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Brief Communication

A Genetic Method for Selective and Quickly Reversible Silencing of Mammalian Neurons

Hilde A. E. Lechner, Edward S. Lein, and Edward M. Callaway

Systems Neurobiology Laboratories, The Salk Institute, La Jolla, California 92037

Genetic methods for neuronal silencing have great promise for allowing selective inactivation of specific cell types within complex neural systems. Present methods, however, are limited in their reversibility by the slow time scale (days) of transcriptional regulation. We report the rapid and reversible inactivation of mammalian cortical neurons expressing the insect G-protein-coupled receptor AlstR (*Drosophila* allatostatin receptor) after application of its peptide ligand allatostatin (AL). The onset and reversal of inactivation could be achieved rapidly, within minutes. Moreover, the effects of AL were selective for AlstR-transfected neurons. The AlstR/AL system is therefore a promising genetic method for selective and quickly reversible silencing of neuronal activity.

Key words: AlstR; allatostatin; GIRK; neural silencing; insect receptor; cortical neurons

Historically, relating neural circuits to perception and behavior has relied heavily on the inactivation of neurons with reversible or irreversible lesion techniques. Conventional inactivation techniques, however, are of limited utility, because even very small regions of neural tissue contain many types of neurons and dendritic and axonal processes that are physically and functionally intertwined. Studies of neural circuits have indicated that different cell types within a single structure have unique patterns of connectivity and play unique roles in information processing (Zemelman and Miesenböck, 2001). Therefore, to relate cellular brain structure to function it is necessary to manipulate neural circuits at the level of individual cell types. Several genetic techniques are currently being developed that use cell type-specific promoters to restrict gene expression to cells of interest (Zemelman and Miesenböck, 2001). For example, Yoshida et al. (2001) have developed a cell type-specific permanent lesion technique by targeting immunotoxins to retinal starburst amacrine cells that express an antigen under the control of the metabotropic glutamate receptor 2 promoter.

An ideal method for inactivation would be not only genetic in nature, to allow for targeting of a specific cell type, but would also allow for reversible manipulation of neural activity on a fast time scale. One potential technique for quickly reversible inactivation is to use G-protein-coupled receptors that activate G-protein-coupled inwardly rectifying K (GIRK) channels (Dascal, 1997; Coward et al., 1998; Redfern et al., 1999; Mark and Herlitze, 2000), thereby hyperpolarizing the cell membrane and silencing neural activity. For example, a modified opiate receptor (Coward et al., 1998) that activates GIRK channels and is activated selectively by a synthetic ligand has been used successfully to slow the heart rate of transgenic mice expressing the receptor (Redfern et al., 1999). In addition to binding RASSL, however, the synthetic ligand binds to endogenous mammalian opiate receptors, which restricts its application to non-neural systems. To manipulate activity selectively in the brain using G-protein-coupled receptors, the receptor should be activated only by a specific ligand that does not cross-react with other endogenous receptors.

The *Drosophila* allatostatin receptor (AlstR) (Birgül et al., 1999) meets many of the criteria mentioned above, and was thus tested as a possible candidate for silencing mammalian neurons reversibly on a fast time scale. AlstR is a G-protein-coupled receptor involved in the regulation of juvenile hormone synthesis in insects. It is activated by the insect peptide allatostatin (AL; Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH_2) and is not activated by the ligands of related mammalian receptors (e.g., somatostatin, galanin, enkephalins, and proctolin) (Birgül et al., 1999). Nevertheless, AlstR has been shown to activate mammalian GIRK channels via $G_{i/o}$ proteins in *Xenopus* oocytes (Birgül et al., 1999). Here we show that the AlstR/AL receptor/ligand system can be used to silence activity in cortical neurons quickly and reversibly. We also show that this effect is selective for neurons that express AlstR. Neurons that do not express the receptor are unaffected by AL, suggesting that the AL does not cross-react with endogenous receptors of cortical neurons.

