Expression and Functional Characterization of GABA Transporters in Crayfish Neurosecretory Cells

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The effect of GABA on membrane potential and ionic currents of X-organ neurons isolated from the crayfish eyestalk was investigated. Under voltage-clamp conditions, GABA elicited an inward Na⁺ current followed by a sustained outward chloride current. Sodium current was partially blocked in a dose-dependent manner by antagonists of GABA plasma membrane transporters such as β-alanine, nipecotic acid, 1-[2([diphenylmethylene]imino)oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO 711), and SKF89976-A at concentrations between 1 and 100 μM. This current was totally blocked by the combined application of NO 711 (5 μM) and β-alanine (50 μM). We obtained an EC₅₀ of 5 μM and a Hill number of 0.97 for the GABA transport mediated response. These results together with studies of immunolocalization using antibodies against neuronal vertebrate GABA transporters (GATs) indicate the presence of GAT-1 and GAT-3-like proteins in X-organ neurons. To isolate the sustained outward Cl⁻ current, extracellular free sodium solution was used to minimize the contribution of GAT activity. We concluded that this current was caused by the activation of GABA_A-like receptors with an EC₅₀ of 10 μM and a Hill number of 1.7.

To assign a functional role to the GATs in the X-organ sinus gland system, we determine the GABA concentration (0.46–0.15 μM) in hemolymph samples using HPLC.

In summary, our results suggest that a sodium-dependent electrogentic GABA uptake mechanism has a direct influence on the excitability of the X-organ neurons, maintaining an excitatory tone that is dependent on the circulating GABA level.

Key words: Procambarus clarkii; crustaceans; crayfish; X-organ sinus gland system; peptidergic neurons; GABA transporters; GABA_A receptors

GABA is the major inhibitory neurotransmitter in both the CNS and PNS of vertebrates (Iversen and Kelly, 1975; Martin, 1976) and invertebrates (Iversen and Kravitz, 1968; Kerkut et al., 1969; Sattelle, 1990). After the neurotransmitter has been released, its postsynaptic action must be terminated by activation of a sodium-dependent high-affinity GABA uptake system, which is present in presynaptic terminals and glial cells (Atwood, 1976; Krnjevic, 1984; Erecinska, 1987; Kanner and Schuldiner, 1987; Dingleidine et al., 1988). Thus, GABA transporters modify the neurotransmitter concentration in the synaptic cleft, producing a reduction in the availability of GABA acting on its receptors. Nevertheless, this is not the only postsynaptic effect, because GABA uptake is an electrogenic process that increases membrane conductance; therefore the transport activity might contribute directly to modify the excitability in postsynaptic neurons. It has been established that GABA transporters (GATs) belong to a sodium chloride-dependent transporter of 12 putative transmembrane helices. This family also includes transporters for serotonin, dopamine, norepinephrine, Gly, and Tau. Mammalian GABA transporters have been cloned and classified into four different members (GAT-1 to GAT-4) by differential amino acid sequences and pharmacological properties (Guastella et al., 1990; Borden et al., 1992; Clark et al., 1992; Liu et al., 1993; Yue et al., 1993; Swan et al., 1994). Two invertebrate GABA transporters that share certain similarities with the mammalian GATs have been cloned, expressed, and pharmacologically characterized. The first one was obtained from Manduca sexta embryos and designated MasGAT (Mbungu et al., 1995); it was functionally tested in Xenopus oocytes by measuring the [³H]GABA transport. The other one, isolated from the cabbage looper Trichoplusia ni and named TrnGAT (Gao et al., 1999), showed high identity with MasGAT as well as GAT-1, however, it was pharmacologically different from GAT-1 by the inability of cyclic GABA analogs, such as nipecotic acid, to inhibit [³H]GABA uptake by TrnGAT.

