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Resource

Remote Control of Behavior through Genetically Targeted Photostimulation of Neurons

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Summary

Optically gated ion channels were expressed in circumscribed groups of neurons in the *Drosophila* CNS so that broad illumination of flies evoked action potentials only in genetically designated target cells. Flies harboring the "phototriggers" in different sets of neurons responded to laser light with behaviors specific to the sites of phototrigger expression. Photostimulation of neurons in the giant fiber system elicited the characteristic escape behaviors of jumping, wing beating, and flight; photostimulation of dopaminergic neurons caused changes in locomotor activity and locomotor patterns. These responses reflected the direct optical activation of central neuronal targets rather than confounding visual input, as they persisted unabated in carriers of a mutation that eliminates phototransduction. Encodable phototriggers provide noninvasive control interfaces for studying the connectivity and dynamics of neural circuits, for assigning behavioral content to neurons and their activity patterns, and, potentially, for restoring information corrupted by injury or disease.

Introduction

Since Galvani (Galvani, 1791), neuroscientists have stimulated neurons directly to probe their function and connectivity, and, more recently, to interface neural and electronic circuits. Because most artificial stimuli are delivered by electrodes or focused light beams (Fork, 1971; Farber and Grinvald, 1983; Callaway and Katz, 1993), they tend to target anatomical locations rather than functional populations of neurons. And because specificity is determined by which site is stimulated, the relative positions of stimulus and target cell(s) must be carefully controlled, making behavioral experiments in unrestrained animals difficult.

These difficulties can potentially be resolved if specificity is encoded biologically (Zemelman and Miesenböck, 2001; Zemelman et al., 2002; Miesenböck, 2004): if only the intended target cells are equipped with a "receiver" that allows them to decode a publicly broadcast stimulus, multiple targets might be addressed simultaneously and selectively, regardless of their number and spatial positions. The capacity to control defined populations of neurons noninvasively would represent a significant step in moving neuroscience from passive observation—which neuronal activity patterns are correlated with a given behavior?—to active and predictive manipulation of behavior.

We report experiments in *Drosophila* that realize this scenario. Unfocused laser light played the part of the publicly broadcast stimulus; genetically encoded "phototriggers" of action potentials (Zemelman et al., 2002; Zemelman et al., 2003) served as the cell type-specific receivers that transduced the optical signal into electrical activity. Brief pulses of laser light allowed us to activate genetically circumscribed groups of central neurons and control specific behaviors in flies moving freely within the optical field. Following validating experiments on the well-defined reflex circuit responsible for escape behaviors, genetically targeted photostimulation was used to investigate the role of dopaminergic neurons in the control of movement. We found that an acute increase in dopaminergic signaling alters the extent of locomotor activity and the walking patterns in which this activity is expressed.

Results and Discussion

Genetically Encoded Phototriggers of Action Potentials

The genetically encoded phototriggers operate according to a photochemical key-and-lock mechanism (Zemelman et al., 2003). Ligand-gated ion channels—the ionotropic purinoceptor $P2X_2$ (Brake et al., 1994; Valera et al., 1994; Zemelman et al., 2003) or the capsaicin receptor TRPV1 (Caterina et al., 1997; Tobin et al., 2002; Zemelman et al., 2003)—are expressed in neurons that normally lack them, and the agonists that gate the conductances of these channels are rendered biologically inert by chemical modification with photoremovable blocking groups (Kaplan et al., 1978; McCray and Trentham, 1989; Zemelman et al., 2003). The initiation of an action potential requires a flash of light that liberates free agonist from the caged precursor (the key) and a target neuron that has been genetically programmed to express the cognate receptor (the lock).

