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REVIEW

Recent Progress in GABAergic Excitation from Mature Brain

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The excitatory effect of γ-Aminobutyric acid (GABA) has been recognized in very young animals and in seizure generation, but not so much in animals after weaning age or in adults. The existence of this phenomenon in mature brain is still controversial. In the course of debate, creative studies have identified and characterized the phenomenon in suprachiasmatic nucleus, cortex, hippocampus and basolateral amygdala, albeit mostly in single neurons. In neural circuit activity, presumed GABAergic excitation was observed in basolateral amygdala during the study of a neuropeptide, cholecystokinin. Though the functional meaning of this phenomenon *in vivo* remains to be uncovered, it may be implicated in epilepsy or anxiety in the adult brain.

Key words: GABA, Bumetanide, Epilepsy, Anxiety

INTRODUCTION

GABA is the major inhibitory neurotransmitter in the nervous system (Sivilotti and Nistri, 1991; Costa, 1998). The effect is mediated by $GABA_A$ and $GABA_B$ receptors (Bowery et al., 2002; Olsen and Sieghart, 2008). The $GABA_A$ receptor is ionotropic receptor and anion-selective channel (Olsen and Sieghart, 2008). The major anion which passes through this channel is the chloride ion (Cl^-) (Olsen and Sieghart, 2008). When the chloride reversal potential is below the resting membrane potential, Cl^- flows into the cell and hyperpolarizes the membrane potential, which in turn inhibits action potential generation. However, if the chloride reversal potential is above the resting membrane potential due to increased intracellular Cl^- concentration ($[Cl^-]_i$), channel opening results in an outward Cl^- current and membrane depolarization (Ben-Ari, 2002).

This review focuses on the controversial, but intriguing phenomenon of depolarization or excitation by GABA in the mature brain. As for the well-known GABAergic depolarization in immature brains and neonatal epilepsy, excellent reviews are available (Ben-Ari, 2002; Sipila and Kaila, 2008; Kirmse et al., 2011; Ben-Ari et al., 2012; Bregestovski and Bernard, 2012; Kahle and Staley, 2012; Loscher et al., 2012; Miles et al., 2012;).

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GABAergic DEPOLARIZATION

In the discussion of depolarization and excitation, it should always be kept in mind that the depolarization does not necessarily lead to excitation, but instead it may be inhibitory to the postsynaptic neurons. Depolarization or hyperpolarization refers to the membrane potential change. However excitation or inhibition refer to the increase or decrease of action potential generation, the end result of membrane potential change. For example, depolarization of membrane potential below the action potential threshold can be inhibitory to action potential generation by subsequent synaptic inputs because increased conductance prevents membrane potential from reaching action potential threshold even if this input alone could have evoked action potentials (Staley and Mody, 1992; Marty and Llano, 2005). In adults, hyperpolarizing GABA action can move the membrane potential away from the action potential threshold, but the conductance increase by GABA can further inhibit the action potential generation.

In early postnatal life, GABA can be an excitatory neurotransmitter (Cherubini et al., 1991). During the first postnatal week, the $GABA_A$ receptor activation depolarizes pyramidal neurons in hippocampus (Ben-Ari et al., 1989; Cherubini et al., 1990). The $GABA_B$ re-

ceptor is also hyperpolarizing at birth, but its effect is small relative to the depolarizing GABA effect mediated by the $GABA_A$ receptor (Janigro and Schwartzkroin, 1988). In this period of early brain development, depolarizing GABA acts as a trophic factor and also is involved in cell migration (Represa and Ben-Ari, 2005; Bortone and Polleux, 2009; Koyama et al., 2012).

The direction of Cl^- movement is determined by the $[Cl^-]_i$ (Ben-Ari, 2002) (Fig. 1). The $[Cl^-]_i$ is maintained partly by the Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1) and the K^+-Cl^- co-transporter 2 (KCC2) (Ben-Ari, 2002; Yamada et al., 2004; Zhu et al., 2005; Blaesse et al., 2009). NKCC1 is a sodium-coupled cation-chloride co-transporter (CCC) that imports Cl^- into the cell using electrochemical gradient of sodium generated by the Na^+-K^+-ATPase, while KCC2 exports Cl^- using the K^+ gradient (Gamba, 2005). In the nervous system, NKCC1 is expressed during early development while KCC2 is expressed in mature neurons (Stein et al., 2004; Wang and Kriegstein, 2011). The developmental change from depolarizing to hyperpolarizing GABA is accompanied by the decrease of NKCC1 and increase of KCC2 which results in a reduction of $[Cl^-]_i$ (Rivera et al., 1999) (Fig. 1). However, the factors regulating the NKCC1 and KCC2 switch are not clear yet. Intracellular signaling involving the transporters is beginning to be unraveled. The intracellular regulation of the co-transporters involve with no K (lysine) kinases (WNK) and STE20 (sterile 20)-like kinases (Delpire and Gagnon, 2008; Kahle et al., 2008).