In this work we have examined the effects of GABA on membrane potential as well as the associated ionic currents in secretory neurons from the crayfish X-organ, defined as neurons with axonal terminals that are specialized for the release of hormones to the circulatory system (Duan and Cooke, 2000). The X-organ sinus gland system is the major neurosecretory structure in crustaceans; it participates in the control of different functions such as molting, regulation of blood sugar levels, tegumentary and retinal pigment position, locomotion, and neuronal activity (García and Aréchiga, 1998). Both spontaneous electrical activity and hormone release in X-organ neurons are regulated by environmental and endogenous influences such as light, stress, and circadian rhythms that are mediated by synaptic and hormonal influences. Recently, it has been shown that GABA and glutamate activate different ionotropic receptors and chloride conductances in crab X-organ neurons (Duan and Cooke, 2000), whereas serotonin, acting on metabotropic receptors, activates calcium-dependent high-conductance potassium channels in a neuronal subpopula-
tion that produces red pigment-concentrating hormone (Alvarado-Alvarez et al., 2000).

In the present paper, we have determined that GABA produces a depolarization associated with neuronal firing, followed by a repolarization that suppresses electrical activity. The excitatory phase was induced by the activation of an electrogenic uptake system, whereas the inhibitory phase was associated with the activation of a ligand-gated chloride conductance. To suggest a physiological role for the transporter-mediated current, we determined the extracellular GABA concentration.

**MATERIALS AND METHODS**

**Chromatography.** Animals were anesthetized in ice and then 200 µl of hemolymph samples were obtained from the coxal membrane articulations of the legs. These samples were precipitated with 0.4 M perchloric acid (1:1, v/v), and the resulting mixture was vortexed and filtered through Millipore membranes of 0.22 µm by centrifugation at 5000 rpm for 15 min. The sample for each animal was mixed with 10 µl of 0.1 M perchloric acid and derivatization reagent (6 µl), which was prepared as follows: 15 mg of o-phthalaldehyde was dissolved in 300 µl of methanol and added with 2.8 ml of 0.4 M tetratopassium borate buffer plus 25 µl of 2-β-mercaptoethanol.

Off-line derivatization procedure was performed, and the samples were vortexed for 120 sec, then they were injected into the HPLC column. The HPLC system consisted of Millenium 32 from Waters with column. The HPLC system consisted of Millenium 32 from Waters with phase A consisted of 40 mM sodium acetate buffer in 10% methanol, phase B consisted of 600 mM perchloric acid and derivatization reagent (6 µl), which was prepared as follows: 15 mg of o-phthalaldehyde was dissolved in 300 µl of methanol and added with 2.8 ml of 0.4 M tetratopassium borate buffer plus 25 µl of 2-β-mercaptoethanol.

Linear regression analysis, using the system manager (Waters).

Calibration curves were adjusted from chromatograms of standards that contained 0.1, 0.3, or 0.5 ng/µl of Asp, Glu, Gln, Gly, Tau, Ala, and GABA (Sigma, St. Louis, MO). The peak area ratios of the standards versus the GABA concentration were adjusted by least-squares analysis, using the system manager (Waters).

**Activation of a ligand-gated chloride conductance.** To suggest a mechanism for the excitatory phase, whereas the inhibitory phase was associated with the activation of a ligand-gated chloride conductance. To suggest a physiological role for the transporter-mediated current, we determined the extracellular GABA concentration.

**RESULTS**

**GABA hemolymph content**

Well-defined chromatographic peaks for Asp, Glu, Gln, Gly, Tau, Ala, and GABA were obtained from standard solutions containing 0.1, 0.3, or 0.5 ng/µl from each amino acid. Their retention times were determined at 5.2, 4.1, 4.9, 6.2, 6.9, 7.7, and 8.3 min, respectively (Fig. 1A). Peaks with identical retention times were identified in hemolymph samples. Figure 1B shows the chromatographic profile of a sample obtained at 6 P.M. that contained on average (n = 6 ± SD) the following concentrations (in µM): 1.6 ± 0.14 Asp, 3.8 ± 1.08 Glu, 3.6 ± 0.41 Gln, 3.3 ± 0.23 Gly, 1.7 ± 0.46 Tau, 2.9 ± 0.46 Ala, and 0.46 ± 0.11 GABA (Fig. 1B). These levels fluctuate during the day, but particularly the GABA levels decrease until 0.15 µM in samples obtained at midnight. To date there have been no published reports about the GABA hemolymph content or changes in its concentration during the day in crustaceans.

**Effects of GABA in cultured X-organ cells**

As illustrated in Figure 2, low GABA concentrations (0.1–0.5 µM) evoked a sustained depolarization that produced neuronal firing, whereas concentrations ranging between 1 and 10 µM evoked a complex response. This consisted of an early transient depolarizing phase associated with neuronal firing followed by a repolarization phase that suppresses the electrical activity. During GABA washout, a later depolarizing phase was evident, suggesting that the ionic current responsible for the early transient depolarizing phase was still active.