The optimal phototrigger for stimulating fly neurons was selected by testing the two candidate channels, $P2X_2$ and TRPV1 (Zemelman et al., 2003), in the *Drosophila* Schneider S2 cell line. Transfected S2 cells responded to applications of 100 μM ATP or 10 μM capsaicin with cytoplasmic Ca^{2+} increases (Figure 1). The $P2X_2$-mediated Ca^{2+} current disappeared in Ca^{2+}-free extracellular solution, consistent with Ca^{2+} entry across the plasma membrane (Figure 1A). The TRPV1 current, in contrast, was insensitive to reductions in extracellular Ca^{2+} but vanished if Ca^{2+} stored in the endoplasmic reticulum (ER) was depleted during a 40 min preincubation with 5 μM thapsigargin, an inhibitor of the ER-localized Ca^{2+} ATPase (Thastrup et al., 1990) (Figure 1B). Unlike mammalian neurons, which transport heterologously expressed TRPV1 to the cell surface (Zemelman et al., 2003), insect cells appeared to retain the channel in the ER, where it was functional and could be gated by membrane-permeable capsaicin (Tominaga et

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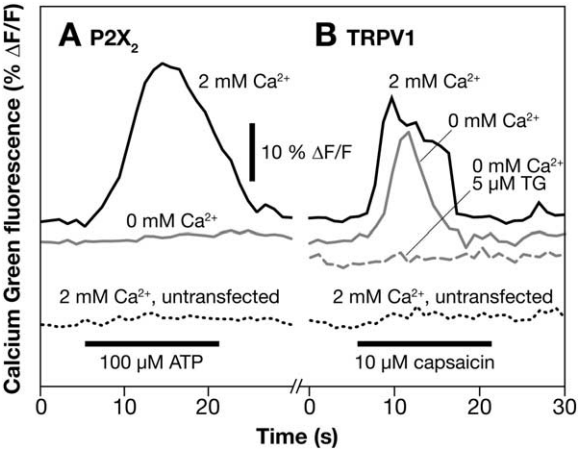

Figure 1. Stimulation of Ca^{2+} Influx in the *Drosophila* S2 Cell Line

S2 cells expressing cDNAs encoding (A) a covalently linked trimer of rat $P2X_2$ or (B) rat TRPV1 were loaded with 10 μM Calcium Green 1-acetoxymethyl ester. Traces represent fluorescence recordings, acquired by wide-field microscopy at 1 Hz, of individual cells in the presence (black lines) or absence (gray lines) of 2 mM extracellular Ca^{2+} or after treatment with thapsigargin (TG) in Ca^{2+}-free solution (dashed gray line in [B]). S2 cells lacking $P2X_2$ or TRPV1 do not express ATP- or capsaicin-gated Ca^{2+} conductances (dotted black lines in [A] and [B]).

al., 1998) to produce cytoplasmic Ca^{2+} increases that were not coupled to voltage changes across the plasma membrane. Of the two phototrigger candidates, only $P2X_2$ is therefore in a position to trigger action potentials in *Drosophila* neurons. Because the fly genome lacks purinoceptor sequences (Littleton and Ganetzky, 2000), photoreleased ATP is expected to act selectively on the genetically designated targets. Experiments presented below confirm this expectation.

Flies carrying a GAL4-responsive *UAS-P2X$_2$* transgene were prepared and crossed with the driver line *Nrv2-GAL4*, which directs $P2X_2$ expression throughout the nervous system (Sun et al., 1999). Development and behavior of flies of genotype *Nrv2-GAL4:UAS-P2X$_2$* were indistinguishable from those of the parental strains, suggesting that the ubiquitous expression of $P2X_2$ did not perturb neuronal function. In a few instances where $P2X_2$ was massively overexpressed, however, characteristic defects—in all likelihood due to a current leak—appeared. These defects ranged from subtle incoordination in the case of the pan-neuronal driver line *elav-GAL4* (Lin and Goodman, 1994) to a striking but mysterious reduction in adult life span, without behavioral deficits, in flies expressing $P2X_2$ under the control of the choline acetyltransferase promoter (Yasuyama and Salvaterra, 1999) in cholinergic neurons (mean survival time after eclosion = 2.58 ± 1.34 days, n = 329). These side effects of $P2X_2$ overexpression are most probably preventable by fine adjustments of expression levels with the help of transcriptional regulators. In the present set of experiments, behavioral or physiological signs of current leaks were detected with two (*elav-GAL4* and *Cha-GAL4*; see above) of the eight *GAL4* driver lines tested (see "*Drosophila* Strains" in Experimental Procedures).

To examine the ability of $P2X_2$ to trigger action potentials in a pharmacologically accessible preparation in vivo, third-instar larvae expressing $P2X_2$ in cholinergic neurons were challenged with purine nucleotides while the membrane potential of an abdominal muscle fiber was recorded. In this experimental configuration, $P2X_2$-mediated stimulation of cholinergic afferents is expected to trigger action potentials in glutamatergic motor neurons. These, in turn, will produce excitatory junction potentials (EJPs) in the recorded muscle.