Though it has been widely accepted that GABA is depolarizing or excitatory in immature brain, a research group (Zilberter group) produced a series of papers asserting that the phenomenon is artifact due to insufficient energy supplies in brain slice preparations (Rheims et al., 2009; Holmgren et al., 2010; Ivanov et al., 2011; Mukhtarov et al., 2011). Not surprisingly, it stimulated a series of interesting papers testing this assertion from multiple groups (Kirmse et al., 2010; Ruusuvuori et al., 2010; Tyzio et al., 2011). The conservative interpretation of the data from the Zilberter group is that energy sources including ketone bodies, lactate and pyruvate can contribute to the intracellular chloride homeostasis in certain conditions (Zilberter et al., 2010). Whether the data is enough to firmly deny the other data for the GABAergic depolarization phenomenon during the neonatal period is up to a rather subjective decision at this time (Khakhalin, 2011; Ben-Ari et al., 2012; Bregestovski and Bernard, 2012). An interesting piece of third party data came from adult neural stem cells in which GABA is depolarizing to the new born neurons in brain slice preparation (Ge et al., 2006).

GABAergic DEPOLARIZATION OR EXCITATION IN INDIVIDUAL NEURONS FROM MATURE BRAIN

It is generally accepted that GABA is inhibitory to most adult neurons except in abnormal cases such as

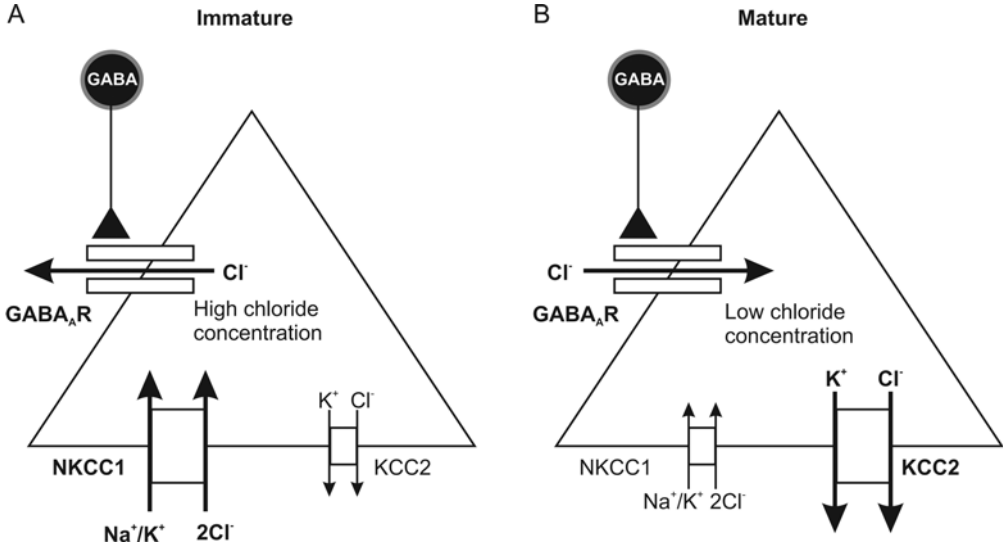

Fig. 1. $[Cl^-]_i$ is determined primarily by NKCC1 and KCC2 at different developmental stages. (**A**) In neurons of immature brain, high expression of NKCC1 leads to high $[Cl^-]_i$ enough for Cl^- reversal potential above resting membrane potential. In this condition, opening of Cl^- channel by $GABA_AR$ activation results in Cl^- efflux and membrane potential depolarization. (**B**) In neurons of mature brain, high KCC2 expression leads to low $[Cl^-]_i$ and Cl^- reversal potential below resting membrane potential. Contrary to the immature brain, Cl^- channel opening by $GABA_AR$ activation causes influx of Cl^- and hyperpolarization. $GABA_AR$: $GABA_A$ receptor.

epilepsy (Costa, 1998). However, there are reports of GABAergic depolarization and excitation in seemingly normal states in multiple brain regions (Marty and Llano, 2005).

