^2$) of yellow light (545–585 nm; 1 s duration) were used to illuminate cortical and hippocampal slices. In both brain regions, illumination of NpHR-positive pyramidal neurons generated outward currents (Fig. 3a). No currents were evoked during illumination of NpHR-negative neurons (data not shown), demonstrating that the currents were due to activation of NpHR. At maximal excitation light intensity, the time constant for activation of these currents during the light pulse was $7.0 \pm 0.5 \text{ ms}$ and the time constant for deactivation of the currents following the end of illumination was $7.0 \pm 0.5 \text{ ms}$.

The magnitude of the NpHR-mediated photocurrent depended upon light intensity, with stronger illumination yielding larger currents (Fig 3a). This relationship arises from progressive activation of more NpHR pumps as the light intensity increases. The relationship between light intensity and peak amplitude of the photocurrent could be described by the Hill equation (Fig. 3b):

$$Y = I_{\max} \frac{X^n}{K^n + X^n}$$

where X is light luminance and K represents the light level where the photocurrent was half-maximal ($15 \pm 0.2 \text{ mW/mm}^2$). $I_{\max}$, the maximum current amplitude, was $68 \pm 5.5 \text{ pA}$ and n , the Hill coefficient, was 1.1 ± 0.1 . The Hill coefficient of approximately 1 indicates that absorption of a single photon is sufficient for activation of NpHR, even though NpHR is known to trimerize (Kolbe et al., 2000).

In current-clamp conditions, illumination (1 s long light flashes) of NpHR produced small hyperpolarizations of the membrane potential (Fig. 3c). At maximal excitation light intensity, the time required to reach the half-maximal change in membrane potential was $23 \pm 4 \text{ ms}$ and the half-time for repolarization of the membrane potential after illumination ended was $19.3 \pm 3.2 \text{ ms}$. As was the case for NpHR-mediated currents, the magnitude of these hyperpolarizations depended upon light intensity. The relationship between the amplitude of these potential changes



and light luminance again was a saturable function that could be described by the Hill equation (Fig. 3d). The derived values of K ($13 \pm 0.1 \text{ mW/mm}^2$) and Hill coefficient (0.8 ± 0.1) were similar to those determined for the light-induced photocurrents (1.1 ± 0.1), while the maximal voltage change was $8.9 \pm 2.5 \text{ mV}$ ($n = 5$).

To examine the effects of NpHR activation on neuronal excitability, NpHR-positive pyramidal neurons in the cortex and hippocampus were stimulated with depolarizing current pulses (2 s duration) to evoke trains of action potentials (Fig. 3e, top). Subsequent illumination reduced the AP frequency in a light-dependent manner (Fig. 3e, lower traces; $n = 11$). Fits of the Hill equation (Fig. 3f) indicated a half-maximal light intensity of $18 \pm 0.1 \text{ mW/mm}^2$ and a Hill coefficient of 1.1 ± 0.1 , again similar to the values determined in Figs. 3b and 3d. The maximum reduction of action potential frequency was 100% during light flashes. Together these data demonstrate that NpHR is an effective tool in silencing neuronal activity when genetically targeted and chronically expressed in transgenic mice.

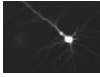
Intracellular blebs represent ER retention of NpHR-YFP

Even though these physiological results demonstrate that NpHR-YFP expressed in neurons of transgenic mice is capable of mediating photoinhibition of neuronal activity, our histological images suggest that this form of NpHR-YFP is not efficiently targeted to the plasma membrane, which would reduce efficiency of photoinhibition. Furthermore, the formation of blebs raises the concern that high levels of NpHR-YFP expression may adversely affect neuronal function. To solve this problem, we began by determining the nature of the intracellular blebs formed by NpHR-YFP. We found that similar blebs were formed when NpHR-YFP was highly expressed in cultured hippocampal neurons as well as in non-neuronal cell lines, such as HEK293T cells and COS7 cells (Fig. 4a–d). Because Thy1-ChR2-YFP mice were generated with a wildtype (codons not humanized) construct, we considered the possibility that the bleb formation in Thy1-NpHR-YFP mice might be caused by a dramatic increase in expression due to codon optimization of NpHR. However, we found that wildtype NpHR-YFP also formed blebs when expressed in cultured neurons at high levels (Fig. 4e, f).

We next used various subcellular markers to determine the compartment in which NpHR-YFP accumulated. Cotransfection of NpHR-YFP and the transferrin receptor (TfR), a marker for recycling endosomes (Harding et al., 1983), did not show any colocalization of the two proteins (Fig. 5a–a’). This indicates that the NpHR-YFP was not accumulating in recycling endosomes. Antibody staining for endogenous GM130, a marker for the Golgi apparatus (Nakamura et al., 1995), also showed that NpHR-YFP blebs did not colocalize with GM130 (Fig. 5b–b’). Thus, NpHR-YFP was not accumulating in the Golgi, either. In contrast, staining with an antibody for the ER marker BiP showed precise colocalization of NpHR-YFP blebs with BiP (Fig. 5c–c’). Even in cells with low levels of NpHR-YFP expression, where no blebs were present, NpHR-YFP and BiP colocalized (Fig. 5d–d’). This suggests that significant amounts of NpHR-YFP ER are retained in the ER even before blebs are formed, with blebs resulting from accumulation of very high levels of NpHR-YFP in the ER.

Improved ER trafficking and membrane targeting of NpHR-YFP

Several sequence features, such as signal peptide sequences, ER retention signals and ER export signals, affect ER trafficking and expression of integral membrane proteins (Derby and Gleeson, 2007). Scanning the amino acid sequence of NpHR did not identify any known ER retention signals except for the MDEL sequence in YFP that is similar to the known ER retention signal KDEL (Derby and Gleeson, 2007). However, mutating the MDEL motif of NpHR-YFP to MDDV did not reduce the bleb formation (data not shown). We also noticed that NpHR does not contain a typical signal peptide sequence (Bendtsen et al., 2004), so we changed the signal peptide sequence



of NpHR-YFP to that of ChR2 or the $\beta 2$ subunit of the neuronal nicotinic acetylcholine receptor (Fig. 6a–c). Expression in cultured hippocampal neurons showed that modifying the signal peptide sequence of NpHR-YFP was insufficient to eliminate bleb formation (Fig. 6d–f).