The GABA depolarizing phase was not blocked by picrotoxin, a well-characterized noncompetitive antagonist of GABA_A receptors (Sattelle, 1990). The experiment illustrated in Figure 3 shows that 5 µM GABA induced only a sustained depolarization associated with neuronal firing when the GABA pulse was applied in the presence of picrotoxin. This result is similar to those...
observed with low GABA concentration (Fig. 2). To explore the ionic nature of the depolarizing responses, the extracellular sodium was substituted with N-methyl-glucamine. Under this condition and even in the presence of picrotoxin, GABA did not evoke any changes in membrane potential (Fig. 3), suggesting that the depolarizing phase was sodium dependent, whereas the repolarizing phase could be attributed to an increase in chloride permeability.

To explore the changes in membrane conductance evoked by GABA, recordings were performed in either current- or voltage-clamp conditions in the perforated-patch configuration. The early depolarizing phase of the GABA response was associated with a transient inward current, whereas the repolarizing phase was associated with a sustained outward current that drastically reduced the membrane resistance, which in turn induced suppression of the neuronal firing in current-clamp recordings. As shown in Figure 4, the current amplitude and its corresponding decay in membrane resistance were dependent on the GABA concentration; the average reductions of the input resistance during GABA superfusion were 27, 80, and 90% for concentrations of 1, 10, and 100 μM, respectively. These effects are representative of those observed in six other neurons.

We next examined the voltage dependence of the GABA-induced currents using the perforated-patch configuration by plotting peak currents against membrane potentials from −120 to 20 mV. Figure 5A shows that the inward current progressively decreased as the holding potential was changed to positive values; although this current did not reverse, it was reduced to undetectable levels. Figure 5B depicts pronounced inward rectification to an extent that was undetectable between 0 and 20 mV (Fig. 5B, ○). This rectification is characteristic of GAT-associated currents (Quick et al., 1997). In contrast, the sustained outward current increased at depolarizing values and reversed at −75 mV (Fig. 5B, ●). This value corresponded to the chloride equilibrium potential (ECl−) estimated in our laboratory when we studied the chloride current generated by inhibitory glutamate receptors in crayfish X-organ neurons in culture. To confirm that the outward current is generated by chloride, in some experiments the cells were incubated with 5 μM picrotoxin during the GABA pulse applications (Fig. 5C, middle traces); such experimental conditions allowed us to isolate the inward current. These results suggest that chloride channels associated with GABA receptors mediate the sustained outward current, whereas the transient inward current could be caused by the activation of an electrogenic GABA uptake mechanism, similar to those described in the crayfish stretch receptor (Kaila et al., 1992) and the horizontal cells of the catfish retina (Cammack and Schwartz, 1993).
ner and Schuldiner, 1987). To demonstrate the Na⁺ dependence on extracellular sodium (Erecinska, 1987; Kan-
seven additional cells. The potential was maintained at
phase, but it was completely abolished when the external solution was
solution was modi-

– methylglucamine chloride. After the return to crayfish saline solution than those observed in low-sodium solu-

To corroborate that the outward current was caused exclusively by an increase in chloride permeability, cells were superfused with sodium-free solution, and current traces were obtained in the standard whole-cell configuration. In addition, two internal solutions were used to establish an \( E_{\text{Cl}^-} \) of 0 mV (perforated patch) or \( \sim -62.6 \) mV (standard whole cell). A voltage ramp was applied from −120 to 30 mV at 7.5 mV/sec from a holding membrane potential of −60 mV before and during GABA superfusion. In Figure 8A, traces a correspond to the steady-state current obtained before GABA superfusion when the \( E_{\text{Cl}^-} \) was 0 mV. The leak current at \( -60 \) mV was \( \sim -20 \) pA, and near \(-30 \) mV a negative slope current was activated that reached its maximum value at \(-20 \) mV; this was followed by an outward current. Traces b and c correspond to the net GABA-induced current obtained at \( E_{\text{Cl}^-} \) of both 0 and \( -62.6 \) mV, respectively. We subtract the control traces from those obtained during GABA superfusion to minimize the influence of the noninduced GABA currents. Figure 8B summarizes the results obtained with 16 (口) and 10 (●) neurons recorded under the two different \( E_{\text{Cl}^-} \) values. The average values were fitted to a linear regression that crossed the voltage axis at \(-62.6 \) and \(-3 \) mV, respectively.