MATERIALS AND METHODS

Tissue culture. Brain slices were prepared from the visual cortex of 13- to 30-d-old ferrets as described previously (McAllister et al., 1995; Dantzker and Callaway, 1998). Briefly, animals were deeply anesthetized with sodium pentobarbitol (100 mg/kg, i.p.) and decapitated. Brains were extracted under sterile conditions and submerged in 4°C HEPES-buffered artificial CSF (ACSF) (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 24 dextrose, 10 HEPES, and 1 $CaCl_2$, pH 7.4. After the pia was removed, the visual cortex was cut into 400 μm parasagittal slices using a tissue slicer (Katz, 1987). Slices were then transferred onto cell-culture inserts (0.4 μm pore size; Falcon, Franklin Lakes, NJ) in six well culture dishes and fed with medium from below. The medium was composed of 50% basal Eagle's medium without glutamine, 25% HBSS, 330 mM dextrose, 10 mM HEPES, 200 mM L-glutamine, 10 U/ml penicillin–streptomycin (all from Invitrogen, San Diego, CA), and 25% horse serum (Hyclone, Logan, UT). Slices were then transfected using the Helios Gene Gun (Bio-Rad, Hercules, CA) and incubated in 5% CO_2 at 37°C for 1 d before recording. Similar procedures using rat brain tissue did not result in healthy slices.

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Correspondence should be addressed to Hilde A. E. Lechner, Systems Neurobiology Laboratories-C, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037. E-mail: lechner@salk.edu.

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Gene gun DNA transfer. Gold microcarriers (1.6 μm diameter; Bio-Rad) were coated with vector DNA at a concentration of 60 μg of DNA per 12.5 mg of gold, according to the supplier's instructions. The following plasmids were used (in μg): controls: 20 pEGFP-N1 (Clontech, Palo Alto, CA), 20 pcDNA3.1-GIRK1 (Dascal et al., 1993), and 20 pcDNA3.1-GIRK2 (Lesage et al., 1994); experimental: 15 pcDNA3.1-AlstR (Birgül et al., 1999), 15 pEGFP-N1, 15 pcDNA3.1-GIRK1, and 15 pcDNA3.1-GIRK2. All transgenes were expressed under the control of a cytomegalovirus immediate-early promoter. Slices were shot at 90–100 psi. Co-transfection using gold particles coated with multiple plasmids has been reported to be nearly 100% (Arnold et al., 1994). Thus, green fluorescent protein (GFP)-labeled neurons were assumed to also express the allatostatin receptor and/or the GIRK channel subunits.

Recording. Cells were recorded 24–48 hr after transfection. Patch electrodes (8–14 MΩ resistance) filled with (in mM): 140 K-gluconate, 8 NaCl, 10 HEPES, 1.3 EGTA, 2 ATP, and 0.3 GTP, pH 7.7, along with 285 mOsm KOH, were used for whole-cell current-clamp recordings. Slices were perfused with ACSF (in mM): 124 NaCl, 5 KCl, 1.3 $MgSO_4$, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, and 11 dextrose at room temperature. The peptide AL (Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH_2; 1 nM in ACSF) was applied by perfusion at a rate of ~800 μl/min. AL was washed out by replacement with normal ACSF at a flow rate of ~200 μl/min, corresponding to a complete exchange of the bath volume (3 ml) every 15 min. Physiological measures before, after 8 min of perfusion with AL, and 15–20 min after washout were compared.

RESULTS

AlstR was expressed in neonatal [postnatal day 13 (P13) to P30] ferret visual cortex slices using "Biolistics" particle-mediated gene transfer. Neurons were transfected with AlstR, GIRK channel subunits 1 and 2 (Dascal, 1997; Mark and Herlitze, 2000), and enhanced GFP (EGFP). Control neurons were transfected with GIRK1, GIRK2, and EGFP. The overexpression of GIRK channel subunits was necessary, because their natural expression is under developmental regulation. Newborn cortical neurons of mice do not reach adult levels of GIRK channel expression before P10 (Chen et al., 1997), and expression is likely to be further delayed in the more slowly developing ferret cortex. Indeed, neonatal cortical neurons of the ferret did not respond to the $GABA_\beta$ agonist baclofen (data not shown), suggesting that GIRK channels were not yet expressed at sufficient levels. At 24–36 hr after transfection, the membrane potential, input resistance, and excitability (current required to reach spike threshold) of AlstR-transfected and control neurons were determined before, during, and after addition of 1 nM allatostatin to the bath (Fig. 1*A,B*).