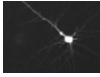
We next determined whether addition of a conserved ER exporting sequence to NpHR-YFP would reduce bleb formation. For this purpose, we attached the ER export sequence NANSFCYENEVALTSK (Ma et al., 2001) to the carboxyl terminus of NpHR-YFP. NpHR-YFP containing the ER export signal (eNpHR-YFP) showed dramatically improved membrane targeting and diminished bleb formation in COS7 cells (Fig. 6g–i). Most importantly, expression of eNpHR-YFP in cultured hippocampal neurons did not yield blebs and showed improved plasma membrane targeting (Fig. 6j–l). Thus, by modifying the signal peptide sequence and adding an ER export signal, we generated a version of NpHR-YFP that has much-improved trafficking and should therefore be better for light-induced inhibition of neuronal activity.

Discussion

Several chemical genetic approaches have been developed to silence neuronal activity in mammalian neurons (Tervo and Karpova, 2007). These methods allow perturbation of neuronal activity in specific subpopulations of neurons by genetic targeting and administration of chemical inducers, enabling neuroscientists to probe how individual groups of neurons regulate circuitry activity and behavior (Karpova et al., 2005; Tan et al., 2006; Armbruster et al., 2007; Lerchner et al., 2007; Wulff et al., 2007). The major advantages of these chemical genetic approaches are their on-and-off inducibility, their ability to silence large numbers of neurons and their ability to reach deep brain regions through systemic administration of chemical inducers. The major disadvantages are the slow time course of onset/offset (minutes in cultured neurons and hours to days *in vivo*) and the potential toxicity or interference with physiological processes by chemical inducers (Tervo and Karpova, 2007).

The recent development of optogenetic tools for manipulating neuronal activity with spatial and temporal precision provides an unprecedented opportunity to dissect functional circuitry connectivity and to probe the neural basis of complex behaviors (Nagel et al., 2005; Schroll et al., 2006; Adamantidis et al., 2007; Zhang et al., 2007b; Huber et al., 2008). As a light-induced silencer, NpHR has the advantages of rapid onset/offset (in milliseconds) and spatial precision determined by the size and position of the light source. To complement the existing Thy1-ChR2-YFP transgenic mice for photoactivation of neurons (Arenkiel et al., 2007; Wang et al., 2007), in this study we generated Thy1-NpHR-YFP transgenic mice for light-induced silencing of neuronal activity. We demonstrated that illumination of NpHR-positive neurons in acute brain slices led to hyperpolarization of neurons and produced rapid, reversible inhibition of neuronal firing. Thus, in principle NpHR in transgenic mice silences neurons in a light-inducible manner, raising the potential for its targeting to genetically distinct subpopulations of neurons in order to determine how neuronal activity in each neuron subtype shapes circuitry function and behavior.

Despite this great promise, we found that the current form of NpHR has two major problems when expressed in transgenic mice: poor membrane targeting and bleb formation due to ER retention. The poor expression on the plasma membrane could lower the efficiency of light-induced inhibition of neuronal activity. In cultured hippocampal neurons transfected with NpHR-YFP, the light intensity required to achieve near 100% inhibition of neuronal firing is 21.7 mW/mm^2 at the sample in cultured neurons (Zhang et al., 2007a). While it is difficult to directly compare these measurements to the slice experiments described here, it is likely that lower membrane expression in Thy1-NpHR-YFP line 6 mice contributed to our observation that higher light intensities ($\geq 186 \text{ mW/mm}^2$) were required for 100% inhibition of cortical pyramidal cells. However, higher levels of expression in transgenic mice led to the formation of numerous intracellular blebs, discouraging us from using these mice for further experiments. In addition, we found that if NpHR is highly expressed in cultured neurons or even in non-neuronal cell lines it forms similar blebs as observed in neurons of the transgenic mice, suggesting a general defect in trafficking of NpHR to the plasma membrane. Although the mechanism of ER retention is



unclear— we did not find any known ER retention signals in the NpHR sequence— our modification, through replacement of the signal peptide and the addition of an ER export signal, has resulted in a much improved version of NpHR-YFP for light-induced silencing of neuronal activity. A similarly modified eNpHR has also been shown to have increased peak photocurrent compared to the original NpHR (Gradinaru et al., 2008). Thus, future transgenic mice expressing the improved eNpHR in neurons will likely be a valuable tool for probing circuitry function.

Materials and Methods

All animal procedures listed here were approved by the Duke University Institutional Animal Care and Use Committee.

Generation of Thy1-NpHR transgenic mice

Humanized NpHR from pcDNA3.1/NpHR-EYFP (Zhang et al., 2007a) was cut out with *NheI* and *XbaI* digestion, blunted, and subcloned into the mouse Thy1 vector (Caroni, 1996; Feng et al., 2000). Transgenic mice were generated by the injection of the gel purified Thy1- NpHR-YFP DNA construct into fertilized oocytes, using standard pronuclear injection techniques (Feng et al., 2004). Fertilized eggs were collected from the matings between C57BL/6J and CBA F1 hybrids. Transgenic founders were identified through tail DNA PCR using primers for Thy1 and NpHR (Thy1F1, TCTGAGTGGCAAAGGACCTTAGG; NpHRR1, TCCACCAGCAGGATATACAAGACC), which amplify a 750 bp fragment. The transgenic lines generated were backcrossed to C57BL/6. Of 7 founder lines established, 5 showed NpHR-YFP expression.