Suprachiasmatic nucleus

Hypothalamic suprachiasmatic nucleus (SCN) is where circadian oscillation originates (Reppert and Weaver, 2001; Hastings and Herzog, 2004). In SCN, GABA increased firing rate recorded extracellularly during the day, but inhibited firing at night in the majority of cells from 3-to-8-week-old rats (Wagner et al., 1997). From whole cell patch recording, the change in $[Cl^-]_i$ seemed to be the cause of the circadian GABA effect. The $[Cl^-]_i$ was high during the day and low during the night (Wagner et al., 1997). The use of whole cell patch technique limited the interpretation of the data because the neuron's original physiological chloride concentration was perturbed by the recording solution. The chloride concentration of the neuron was not measured separately. To minimize the disturbance of chloride concentration, gramicidin-perforated patch-clamp technique was subsequently used (De and Pennartz, 2002). In this study, GABA was mostly inhibitory during the day time, but at night time was excitatory in about half of the neurons, which is opposite pattern to that of Wagner et al. (1997). However, GABA remained excitatory to some neurons in SCN in both studies.

Contrary to the two reports above (Wagner et al., 1997; De and Pennartz, 2002), GABA was mostly inhibitory to the firing rate measured with a multiunit activity recording technique in SCN (Gribkoff et al., 2003). The advantage of extracellular multiunit recording is the stable recording of a group of neurons for a long time compared with single unit recording. The drawback is that individual neurons' characteristics can be lost due to averaging the population together. If a small subset of cells were excited by GABA, their response could be masked by the inhibitory effect of GABA on the majority.

This masking possibility was probably a real one as shown in a later study employing multiple experimental techniques to a massive number of neurons (n = 786) (Choi et al., 2008). Exogenously applied GABA was mostly inhibitory, but in a subset of cells, the firing rate increased (15%) or appeared biphasic (13%) in extracellular recording from rats (Choi et al., 2008). Unlike Wagner et al. (1997), the excitation was more common at night instead of the day time (Choi et al., 2008). It was also region-dependent such that the dorsal part of the SCN showed a higher percentage of excitation than the ventral region did. The excitatory effect could be reduced by application of bumetanide, NKCC1 inhibitor, or by recording in *Nkcc1* knock-out mice. This study provides strong evidence that GABA can be excitatory to a small, but significant portion of neurons in SCN. However, the possibility of weakened chloride extrusion by KCC2 was not explored (Belenky et al., 2008). An important future task is to show the *in vivo* functional importance of this effect in those minority of cells excited by GABA.

Cortex, hippocampus and basolateral amygdala

In cortical structures, the presence of GABAergic depolarization has been in debate as summarized below. GABA is depolarizing and excitatory depending on the location of GABA application (dendrite or soma) and the timing of GABA application relative to the action potential or glutamatergic excitation in layer 5 pyramidal neurons from 3-to-4-week-old rats (Gulledge and Stuart, 2003). In this work, GABA was exogenously supplied, rather than using the interneurons as a GABA source.

Dual recordings from synaptically connected pairs of interneurons and pyramidal neurons enable a controlled physiological GABA release after an interneuron action potential. Axo-axonic inhibitory cells (also called Chandelier cells in cortex) are a unique subpopulation of GABAergic interneurons that synapse specifically on the axon initial segment (AIS) of pyramidal neurons in cortex (Lewis, 1998; Somogyi et al., 1998; DeFelipe, 1999). Because action potentials are generated near the AIS, this GABAergic synapse would strongly inhibit action potential generation (Howard et al., 2005). However, the recorded axo-axonic cells could excite two out of 17 pyramidal neurons in 3-to-5-week-old rats although disynaptic depolarization after an action potential from the axo-axonic cell was more common (22 of 48) (Szabadics et al., 2006). The disynaptic depolarization implies that released GABA excites glutamatergic neurons strongly enough for action potential generation. This phenomenon was correlated with low expression of KCC2 in the AIS, which suggests that the increased chloride concentration in AIS is due to reduced extrusion of Cl^-. A similar conclusion is also possible if NKCC1 were highly expressed in the AIS.