As illustrated in Figure 8C (concentration–response curve), the chloride current started at 2 \( \mu \)M GABA, the \( EC_{50} \) corresponded to 10 \( \mu \)M, and the saturating concentration was reached between 50 and 100 \( \mu \)M. The linearized concentration–response curve yielded a Hill coefficient of 1.7 (Fig. 8D). This value indicates that at least two GABA molecules are necessary to activate a single GABA receptor, in agreement with previous studies (Sakmann et al., 1983; Hattori et al., 1984; Bormann and Clapham, 1985; White, 1992).

### Pharmacology of the GABA-induced transient inward current

Several compounds that block GABA plasma membrane transport in mammalian CNS were tested for their ability to antagonize the sodium inward current in X-organ neurons. The inhibitors of GAT-1, nipeptocic acid, NO 711, and SKF89976-A as well as \( \beta \)-alanine, a potent and selective GAT-3 inhibitor, blocked the sodium inward current in a dose-dependent manner. However, none of them alone was able to block fully the sodium current. In fact, the superfusion of nipeptocic acid (10 \( \mu \)M) induced a sustained inward current that reduced the amplitude of the GABA-induced inward current by 50% (Fig. 9A, top traces). As a consequence of

### Sodium dependence of the GABA-induced transient inward current

An important feature of GABA transport systems is their absolute dependence on extracellular sodium (Erecinska, 1987; Kanner and Schuldiner, 1987). To demonstrate the Na⁺ dependence of the inward current, we explored the GABA response in different extracellular sodium concentrations. Neurons were superfused with crayfish saline, low-sodium or free-sodium solutions, and for each condition 10 \( \mu \)M GABA was tested on both voltage- and current-clamp modes. In the low-sodium solution, the amplitude of inward current decreased consistently by 45%, whereas the sustained outward current increased by 25%. Furthermore, in the free-sodium solution the inward current was not detectable, and the sustained outward current increased by 70% (Fig. 6A, current traces). It was evident that during GABA removal, the time course of the later depolarization was more prominent in the crayfish saline solution than those observed in low-sodium solu-

Figure 3. Role of sodium and effect of picrotoxin on the GABA-evoked response in X-organ neurons. The GABA response in crayfish saline solution was modified by picrotoxin, which suppressed the repolarizing phase, but it was completely abolished when the external solution was switched to one in which all sodium chloride had been replaced with N-methylglucamine chloride. After the return to crayfish saline solution (bottom trace), the response to GABA was restored. The traces were obtained in the gramicidin–perforated-patch configuration; the washout interval between each GABA application was 3 min, and the membrane potential was maintained at \( -60 \) mV. Similar results were obtained from seven additional cells. The horizontal bars represent 1 min.

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**Isolation of the outward Cl⁻ current**

To corroborate that the outward current was caused exclusively by an increase in chloride permeability, cells were superfused with sodium-free solution, and current traces were obtained in the standard whole-cell configuration. In addition, two internal solutions were used to establish an \( E_{\text{Cl}^-} \) of 0 mV (perforated patch) or \( \sim -62.6 \) mV (standard whole cell). A voltage ramp was applied from −120 to 30 mV at 7.5 mV/sec from a holding membrane potential of −60 mV before and during GABA superfusion. In Figure 8A, traces a correspond to the steady-state current obtained before GABA superfusion when the \( E_{\text{Cl}^-} \) was 0 mV. The leak current at \(-60 \) mV was \( \sim -20 \) pA, and near \(-30 \) mV a negative slope current was activated that reached its maximum value at \(-20 \) mV; this was followed by an outward current. Traces b and c correspond to the net GABA-induced current obtained at \( E_{\text{Cl}^-} \) of both 0 and \(-62.6 \) mV, respectively. We subtract the control traces from those obtained during GABA superfusion to minimize the influence of the noninduced GABA currents. Figure 8B summarizes the results obtained with 16 (口) and 10 (●) neurons recorded under the two different \( E_{\text{Cl}^-} \) values. The average values were fitted to a linear regression that crossed the voltage axis at \(-62.6 \) and \(-3 \) mV, respectively.