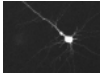
Anatomy

To assess the expression of NpHR-YFP, mice were anesthetized with an overdose of isoflurane and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were cut into 50 μ m thick slices on a vibratome. Fluorescence images (4X objective) were obtained with an AxioImager microscope (Zeiss) and Axiocam HRC camera or a Nikon confocal microscope (20X objective). Image montages were assembled with Photoshop Elements software.

Brain slice recording and photoinhibition

For recordings of neuronal electrophysiological properties, 250–350 μ m thick slices were prepared from the cortex and hippocampus of 13- to 36-d-old line 6 mice using procedures approved by the Animal Care and Use Committee of Duke University. Whole-cell patch clamp recordings were made with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), acquired with Clampfit (Axon Instruments), and analyzed with Igor Pro. Recording pipettes had resistances of 2–7 M Ω and contained 130 mM K-gluconate, 2 mM NaCl, 4 mM MgCl₂, 20 mM Hepes, 4 mM Na₂ATP, 0.4 mM NaGTP, and 250 μ M K- EGTA (pH 7.3). The extracellular solution consisted of 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 or 1.3 mM MgCl₂, 20 mM dextrose or D-glucose, 1.25 mM NaH₂PO₄, and 26 mM NaHCO₃, (pH 7.4 after bubbling with 95% O₂/5% CO₂). A junction potential of 10 mV was taken into account when reporting membrane potentials. Experiments were performed at room temperature (21–24°C). Slices were examined on an upright epifluorescence microscope (Eclipse E600-FN; Nikon). For identifying NpHR-expressing neurons, the fluorescence of YFP fused to NpHR (515–555 nm) was detected with a CoolSNAP-fx camera (Photometrics, Tucson, AZ). For activating NpHR, the same arc lamp was used to create bandpass-filtered light pulses (545–585 nm), with light pulse duration controlled by an electronic shutter (Uniblitz; Vincent Associates, Rochester, NY).

Constructs



Information for pcDNA3.1/wildtype NpHR-YFP and pcDNA3.1/NpHR-EYFP can be obtained from the Karl Deisseroth lab website at Stanford University (http://www.stanford.edu/group/dlab/optogenetics/sequence_info.html). pCMV-ChR2SP- NpHR-YFP was produced by the replacement of the first 27 amino acids of NpHR with the signal peptide of hChR2 (MDYGGALSAVGRELLFVTNPVVVNGS). pCMV- β 2SP- NpHR-YFP was prepared by the replacement of the NpHR signal peptide with the β 2 subunit signal peptide of the neuronal nicotinic acetylcholine receptor (MAGHSNSMALFSFSLLWLCSGVLGTEF). pCMV/eNpHR-YFP, pCMV/ChR2SP- eNpHR-YFP, and pCMV/ β 2SP-eNpHR-YFP were prepared by fusing the 16 amino acids from 373 to 385 of the potassium channel Kir2.1 (NANSFCYENEVALTSK) to the C- terminus of NpHR-YFP.

Antibodies

Mouse anti-GM130 monoclonal antibody (ab1299, 1:500 dilutions) and Rabbit anti-GRP78 BiP (ab21685, 1:200 dilutions) were from Abcam.

Cell culture and transfection

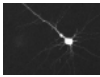
293T cells were cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. COS7 cells were cultured in DMEM with 10% FBS, non-essential amino acids and 1% penicillin/streptomycin. Hippocampal neuron cultures were prepared from E18 rat embryos as described (Ehlers, 2000). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Acknowledgments

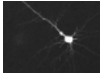
We thank João Peça and Mary (Molly) Heyer for their help in the preparation of this manuscript. We thank members of the Feng lab for their support. We also thank members of the lab of Michael Ehlers for technical help. This work was supported by NIH grants to KD, GJA and GF.

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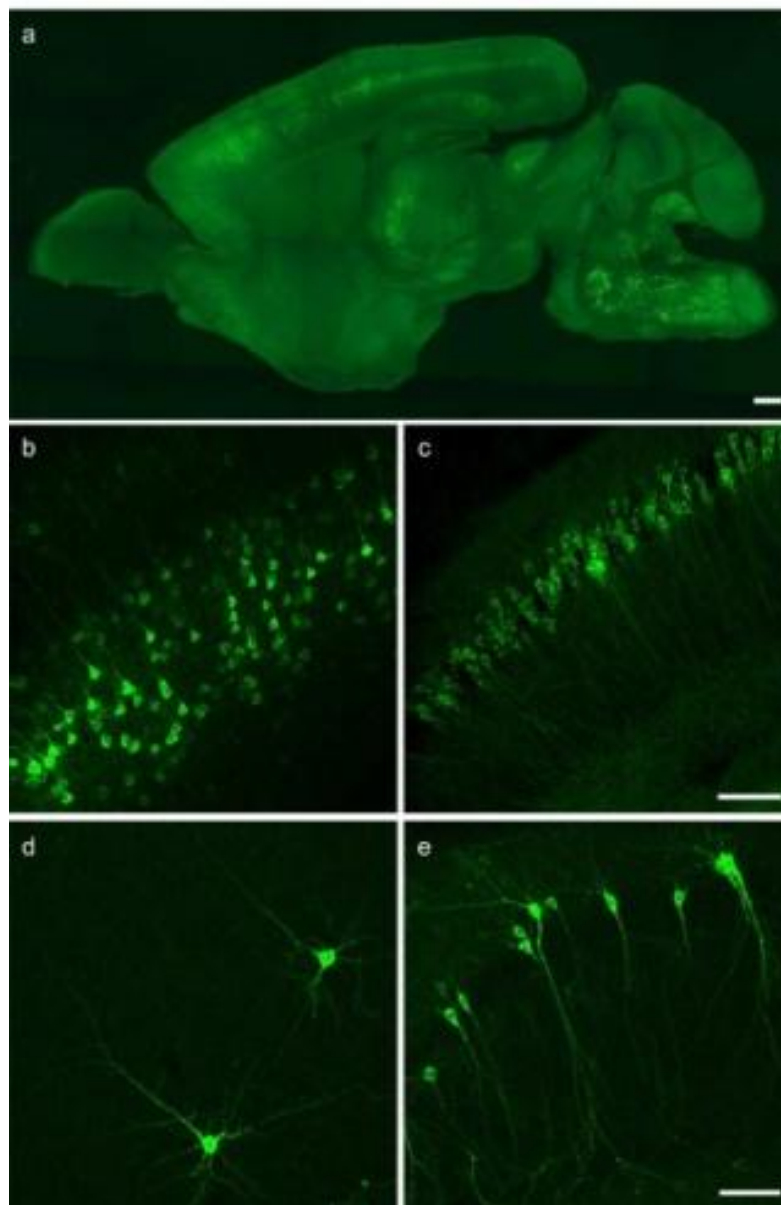
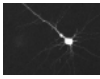