In the absence of nucleotide, and indistinguishably in the presence of 200 μM GTP, which lacks agonist activity on $P2X_2$ (Valera et al., 1994), the membrane potential of the muscle fiber showed miniature EJPs (Figure 2A, left inset). Full-scale EJPs were seen exclusively during occasional spontaneous bursts of activity (not shown) and in the presence of 200 μM ATP (Figure 2A, right inset). Pulsed ATP applications caused trains of EJPs at frequencies of 18.6 ± 1.18 s^{-1} (mean ± SD) to appear and disappear with perfusion-limited latencies. The EJPs were action potential driven, as they could be blocked by 200 nM tetrodotoxin (not shown). Their amplitudes (mean ± SD = 22.0 ± 0.24 mV; n = 515) lay at the lower end of the EJP amplitude distribution reported for direct electrical stimulation of the segmental nerves innervating muscle fibers 6 or 7 (20–40 mV; Broadie, 2000). Because these nerves each contain two motor neurons (Hoang and Chiba, 2001) that generate coincident EJPs during electrical stimulation, the amplitudes of the ATP-triggered EJPs are consistent with synchronized or unsynchronized spikes in one or both of these neurons.

ATP exerted its effect exclusively through $P2X_2$: larvae lacking expression of the receptor transgene also lacked responsiveness to the nucleotide (Figure 2B). The comparison with control animals (Figure 2B) revealed that larvae expressing $P2X_2$ in cholinergic neurons (Figure 2A) exhibited an ~2.5-fold higher minature EJP (mEJP) frequency (mean ± SD = 5.7 ± 2.64 s^{-1} versus 2.2 ± 1.85 s^{-1} in controls; n = 602 and 514, respectively; $p < 0.001$) and a higher average mEJP amplitude (mean ± SD = 1.2 ± 0.65 mV versus 0.8 ± 0.32 mV in controls; $p < 0.001$), possibly due to the more common occurrence of composite events. We attribute the increased mEJP rate, like the brevity of adult life discussed above, to the unusual strength of the choline acetyltransferase promoter (Yasuyama and Salvaterra, 1999), which causes the expression of high $P2X_2$ levels and, presumably, a small Ca^{2+} current leak that could be remedied by titration of $P2X_2$ expression levels.

"Command System" Control of Movement

Invertebrates display a variety of stereotyped motor behaviors thought to be controlled by small sets of neurons. The most clearly defined of these so-called "command neuron systems" (Wiersma and Ikeda, 1964; Kupfermann and Weiss, 1978; Kupfermann and Weiss, 2001) in insects is the giant fiber (GF) system responsible for escape movements such as jumping and the initiation of flight (Koto et al., 1981; Thomas and Wyman, 1984; Wyman et al., 1984). The circuit (Figure 3, left) consists of the GF neurons proper, a pair of large interneurons in the brain (Koto et al., 1981), and their

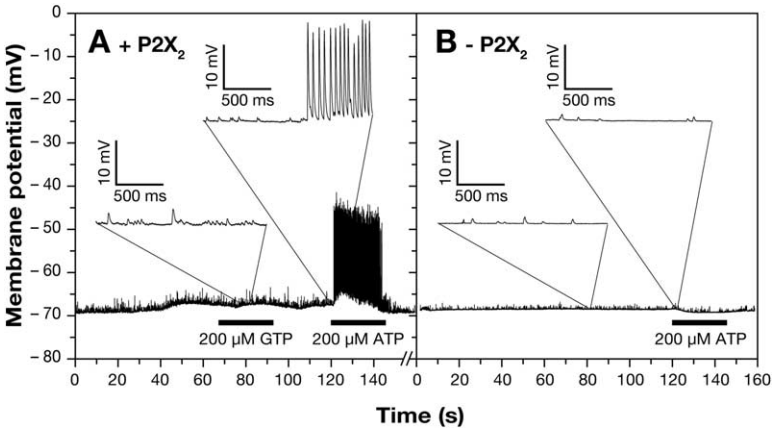

Figure 2. Stimulation of Excitatory Junction Potentials at the Larval Neuromuscular Junction

The membrane potential of muscle fiber 6 was recorded during superfusion of purine nucleotides in third-instar larval filets.