Specificity of AL-induced effects

Before perfusion with AL, AlstR-transfected neurons and control cells did not differ in resting membrane potential, input resistance, or excitability (Table 1). After application of AL, however, AlstR-transfected neurons were quickly hyperpolarized, whereas untransfected neurons remained unaffected (Fig. 1). In AlstR-transfected cells, application of 1 nM AL produced a change in resting membrane potential of -6.7 ± 0.7 mV compared with baseline values ($n = 15$; $p < 0.0001$), and a decrease in input resistance to $48 \pm 7\%$ of the initial value ($n = 15$; $p < 0.001$) within several minutes (Fig. 2*A1,2*). Control cells showed no change in resting membrane potential (0.1 ± 0.9 mV; $n = 9$) and a small but nonsignificant increase in input resistance ($116 \pm 17\%$; $n = 9$), possibly as a result of dialysis during whole-cell patch recordings. The amplitude of depolarizing current pulses necessary to elicit an action potential increased 13-fold (13.0 ± 4.1; $n = 15$; $p < 0.05$) in AlstR-transfected cells, indicating greatly reduced excitability (Fig. 2*A3*). Control cells, in contrast, showed no change in excitability (1.1 ± 0.1; $n = 9$). These results indicate that AL can be used to, in effect, silence AlstR-transfected cortical neurons. Moreover, these results suggest that the effects of AL are specific and that the physiological properties of untransfected neurons remain unchanged.

Reversibility of AL-induced effects

In a subset of AlstR-transfected cells ($n = 8$) we tested whether, and how quickly, silencing was reversible. The effects of AL typically subsided within 15 min after AL removal (Fig. 1*A2*). At a perfusion rate of ~200 μl/min and a bath volume of ~3 ml of saline, this time was approximately equivalent to one exchange of the bath volume. At 15 min after initiation of AL washout, the membrane potential of AlstR-transfected cells returned to within $4.4 \pm 1.1\%$ of its original value (Fig. 2*B1*; Table 1). Input resistance and excitability also returned to approximately original values (Fig. 2*B2,3*; Table 1). Considering the limitations for efficiently removing AL by perfusion, it is likely that the effects of AL could, in principle, be reversed even faster.

DISCUSSION

Together, these experiments demonstrate the potential of AlstR/AL as a genetic system for selectively and quickly hyperpolarizing mammalian neurons in a reversible manner. Given the large decreases in excitability that we observed in our *in vitro* preparation, it is likely that AlstR/AL will also be able to, in effect, silence cortical neurons under physiological conditions *in vivo*.

Quickly reversible silencing versus transcriptionally regulated inactivation

The AlstR/AL system represents an important new tool among the growing number of techniques for neuronal inactivation. First, because AlstR is not known to be activated by mammalian ligands (Birgül et al., 1999), its constitutive expression in the mammalian brain should not result in complications related to long-term plasticity or developmental compensation mechanisms. Second, the activation of the receptor by its ligand allows for much faster manipulations of neural activity than can be achieved by genetically regulated methods of silencing, such as the inducible expression of K+ channels (Johns et al., 1999; Zemelman and Miesenböck, 2001). Although transcriptional inactivation methods are well suited for long-term silencing of neurons (e.g., in developmental studies), the high degree of selectivity and temporal control provided by ligand-induced silencing via the AlstR/AL system is uniquely suited for switching identified populations of neurons off and on quickly (e.g., during *in vivo* neurophysiological experiments and in awake behaving animals).