Fig. 1. Thy1-NpHR-YFP transgenic mice

(a) An image of a sagittal brain section from an adult Thy1-NpHR-YFP transgenic mouse (line 1). (b, c) Confocal images showing the expression of NpHR-YFP in layer V pyramidal neurons of the cortex (b) and CA1 pyramidal neurons of the hippocampus (c) in Thy1- NpHR-YFP mice (line 1). (d, e) Confocal images showing that NpHR is expressed sparsely in cortex and CA1 of the hippocampus in line 6 Thy1-NpHR-YFP transgenic mice. Scale bars, 500 μm in a, 100 μm in c for b and c, and 100 μm in e for d and e.

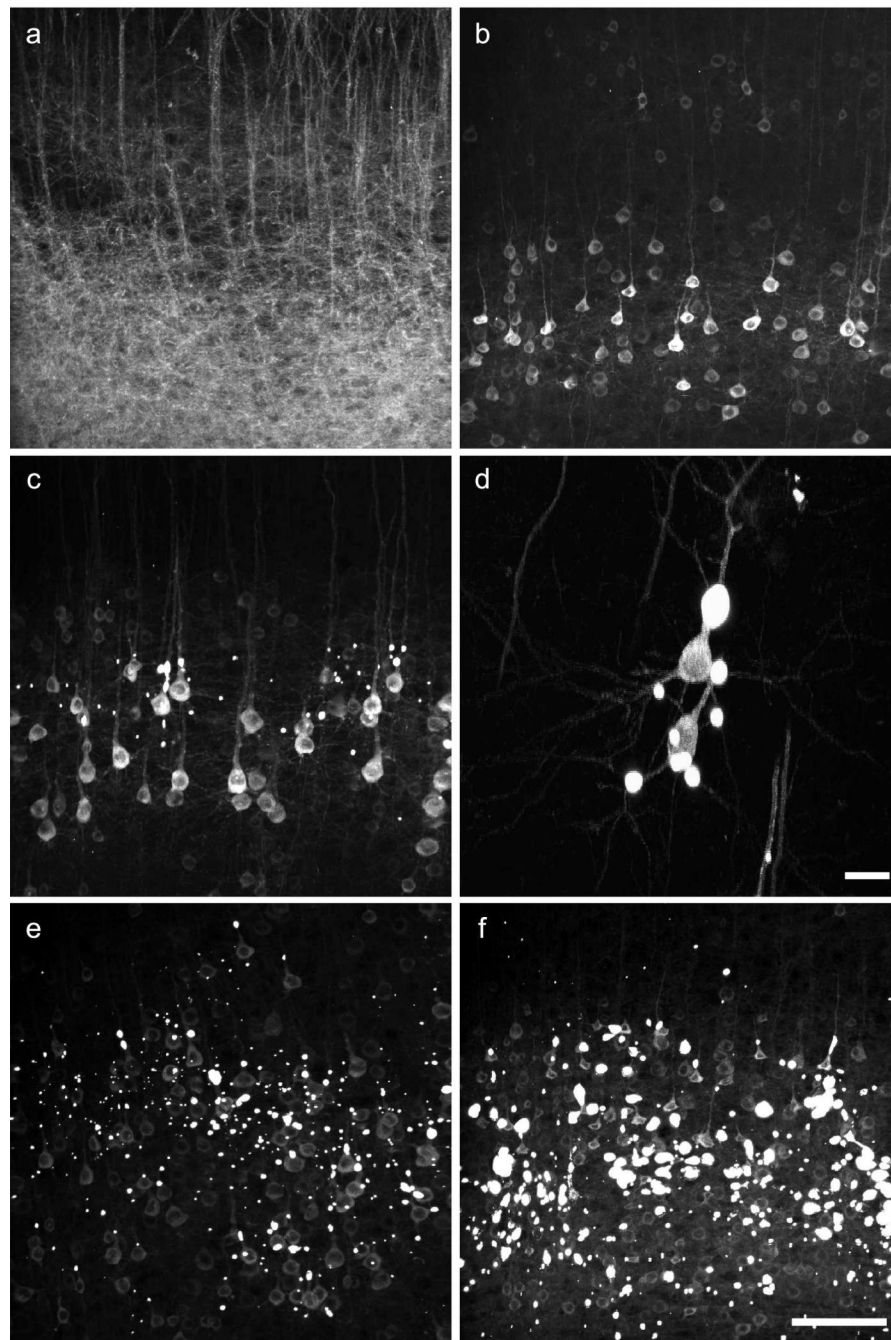
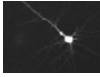


Fig. 2. Poor membrane expression and intracellular blebs in Thy1-NpHR-YFP mice
 (a) In Thy1-ChR2-YFP transgenic mice, ChR2-YFP is efficiently targeted to the plasma membrane, as the intensity of YFP fluorescence signal is highest where the cells have a high surface/volume ratio such as in dendrites. (b) In Thy1-NpHR-YFP transgenic mice, NpHR- YFP is mostly retained in cell bodies and proximal dendrites (line 1). (c–f) NpHR-YFP forms numerous intracellular blebs in neurons of Thy1-NpHR-YFP transgenic mice. Images are from the cortex of line 1 (c), 6 (d), 3 (e), and 7 (f) mice. Scale bars, 20 μ m in d, 100 μ m in f for a–c, and e, f.