In basolateral amygdala, when pairs of two parvalbumin-positive interneurons were recorded, an action potential in one neuron caused an outward current followed by an inward current in the postsynaptic interneuron (Woodruff et al., 2006). The AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid) receptor antagonist (NBQX) blocked only the inward current, but the $GABA_A$ receptor antagonist (bicuculline) blocked both currents. This indicates that GABA excited

a glutamatergic neuron synapsing on the postsynaptic interneuron. This idea is very similar to the disynaptic depolarization seen in the cortex (Szabadics et al., 2006).

Instead of KCC2, the role of NKCC1 in the chloride reversal potential was tested in dentate gyrus (DG) of hippocampus and layer 2/3 cortex (Khirug et al., 2008). They used GABA uncaging to measure the reversal potential from multiple locations on the AIS, soma and dendrite. The relatively depolarized chloride reversal of AIS compared with soma was abolished in *Nkcc1* knock-out mice and by bumetanide application both in mice and rats. The importance of NKCC1 is a bit of surprise because according to previous data, NKCC1 expression decreases and KCC2 expression increases across development (Rivera et al., 1999; Payne et al., 2003; Yamada et al., 2004). However, recent data demonstrate enriched NKCC1 in AIS (Wang and Sun, 2012).

The GABAergic excitation as a general phenomenon was challenged in the CA1 region of the hippocampus (Glickfeld et al., 2009). To avoid invasive recording, they used extracellular recordings at multiple locations along the somatodendritic axis of CA1 pyramidal neurons while GABA was released from an interneuron whose morphology including axonal arbors was reconstructed after recording. As a rule, GABA was hyperpolarizing to the pyramidal neurons when basket, oriens-lacunosum molecular and axo-axonic interneurons generated action potentials. As a minor but important twist, in one of three axo-axonic interneurons, action potentials seemed to excite pyramidal cells. This elegant experiment measured the hyperpolarizing end effect of GABA released from interneurons in CA1. A similar conclusion was obtained from an experiment in CA3 (Bazelot et al., 2010).

However, the result from Glickfeld et al. (2009) should be interpreted with caution. First, the axo-axonic interneurons were not the primary target of interest in this work, though this type of interneuron was the major subtype in the discussion of GABAergic depolarization in cortex (Szabadics et al., 2006). Second, the depolarizing effect on the AIS is difficult to measure via extracellular recording. Third, GABA release from a single action potential is phasic (Marty and Llano, 2005). The effect of sustained GABA release from multiple action potentials was not tested. Fourth, in an abnormal state, the seemingly insignificant excitatory GABA machinery can be amplified and cause significant alterations to the circuit activity.

Depending on the active state of the postsynaptic neurons, the GABA released from an interneuron can be both excitatory and inhibitory in cortical layer 2/3 (Woodruff et al., 2011). At the resting membrane potential of pyramidal neurons, action potentials from Chandelier cells were depolarizing and facilitatory for action potential generation in pyramidal neurons. However, during a heightened activity state of pyramidal neurons, an action potential from a Chandelier cell was inhibitory to the pyramidal neuron. This points out that the dynamic role of GABA is determined by the temporal context of the recipient.

In sub-regions of hippocampus, differential intrinsic properties of the neurons were found to be a determinant for excitatory GABA (Sauer et al., 2012). Using the same technique as Glickfeld et al. (2009) and perforated-patch recordings, they showed that an action potential from an interneuron synapsing on a DG pyramidal soma was depolarizing, but in CA1 similar perisomatic interneuron synapses were hyperpolarizing. The GABA reversal potentials were similar in CA1 and DG, but the resting membrane potential of DG pyramidal neurons was more negative than the GABA reversal potential while that of CA1 pyramidal neurons was slightly positive relative to the GABA reversal potential. Under this condition, GABA is depolarizing to DG but hyperpolarizing to CA1 pyramidal neurons. However, the mechanism underlying how GABA depolarization becomes excitatory in DG was not resolved in this study (Sauer et al., 2012). Another new finding from this work is that perisomatic interneurons can also be excitatory in addition to the axo-axonic interneurons (Szabadics et al., 2006; Woodruff et al., 2006). The functional meaning of this variable GABA effect between CA1 and DG remains to be investigated.