The advantages and disadvantages of each technique have to be weighed for each application. For example, the sustained increase in K+ conductance associated with slow transcriptional regulation of K+ channel expression may lead to irreversible silencing and cell death in some systems (Nadeau et al., 2000). AL-induced silencing, in contrast, is quickly turned on and off, which should limit any cytotoxic effects associated with increased K+ conductance. For long-term silencing, however, the AlstR/AL system may not be useful. Like other G-protein-coupled receptors, it is likely that the AlstR may be desensitized or internalized with prolonged exposure to its ligand.

The effectiveness of inactivation by the AlstR/AL system, in some cases, may also be dependent on the level of endogenous GIRK channel expression (e.g., in a given cell type or at an early developmental stage). In such systems, cotransfection with GIRK channel subunits, as in this study, may be necessary to effectively inactivate neurons using AlstR/AL. The requirement to coexpress GIRK channels in such cases should not preclude the effective use of the AlstR/AL system. The *in vitro* expression of

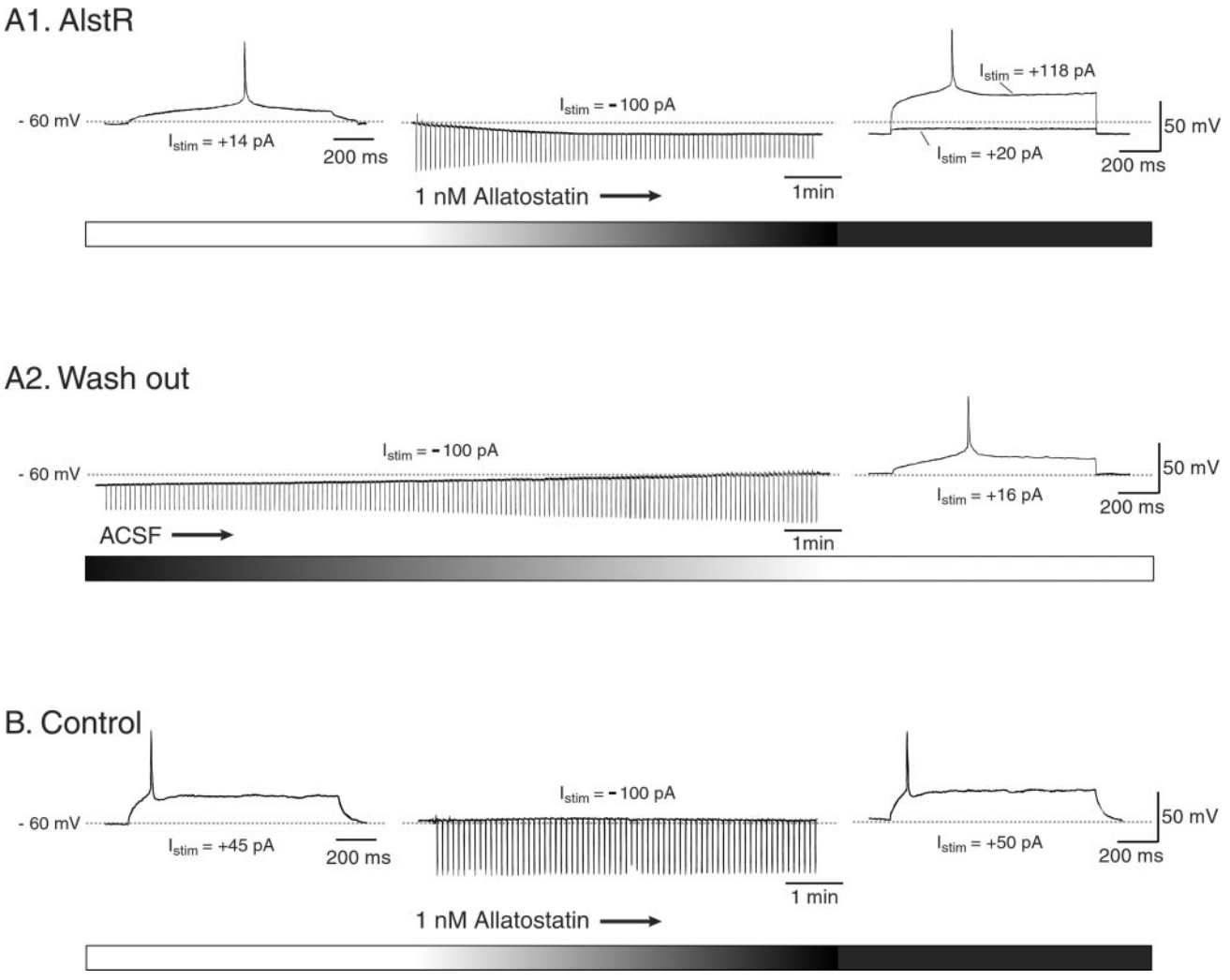

Figure 1. Silencing of cortical neurons with the AlstR/AL receptor/ligand system. *A1*, The spike threshold of a representative AlstR-transfected neuron was determined by a series of depolarizing current pulses (I_{stim}, 1 sec duration; *left panel*) before addition of allatostatin to the bath (*gradually shaded bar*). In this example, the spike threshold was found to be at +14 pA. Before and after the onset of perfusion with 1 nM AL, input resistance was monitored by hyperpolarizing current pulses at 5 sec intervals (*middle panel*). Input resistance and resting membrane potential decreased within minutes of AL application. The amount of current necessary to elicit an action potential in the presence of AL (+118 pA) was greatly increased with respect to the initial values (*right panel*). *A2*, The effects of AL were reversible over the course of several minutes by washing out AL with normal ACSF. Input resistance, membrane potential (*left panel*), and spike threshold (*right panel*) returned to approximately initial values after a perfusion time approximately equivalent to the time required for the exchange of one bath volume (see Results). *B*, Input resistance, membrane potential, and spike threshold of control neurons were unaffected by application of AL.

Table 1. Electrophysiological properties of AlstR-transfected and control neurons before and during application of AL

	Membrane potential (mV)			Input resistance (MΩ)			Threshold current (pA)		
	ACSF	1 nM AL	Wash	ACSF	1 nM AL	Wash	ACSF	1 nM AL	Wash
Control (n = 9)	−59.8 (1.0)	−59.8 (1.2)	—	448 (74)	472 (54)	—	41.4 (10.2)	44.2 (11.6)	—
AlstR (n = 15)	−61.2 (0.9)	−67.9 (1.0)***	See subset	455 (50)	197 (29)***	See subset	28.9 (3.2)	321.3 (76.1)*	See subset
AlstR subset (n = 8)	−62.0 (1.2)	−70.1 (1.1)**	−62.4 (1.8)	493 (71)	147 (20)**	591 (185)	26.5 (3.7)	452.3 (95.1)*	54.0 (27.0)

A subset of AlstR-transfected neurons was also tested for reversibility of AL-induced effects (wash). Data are given as means ± SEM. Statistically significant changes are indicated as follows: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

GIRK subunits in cortical neurons of the neonatal ferret visual cortex did not affect the membrane potential or survival of those neurons compared with EGFP-transfected neurons (data not shown). In any case, we expect that these limitations will not be common in the application of the AlstR/AL system to adult brain tissue, because endogenous expression of GIRK channels is common to the great majority of adult mammalian brain areas (Karschin et al., 1996). However, it will be necessary for any given system to determine whether the neurons to be inactivated express GIRK channels or are inactivated by drugs (e.g., baclofen) whose effects are mediated by GIRK channels.