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this reduction, an increase in the amplitude of GABA-induced outward chloride current was observed. Note in the current-clamp traces that during the nipecotic acid superfusion, the resting membrane potential was depolarized and the repolarizing phase reached more negative potentials (Fig. 9A, bottom traces). This effect can be attributed to a competitive blockage excerpted on the GABA transporters.

The pharmacological results are summarized in Figure 9B. Note that the combination of NO 711 (5 μM) with β-alanine (50 μM) totally abolished the sodium inward current. These observations strongly suggest that X-organ neurons expressed GABA transporters that have a pharmacological profile similar to that described in mammalian CNS. To confirm these results, we performed immunocytochemical studies for plasma membrane transporters GAT-1 and GAT-3.

**Immunocytochemistry**

Additional evidence for the presence of GABA receptors and transporters in the crayfish X-organ neurons is depicted in Figure 10. The antibody directed to GAT-1 recognizes the sequence located between aa 270 and 288, whereas the antibody directed to GAT-3 recognizes the C-terminal region (aa 613–627). Finally, the antibody directed to the GABA receptor recognizes the α1 subunit. Nonspecific binding of antibodies was determined as indicated in Materials and Methods. From the observation of 640 cells, we concluded that all neurons in X-organ expressed GABA-like receptor as well as the GAT-1- and GAT-3-like transporters.

**DISCUSSION**

Various excitatory actions of GABA have been described in crustaceans. In the visual system of the crayfish, GABA induces a depolarization of tangential cells in the medulla externa of the optic peduncle while decreasing their membrane conductance (Pfeiffer-Linn and Glantz, 1989). In a selected population of neurosecretory cells in the X-organ of the crayfish isolated eyestalk, GABA elicits depolarizing responses and bursts of action potentials; these effects are blocked by picrotoxin but not by bicuculline, and they involve a reduction of the input resistance (García et al., 1994). Recently, in the stomatogastric ganglion neurons, lateral pyloric and piloric, of the crab *Cancer borealis*, it was shown that GABA and muscimol elicits a picrotoxin-sensitive depolarizing response, the ionic nature of which remains unre-
sodium solution. In the low-sodium solution and was completely abolished in the free-
different sodium concentrations. Note that the inward current decreased
mean values increased when the inward current diminished.

Effects of GABA at the chloride equilibrium potential; isolation
of the sodium inward current. A. Responses to different GABA
concentrations obtained in the whole-cell voltage-clamp mode at –62.6
mV holding potential. B. Dose–response curve. Each point corresponds
to the mean ± SEM; the numbers of cells explored are in parentheses.
The mean values were adjusted at sigmoidal function, the EC$_{50}$ of which was 5 μM. C. Hill plots with a coefficient equal to 0.95.

To provide evidence that the depolarizing effect induced by
GABA in the X-organ neurons is mediated by a Na$^+$-dependent
GABA transport system, we reject the proposed possibilities as
follows. (1) The response cannot be attributed to bicarbonate
(Kaila and Voipio, 1987) because the saline used did not contain
bicarbonate, and the cells were recorded with patch pipettes filled
with saline buffered with HEPES. (2) The response cannot be
attributed to positive chloride equilibrium potential (Hales et al.,
1992, 1994) because the isolated chloride response caused by the
activation of GABA$_A$ receptors observed in the absence of extracellular Na$^+$ reversed at the expected values, as we have shown in
Figure 8. (3) Finally, the possibility that the depolarizing response could be caused by an increase in cation conductance
(Yarowsky and Carpenter, 1978; Swensen et al., 2000) seems
improbable because in the absence of extracellular sodium, the
potassium driving force operating in the opposite direction would
be capable of generating an outward transient current at any
membrane potential above its equilibrium potential.