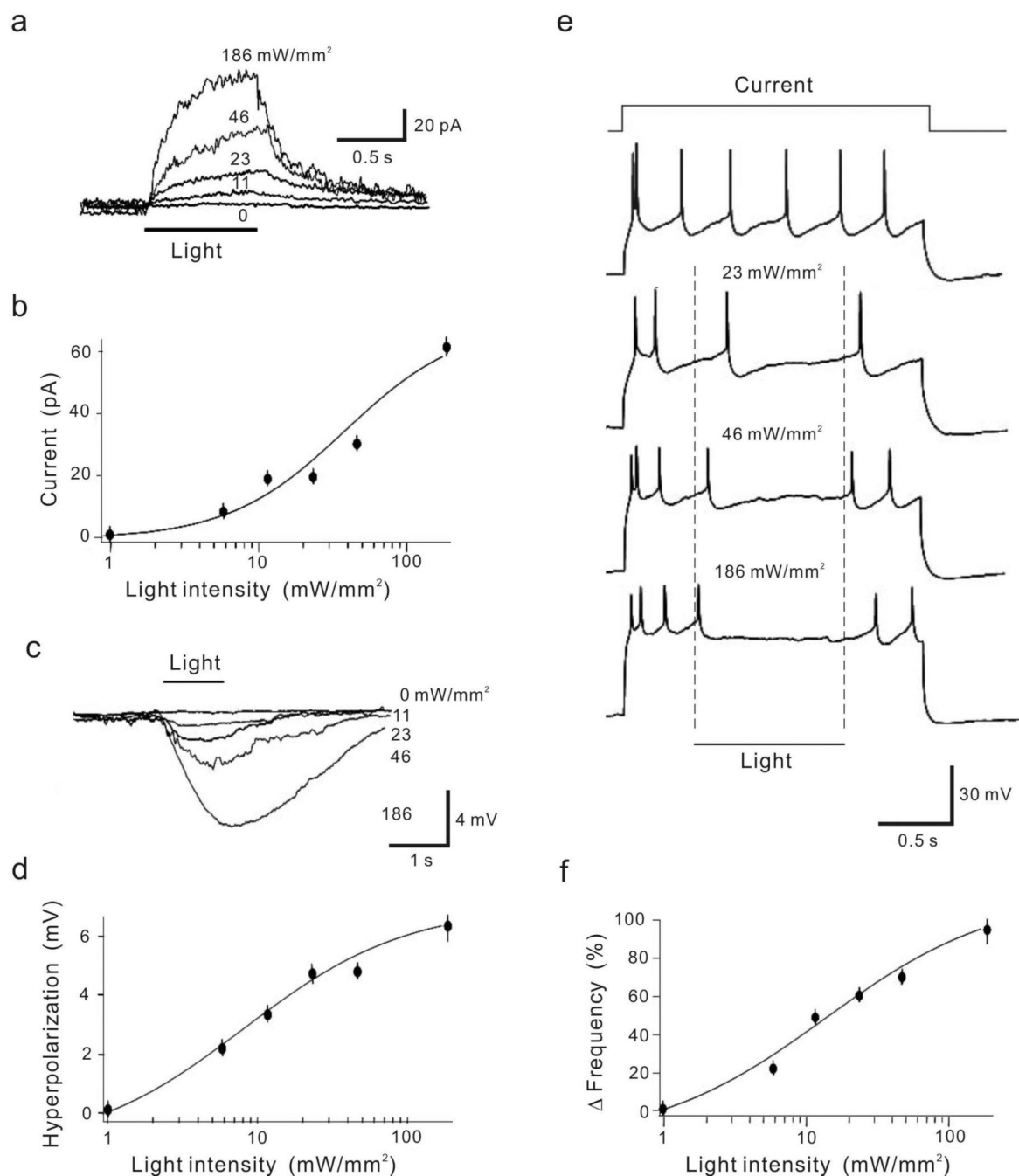
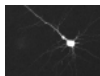


Fig. 3. Light-induced photoinhibition in NpHR-expressing neurons of Thy1-NpHR-YFP mice
 (a, b) Illumination evokes photocurrents in NpHR-positive neurons ($n = 5$). (a) Light pulses of 1 s duration generated photocurrents whose amplitude depended upon light intensity. (b) Relationship between photocurrent amplitude and light intensity. Curve is fit of the Hill equation. (c, d) Light induced hyperpolarization in NpHR-positive neurons ($n = 5$). (c) Illumination of neurons with light of variable intensity induced proportionate changes in the membrane potential of NpHR-positive neurons. (d) Relationship between light intensity and voltage changes. Curve is fit of Hill equation. (e, f) Illumination controls frequency of action potentials. (e) Light of varying luminance inhibited neuronal firing in a light intensity-dependent manner. (f) Relationship between light intensity and light-induced reduction of the AP frequency ($n = 11$). Curve is fit of the Hill equation.