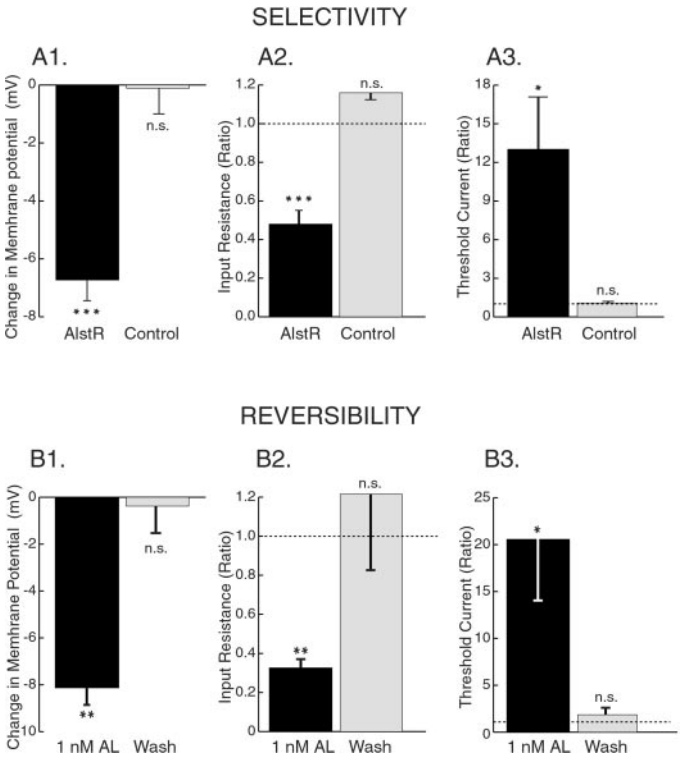

Figure 2. Selectivity and reversibility of AL-induced effects. *A1*, Effect of AL on membrane potential in AlstR-transfected ($n = 15$; *black bar*) and control neurons ($n = 9$; *light bar*). AL produced a significant decrease in membrane potential in AlstR-transfected neurons (-6.7 ± 0.7 mV; $^{***}p < 0.0001$). Control neurons showed no change (0.1 ± 0.9 mV). *A2*, Ratio of input resistance before and after application of 1 nM AL in AlstR-transfected and control neurons. Input resistance decreased to $48 \pm 7\%$ of the original value ($^{***}p < 0.0001$) for AlstR-transfected neurons ($p < 0.001$), but control neurons were unaffected ($116 \pm 17\%$). *A3*, The ratio of spike threshold (current amplitude necessary to elicit an action potential) before and after application of 1 nM AL in AlstR-transfected and control neurons. Spike threshold increased 13-fold for AlstR-transfected neurons ($1300 \pm 410\%$; $p < 0.05$), but control neurons were unaffected ($110 \pm 10\%$). *B1*, After removal of AL from the bath, the decrease in membrane potential induced by 1 nM AL for a subset of eight AlstR-transfected neurons tested (-8.1 ± 0.7 mV; $p < 0.001$) recovered to its original value (-0.4 ± 1.1 mV). *B2*, Input resistance also returned from reduced levels ($33 \pm 43\%$; $p < 0.001$) to approximately original values ($122 \pm 39\%$). *B3*, The reversal of AL-induced effects on membrane potential and input resistance was accompanied by a recovery of excitability, as measured by the current amplitude for eliciting an action potential, from $2058 \pm 654\%$ ($p < 0.05$) to approximately original values ($186 \pm 75\%$). $^{***}p < 0.0001$; $^{**}p < 0.001$; $^{*}p < 0.05$. *n.s.*, Not significant. *Dotted lines* indicate 100% (no change).

Targeted delivery of "silencing genes"

Techniques for delivering AlstR to targeted cell types are currently being developed using cell type-specific promoters to drive the expression of AlstR. Cell type-specific promoters have been used successfully in transgenic mice to restrict the expression of transgenes to brain areas and defined neuronal populations of interest (Mayford et al., 1996; Tsien et al., 1996; Yoshida et al., 2001; Zemelman and Miesenböck, 2001). In combination with viral delivery methods that are currently being developed in this and other laboratories, cell type-specific expression could also be achieved in animals that are not amenable to transgenic approaches (McCown et al., 1996; Ehrengruber et al., 2001).

Ligand application

Additional experiments will have to explore the best way to administer allatostatin *in vivo*. For example, direct and local application of the peptide into brain areas of interest may be preferable to intravenous injection, because this should allow for greater temporal control over the onset and reversal of AL-induced effects.

The results presented here, together with recent improvements in targeting specific cell types in a variety of animal models, suggest that the AlstR/AL system can become a powerful tool for studying the contribution of defined populations of a particular neural cell type to information processing, cognition, and behavior.

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