uptake on the excitability of the X-organ neurons, which are
postsynaptic, because their terminals are specialized for hor-
monal release and do not establish synaptic contacts with other
cells. Our results suggest that circulating GABA levels could be
acting tonically on these neurons by operating electrogenic
GABA transporters capable of evoking a sustained depolarization
associated with neuronal firing.
The time course of GABA response in our experimental conditions could be explained by considering the EC_{50} values. We have found an EC_{50} of 5 \mu M for GABA transporters, whereas GABA_A receptors required higher concentrations (EC_{50} 10 \mu M) to be activated. The superfusion method that we have used enhanced this behavior, because the neurons were exposed initially to a low GABA concentration and because the transport system is more sensitive; it activated first, and later, when the steady-state concentration was reached, both sodium and chloride currents were present. However, at concentrations between 0.1 and 1.0, \mu M the sodium current generated by the transporters appears to be predominate (Fig. 2). Most of the radioligand binding studies for GABA_A receptors have characterized two high-affinity binding sites with K_d values in the low and high nanomolar range (Olsen et al., 1981; Olsen and Snowman, 1983). However, most electrophysiological studies have focused on the low-affinity site (micromolar range) (Anthony et al., 1993; Hevers and Lüddens, 1998). Because micromolar concentrations of GABA are generally required for the activation of receptor-gated chloride conductances in electrophysiological experiments (Krespan et al., 1984; Maconochie et al., 1994), it is reasonable to suppose that the lower-affinity agonist binding sites are physiologically relevant (Anthony et al., 1993). The EC_{50} value for the GABA receptor determined here corresponds to the low-affinity binding site. Concentration–response curves for GABA_A receptors are sigmoidal, with Hill coefficients between 1 and 2 (Sakmann et al., 1983; Hattori et al., 1984; Bormann and Clapham, 1985; Pinnock et al., 1988; Sattelle et al., 1991; White, 1992), suggesting that the binding of at least two GABA molecules is required to open the channel. In agreement with these findings, the Hill coefficient for the GABA receptor in X-organ neurons corresponded to 1.7.

The EC_{50} value estimated for GABA transport in X-organ neurons
neurons is comparable to the 5 μM Kₘ value obtained for GABA uptake both in brain tissue (Martin, 1976; Lewin et al., 1992) and in cells heterologously expressing GAT-1 (Guastella et al., 1990; Keynan et al., 1992; Ye and Sontheimer, 1996). In agreement with previous reports in which the electric uptake of GABA by GAT-1 expressed in Xenopus oocytes was explored (Kavanaugh et al., 1992; Mager et al., 1993), our study demonstrates that the Hill coefficient for GABA transporters in X-organ neurons is correlated with voltage-clamp current measurements, suggesting a stoichiometry of one net positive elementary charge per GABA molecule transported.

On the basis of the alternating access model proposed by Hilgemann and Lu (1999), an additional support for the short latency observed in the activation of the inward sodium current is the fact that those ions required for GABA transport are not limiting; they are available because of the superfusion of external solution. The binding of sodium to transporters facilitates GABA binding, and a new conformational state is induced immediately that translocates GABA together with its co-ions. Previous experiments suggest that the ions bond to the transporter despite the absence of GABA, converting the transporter to a state with higher affinity for GABA (Mager et al., 1993, 1996; Cammack et al., 1994). The sodium inward current generated by GABA transport in X-organ neurons was sustained when GABA receptors were blocked (Fig. 7). However, this kinetics depends on the superfusion velocity system used, because faster applications of GABA revealed a biphasic kinetics, as shown previously by Cammack et al., (1994).

In conclusion, GABA evoked on X-organ cells a sodium-dependent inward current sensitive to inhibitors of the GABA transport with an EC₅₀ of 5 μM, and simultaneously, but with an EC₅₀ of 10 μM, it activates a ligand-gated chloride current sensitive to picrotoxin. The blockage of the sodium-dependent inward current by GABA transport inhibitors supports this notion. In addition, the immunocytochemical evidence suggests that the antibodies against GAT-1, GAT-3, or GABA receptors from vertebrates recognized GABA transporters as well as GABA receptors present in X-organ neurons. Our findings provide experimental support for the hypothesis that hemolymph GABA levels can induce the expression of functional GABA transporters. The expression of GABA transporters is upregulated by extracellular GABA concentration (Bernstein and Quick, 1999). Therefore, the GABA hemolymphatic content in the crayfish could be induced by the expression of GATs in the X-organ sinus gland system. The study of the cirradian fluctuations in hemolymph GABA concentration and its relation to the expression of the GATs could be the key to understanding the mechanisms involved in its regulation.

REFERENCES