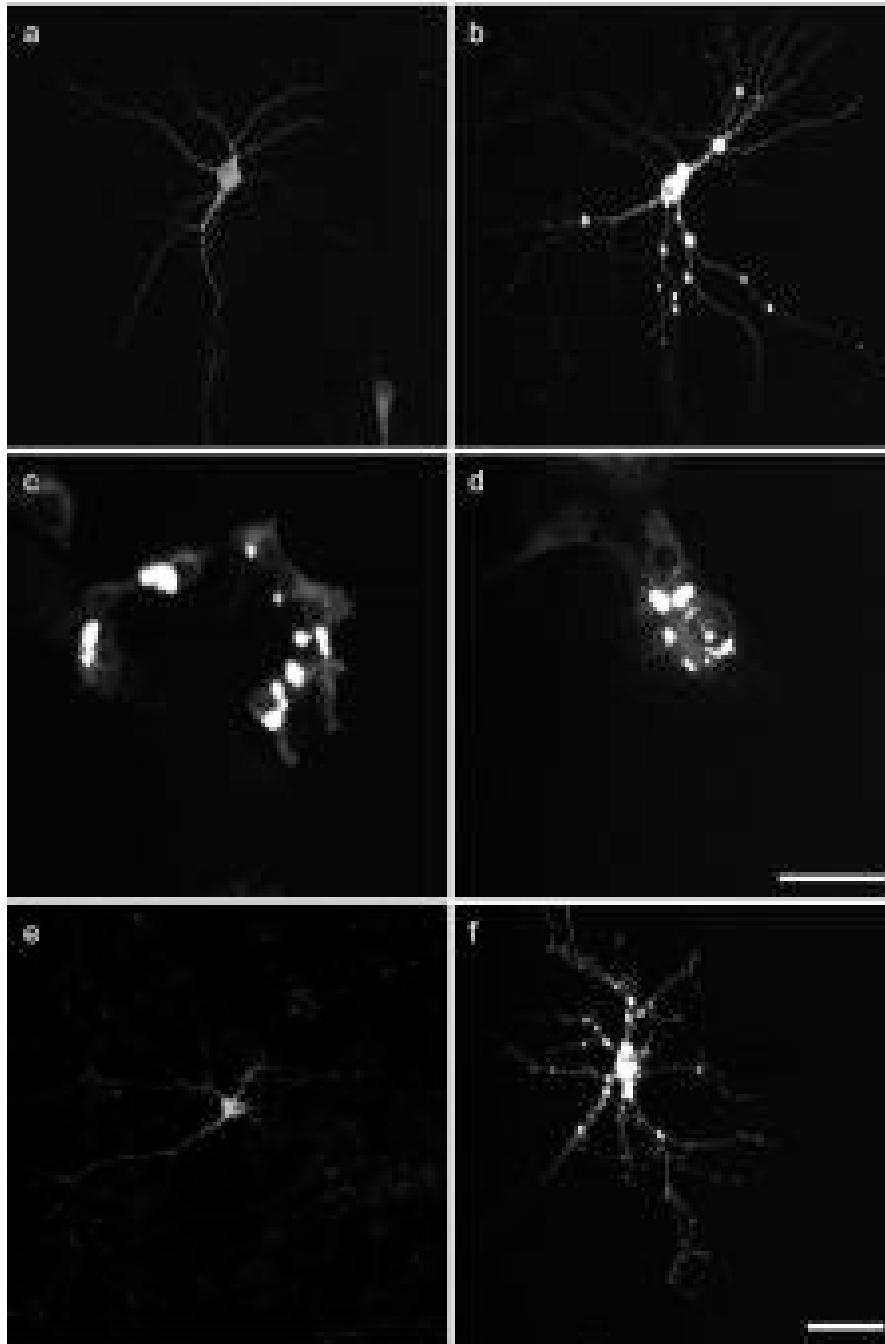
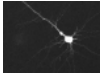


Fig. 4. High levels of NpHR-YFP expression form intracellular blebs in vitro

(a) NpHR-YFP expressed at low levels in cultured hippocampal neurons. (b) High levels of NpHR-YFP expression in cultured hippocampal neurons leads to the formation of numerous intracellular blebs. (c, d) Blebs also form in HEK293T cells (c) and COS7 cells (d) expressing NpHR-YFP. (e, f) Wildtype (codon not humanized) NpHR-YFP forms blebs at high levels of expression (f) but not at low levels of expression (e). Scale bar, 50 μm in d for c and d, 100 μm in f for a, b, e and f.

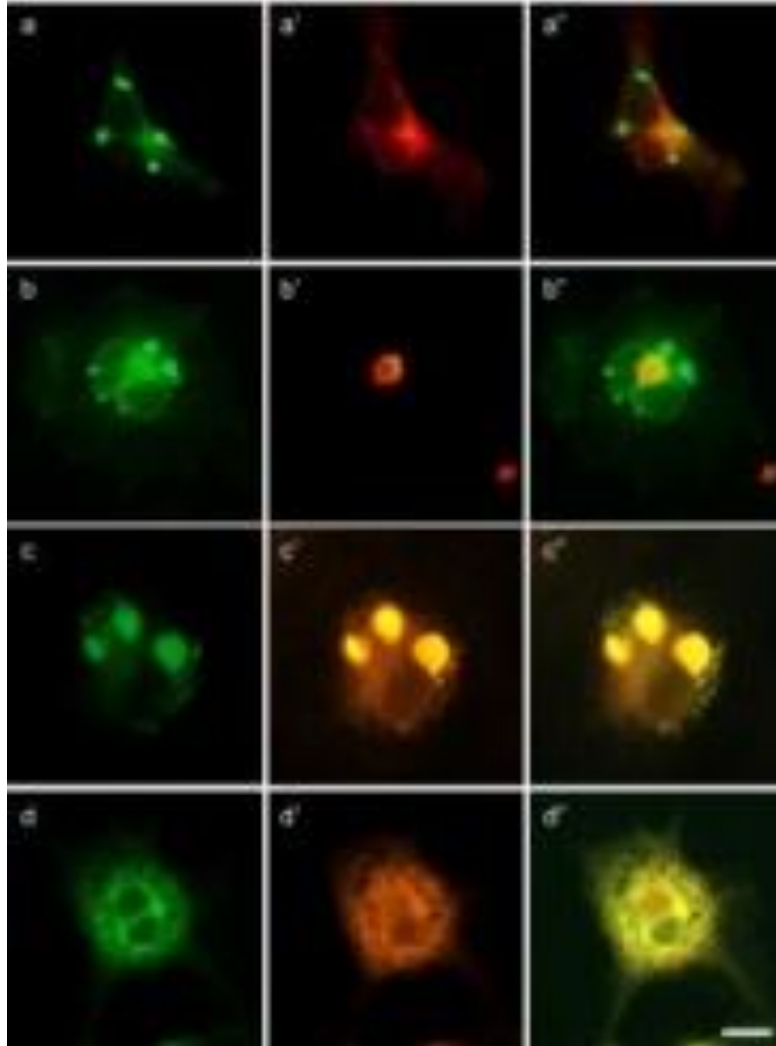
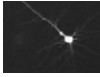