It should be noted that most studies above used a single cell with exogenously applied GABA or a pair of neurons. In this condition, GABA release rarely evoked action potentials in the postsynaptic pyramidal neuron. However, disynaptic excitation after an action potential from the axo-axonic interneuron was 46% (Szabadics et al., 2006). This indicates that GABAergic excitation could be more prevalent in neuronal circuit consisting of multiple neurons. To study this further in the future, the essential approach is noninvasive and simultaneous monitoring of multiple interneurons and pyramidal neurons.

GABAergic EXCITATION IN NEURAL CIRCUIT ACTIVITY FROM MATURE BRAIN

Amygdala is a key brain region for the experience of emotions including fear and anxiety (Ledoux, 2000; Johansen et al., 2011). Amygdala and related brain circuits mediate positive emotions as well, such as

reward (Baxter and Murray, 2002; Everitt et al., 2003). Abnormal function of this circuit results in anxiety and addiction (Davis et al., 2010; Mahan and Ressler, 2012). Synchronous neuronal activity within amygdala contributes to the generation of emotional states (Pare et al., 2002).

In basolateral amygdala, an interesting form of neuronal circuit activity has been observed for a while, but no testable explanation had been suggested until recently. During voltage clamp recording, bursts of inhibitory postsynaptic current (IPSC) were blocked by an AMPA receptor antagonist leaving individual IPSCs intact (Smith and Dudek, 1996). This implies that glutamatergic neurotransmission is involved for burst IPSC generation. The bursts of IPSC could come from outside basolateral amygdala or from the GABAergic interneurons inside the basolateral amygdala. Another possibility is that burst firing of glutamatergic inputs inside or outside the basolateral amygdala drives GABAergic interneurons to fire a group of action potentials accordingly.

In a subsequent study, spontaneous compound inhibitory postsynaptic potential/current (cIPSP/IPSC) with excitatory postsynaptic current (EPSC) was observed in projection neurons and bursts of action potentials in interneurons (Rainnie, 1999). From this, it could be postulated that GABA from bursting interneurons were the source for the cIPSCs. The question remains: what drives the depolarization and action potentials in interneurons? Because bursts of action potentials in projection neurons in basolateral amygdala were rare, the origin was speculated to be outside the nucleus. An important, but uncharacterized detail in this study was about the presumed EPSCs during the cIPSP/Cs. It could be inward current, but it was difficult to isolate the inward current because blocking $GABA_A$ current also removed all the cIPSCs themselves. Also the interneuron type for this phenomenon was not further pursued, hence it was unclear if any specific type of interneurons were involved or not (Klausberger and Somogyi, 2008). This phenomenon appeared in subsequent studies, but the mechanism was not tackled (Rainnie et al., 2004; Ohshiro et al., 2011; Popescu and Pare, 2011).

The clue for the generation mechanism came from an unexpected direction. Cholecystokinin (CCK) is an anxiogenic neuropeptide in animal and human behavior (Rotzinger and Vaccarino, 2003). When CCK was applied to the amygdala circuit in brain slices, it was strongly expected to increase excitatory neurotransmission. To the contrary, CCK increased inhibitory neurotransmission in projection neurons (Chung and Moore, 2007). In addition to the IPSPs of typical small amplitudes, they observed puzzling large amplitude IPSPs in the beginning part of the CCK effect (Fig. 2A).

To characterize the source of IPSPs further, they directly targeted interneurons in the next study (Chung and Moore, 2009a). Among the four distinct types of interneurons, CCK excited three types except one. Among the three types, spontaneous burstings of action potentials occurred only in one type of small proportion (about 5%). The burstings were of synaptic origin because the burstings were gone when synaptic transmission was blocked. The burstings from this type of interneurons could be the source of the large IPSPs in the previous report (Chung and Moore, 2007).

Because the anxiogenic effect and inhibitory neurotransmission induced by CCK were difficult to reconcile, the large IPSP was targeted for further investigation. However, it was difficult to study the large IPSPs separately because they were present only in half of the projection neurons after CCK and lasted less than 30 seconds (Chung and Moore, 2007). The phenomenon could be prolonged by increasing the extracellular K^+ concentration from 2.5 mM to 5 mM in the ACSF. Thus, the large IPSPs evoked by CCK could be associated with the spontaneous cIPSP/IPSC of the previous report (Chung and Moore, 2009b; Rainnie, 1999).

The cIPSP is infact cPSP because a group of EPSPs were always present during the IPSP (Chung and Moore, 2009b) (Fig. 2B). The temporal order of the EPSPs and the large amplitude IPSP was variable, but there should be a mechanism for this temporal relationship. This new concept of a concurrent reciprocal relationship between excitation and inhibition made the authors suggest that GABAergic input from the bursting interneuron excites the glutamatergic projection neuron within the basolateral amydala (Chung and Moore, 2009b) (Fig. 2C). In this model, GABA released from the interneuron after action potentials by CCK or serotonin opens the chloride channels by $GABA_A$ receptor activation. The effect to the postsynaptic glutamatergic neuron is not hyperpolarizing, but depolarizing and excitatory because of the high $[Cl^-]_i$ and efflux of negative Cl^-. The action potential from the glutamatergic neuron further excites the interneuron by activation of AMPA and NMDA type glutamate receptors on the interneuron to form a positive feedback loop.

If depolarizing GABA was a necessary condition for the generation of cPSP as predicted from this hypothesis, lowering $[Cl^-]_i$ will reduce the GABAergic depolarization, thus inhibiting the cPSP generation. Alternatively, if GABA was hyperpolarizing, lowering the chloride concentration will facilitate the cPSP. Bumetanide

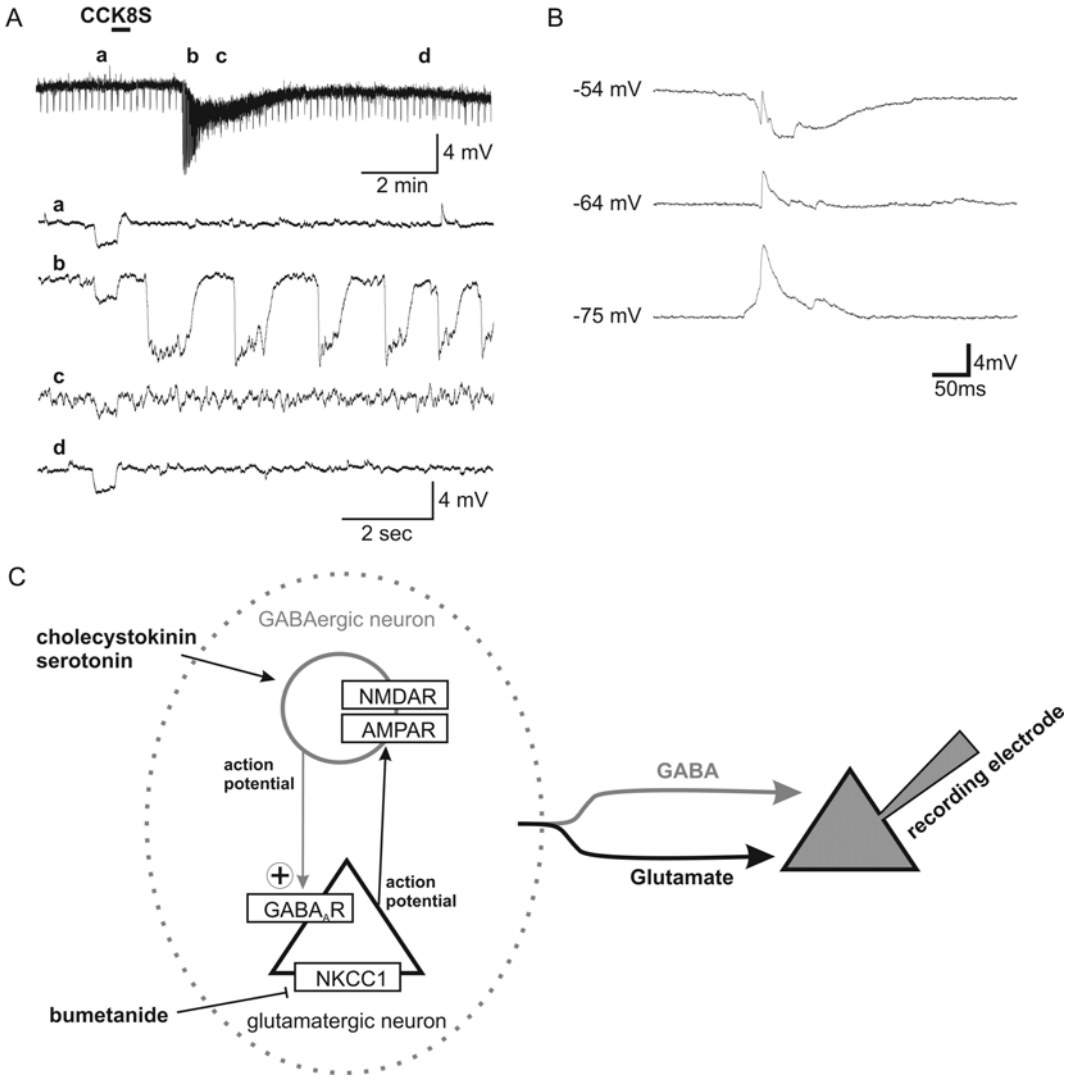

Fig. 2. Summary of a circuit activity in amygdala. (**A**) CCK8S (CCK receptor agonist) increases spontaneous IPSP frequency in basolateral amygdala neurons (Chung and Moore, 2007). CCK8S (1 μM, 30 sec) increases IPSP frequency with slight hyperpolarization. Recordings before (a), during (b and c), and after (d) CCK8S effect in slow time scale. Note the big IPSPs in (b). (**B**) In a single cPSP, hyperpolarizing and depolarizing PSPs occur together (Chung and Moore, 2009b). Membrane potential was manually adjusted with current injection. (**C**) Schematic model (Chung and Moore, 2009b). cPSPs are generated by positive feedback between GABAergic interneurons (e.g., axo-axonic cells) and a subset of glutamatergic cells. Increasing GABAergic transmission (e.g., by CCK or serotonin) initiates action potentials in a subset of glutamate cells (possibly via synapses on the axon initial segment), which in turn further excite interneurons by activation of AMPA and NMDA receptors. Thus, projection cells outside the cPSP generation loop (right side) may receive both increased GABAergic and glutamatergic input. However, bumetanide reduces cPSPs generation by NKCC1 inhibition. NKCC1 inhibition decreases Cl^- influx, lowers Cl^- reversal potential, thus makes the $GABA_AR$ activation by the interneuron be inhibitory or less excitatory at least. $GABA_AR$: $GABA_A$ receptor, AMPAR: AMPA type glutamate receptor, NMDAR: NMDA type glutamate receptor.