Fig. 5. The blebs correspond to retention of NpHR-YFP in the ER

(a-a'') NpHR-YFP (a) co-expressed with transferrin receptor (TfR, red in a') in COS7 cells. Their colocalization is not detectable (merged image in a''). (b-b'') COS7 cells transfected with NpHR-YFP (b) and stained with Golgi marker GM130 (b'). GM130 does not colocalize with the blebs (merged image in b''). (c-d'') NpHR-YFP precisely colocalizes with the ER marker BiP in COS7 cells with (c-c'') or without (d-d'') blebs. Scale bar, 20 μm in d'' for a-d''.

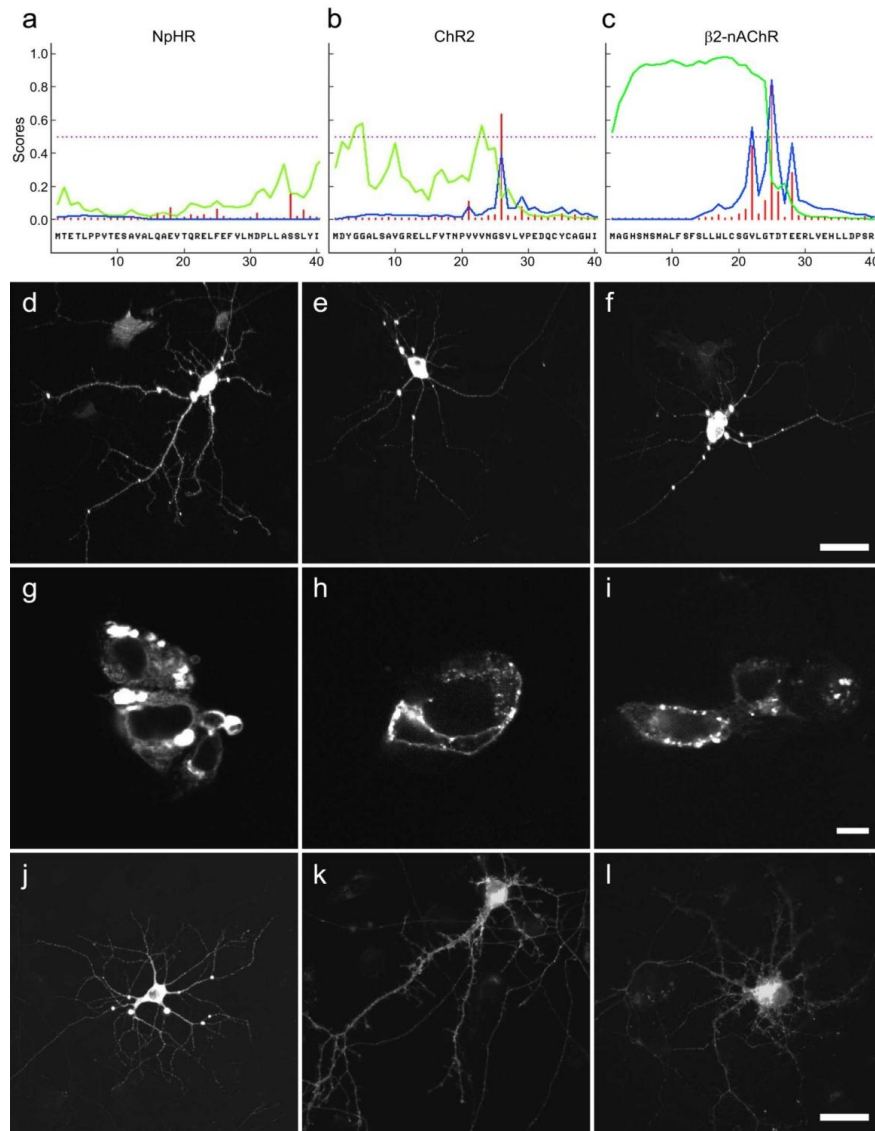
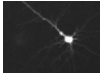


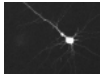
Fig. 6. Adding an ER export motif to NpHR eliminates the formation of blebs

(a) Signal peptide prediction using SignalP 3.0 software (Bendtsen et al., 2004) gives a poor score for NpHR. (b) ChR2 has a much better score for the prediction of a signal peptide than NpHR. (c) The nAChR $\beta 2$ subunit has a near-perfect signal peptide sequence. Green plot indicates signal peptide prediction score (S-score); red plot indicates cleavage site prediction score (C-score); blue plot indicates the combined cleavage site prediction score from S-score and C-score (Y-score). (d–f) Improvement of signal peptide sequence alone is not sufficient to eliminate the bleb formation of NpHR-YFP in cultured hippocampal neurons; (d) NpHR- YFP with native signal peptide, (e) NpHR-YFP with ChR2 signal peptide, and (f) NpHR- YFP with the nAChR $\beta 2$ subunit signal peptide. (g–i) Adding an ER export signal sequence to NpHR-YFP eliminates bleb formation in COS7 cells; (g) original NpHR-YFP without ER export signal, (h) original NpHR with ER export signal, (i) NpHR with the $\beta 2$ nAChR signal peptide and ER export signal. (j–l) Adding an ER export signal sequence to NpHR-YFP eliminates bleb formation in cultured hippocampal neurons; (j) original NpHR-YFP without ER export signal, (k) original NpHR with ER export signal, (l), NpHR with the $\mu 2$ nAChR signal peptide and ER exporting signal. Scale bars, 100 μ m in f for d–f, 20 μ m in i for g–i, 100 μ m in l for j–l.