inhibited the cPSP generation which supports the idea of GABAergic depolarization (Chung and Moore, 2009b).

Implications

Unexpectedly, the cPSP in amygdala is a very similar phenomenon to that observed in GABAergic depolarization in neonatal hippocampus and in the subiculum from adult human epilepsy patients (Cherubini et al., 1991; Cohen et al., 2002). It should be stressed that the cPSP tested in amygdala occurred neither in neonatal animals nor in animals under seizure. With regard to seizure, amygdala is a place where the threshold for kindling is very low (Goddard, 1967; Okabe et al., 2003; Epps and Weinshenker, 2012). This suggests that amygdala is equipped with circuit components that can produce interictal-like neural

circuit activity with repeated external input. cPSP could be the corresponding circuit activity with the GABAergic depolarization property, which can lead to anxiety or seizure.

This opens the possibility of introducing CCC modulation from hippocampal epilepsy research into emotion related disorders in amygdala. Anxiety drugs, such as benzodiazepines, already modulate inhibitory transmission through the Cl^- current (Skolnick, 2012). The modulation of CCCs can provide another therapeutic target for intervention. This is very analogous to the situation in epilepsy research (Kahle and Staley, 2012). Bumetanide combined with current anti-anxiety drugs (benzodiazepine, for example) may be a better approach than the anti-anxiety drug alone.

Another target of manipulation could be the bursting-type interneurons. If we know the unique characteristics present in that type of interneuron, this knowledge can be used to manipulate the interneuron. For example, if the bursting interneuron has a specific type of receptors, we can target them as shown in other classes of interneurons (Freund, 2003; Freund and Katona, 2007).

CONCLUSION

The idea has gained acceptance that GABAergic depolarization or excitation is present during early development and neonatal epilepsy. In contrast, the possibility of a similar phenomenon in mature brain is under intense investigation in brain regions including SCN, cortex, hippocampus and basolateral amygdala. At neuronal circuit level, the contritubtion of GABAergic excitation in mature brain was suggested rather recently. GABAergic excitation could be an odd phenomenon deviating from the general rule, but sometimes the deviants are important in the world of pathology.

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