Table 1: Comparison of electrical properties of wildtype and NpHR expressing neurons

Genotype	Action potential amplitude, mV	Action potential duration, ms	Input resistance, MΩ	Resting potential, mV	Threshold, mV
NpHR ⁻	55.1 $\pm$ 10.4	2.24 $\pm$ 0.4	147.2 $\pm$ 13.7	-62.5 $\pm$ 3.4	-32.5 $\pm$ 4.3
NpHR ⁺	48.3 $\pm$ 6.3	2.11 $\pm$ 0.3	148.7 $\pm$ 33.6	-62.0 $\pm$ 3.5	-29.3 $\pm$ 3.7

Sample size is 10 cells for each group. There was no statistical significant difference ($P > 0.05$; t -test) in the mean value of any parameter between the two types of neurons.

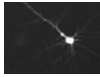


Chapter 5

General Discussion

Our results indicate that GABA_CRs are involved in intra-LA neuronal communication, where they modulate a considerable portion of postsynaptic currents. We interpret this to suggest that GABA_CRs are located on the presynaptic side on the axons of the interneurons and act as autoinhibitors to reduce synaptic GABA release. Auditory Pavlovian fear conditioning under influence of GABA_CR agonists and antagonists showed that GABA_ARs and GABA_CRs play opposing roles on modulations of fear acquisition and consolidation: GABA_ARs impair and GABA_CRs enhance fear learning and memory. Our results demonstrate a novel role of GABA_CRs, which advances our understanding of both the function of GABA_CRs in the brain and in our knowledge of the circuitry of the LA and how fear memories are formed and stored.

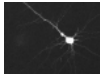
GABAergic agonists (e.g., benzodiazepines) are commonly used drugs to treat anxiety-related disorders, especially by targeting GABA_ARs. However, in addition to their potent anxiolytic properties, these drugs also lead to side effects that include sedation, motor and memory impairments. These side effects are, in part, due to the fact that GABA_ARs are ubiquitously distributed throughout the mammalian brain. Another problem is that these drugs often lead to dependence. A third problem is that GABA_ARs desensitize, possibly explaining why patients with generalized anxiety disorder or panic disorder show lower benzodiazepine binding in some forebrain areas (Kaschka, et al., 1995; Tiihonen et al., 1997; Malizia et al., 1998). Due to the lack of non-specific effects of benzodiazepines, their potential for dependence, and their tendency to desensitize, GABA_ARs are not optimal as a target for long-term treatment of patients with anxiety disorders. As a result, there has been a continued search for new, more specific anxiolytic agents, either by indirect modulation of GABA_ARs via targeting norepinephrine, serotonin, and dopamine, or by research aimed at altering specific GABA_ARs subunits. Given that amygdala processing is altered in anxiety disorders (LeDoux, 2007; Monk, 2008), our results suggest that GABA_CRs in the amygdala might be a useful alternative target for the development of anti-anxiety drugs.



Norepinephrine (NE) is thought to play a key role in fear, but its role in Pavlovian fear conditioning, a major model for understanding the neural basis of fear, is poorly understood. Of the adrenergic receptor subtypes, the α_1 receptor has received the least attention in fear conditioning. We examined the acquisition, consolidation, and extinction of Pavlovian cue fear and the effect of terazosin and prazosin, α_1 -receptor antagonists, on these processes in rats. Terazosin enhances acquisition of both short and long term memory of fear conditioning. We localized this effect to the LA. Terazosin does not affect consolidation of fear memories. Both terazosin and prazosin impair extinction of fear conditioning. This could be problematic for PTSD patients taking prazosin for night terrors if they are also in behavioral therapy. Our data is consistent with the theory that α_1 activity in the LA positively modulates GABAergic inhibition. By blocking the α_1 receptors with terazosin, we are inhibiting GABA function, thereby disinhibiting excitatory processing in the LA fear circuits.

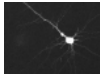
NpHR transgenic mice were shown to provide a valuable platform for probing neuronal circuits in the mouse brain. These animals were shown to be viable, transmitted the transgene at expected Mendelian ratios and did not display abnormal behaviors or gross brain anatomical defects. Electrical properties of neurons expressing the transgene were shown to be similar to those of NpHR-negative neurons. Characterization of NpHR mice revealed expression of NpHR at varying levels and in distinct populations of neurons across different lines. In one line mice presented single-neuron labeling in principal projection neurons, while in another line mice presented very strong expression in large populations of neurons. Neuronal action potentials could be blocked with a time-locked precision, and photocurrents could be controlled by varying illumination levels. NpHR transgenic mice represent a useful tool to study inhibitory circuits in the mammalian brain.

The next step would be to generate NpHR and ChR2 mice with expression in specific cellular subtypes. These will then be useful to investigate the influence of distinct circuits and well defined cellular population on perception and behavior. Another possibility will be to use specific neuronal manipulation to test circuit dysfunction in psychiatric disorders, both by the induction of patterns seen in diseased state, or in the alleviation of the same circuits by functional compensation.



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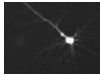
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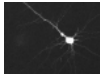
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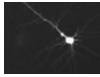
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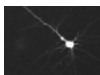
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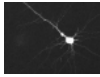
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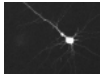
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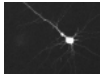
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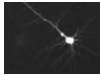
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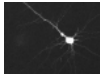
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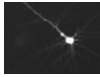
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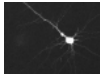
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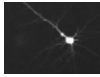
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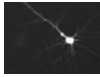
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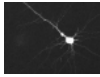
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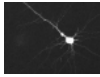
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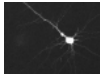
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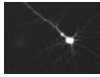
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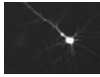
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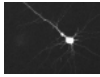
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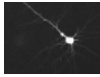
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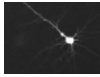
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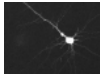
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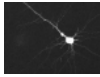
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