(A) The membrane potential of the muscle fiber in an animal expressing $P2X_2$ in cholinergic neurons shows miniature Excitatory Junction Potentials (EJPs) in the presence of GTP (left inset) and full-scale EJPs in the presence of ATP (right inset).

(B) The membrane potential of the muscle fiber in an animal carrying the *UAS-P2X*$_2$ responder transgene but lacking expression (owing to the lack of a *GAL4* driver transgene) shows miniature EJPs (left and right insets). Full-scale EJPs are absent even in the presence of ATP (right inset).

synaptic targets in the thoracic ganglion, the tergotrochanteral (jump) muscle motor neuron (TTMn; Thomas and Wyman, 1982) and the peripherally synapsing interneuron (PSI; Tanouye and Wyman, 1980), which controls motor neurons innervating the dorsal longitudinal flight musculature (Tanouye and Wyman, 1980). Its simplicity, genetic tractability (Allen et al., 1999; Jacobs et al., 2000), and clear-cut function made the GF system an ideal first testing ground for artificially induced behaviors. Experiments were performed in a cylindrical quartz glass arena (diameter 8 mm, height 2 mm) that could be illuminated with 355 nm laser light, a near-optimal wavelength for photoreleasing ATP from the caged precursor (McCray and Trentham, 1989; Zemelman et al., 2003). Caged ATP was microinjected into the CNS of adult males (40–70 mM DMNPE-ATP in 13–41 nl artificial hemolymph), which were analyzed after >10 min of recovery and within a 1 hr window following the injection.

Driver lines *GAL4-c17* (Allen et al., 1999; Trimarchi et al., 1999) and *shakB-GAL4* (Jacobs et al., 2000), respectively, were used to express $P2X_2$ in the GF neurons or their mono- and disynaptic targets in the thoracic ganglion (the group consisting of TTMn, PSI, and DLMns; Figure 3). Flies harboring the phototrigger in either segment of the circuit developed, behaved, and aged like the parental strains or wild-type control animals.

The enhancer trap line *GAL4-c17* expresses $P2X_2$ in only two of the approximately 100,000 neurons in the fly CNS, the GF neurons (Allen et al., 1999; Trimarchi et al., 1999). In addition, the enhancer element is active in eight peripheral sensory neurons of the hair plate, a proprioceptive organ of the prothoracic leg (Trimarchi et al., 1999). Because the hair plate lies outside the blood-brain barrier (Yellman et al., 1997; Carlson et al., 2000) that confines injected DMNPE-ATP to the CNS, only the two GF neurons are possible targets for photostimulation in these flies.

The *shakB-GAL4* line expresses $P2X_2$ in 11 pairs of neurons in the thoracic ganglion (Jacobs et al., 2000). Seven of these pairs are direct or indirect synaptic targets of the GF neurons: the TTMns, the five pairs of DLMns, and the PSIs (Jacobs et al., 2000). In addition, the *shakB* promoter is active in one pair of neurons in the midbrain, two pairs of neurons in the subesophageal ganglion, and a handful of cells in the abdominal ganglion (Jacobs et al., 2000). Together, these neurons represent ~0.05% of the neuronal population of the CNS. Importantly, the expression pattern of *shakB-GAL4* excludes the GF neurons (Jacobs et al., 2000), which are targeted selectively by strain *GAL4-c17* (Allen et al., 1999; Trimarchi et al., 1999).

Brief UV illumination (8 mW mm^{-2} for 150–250 ms) of flies expressing the phototrigger in either of these two small, highly restricted sets of neurons residing in nonoverlapping segments of the GF system elicited the typical GF-mediated escape movements (Thomas and Wyman, 1984; Wyman et al., 1984; Trimarchi and Schneiderman, 1995): leg extension, jumping, wing opening, and high-frequency wing flapping (Figures 3A and 3B; Movies S1 and S2); actual flight was prohibited by the small dimensions of the lidded arena. Laser pulses repeated at 2.5 s intervals caused identical, transient responses (Movie S2), implying that $P2X_2$ did not desensitize appreciably, and that photoreleased ATP was quickly consumed or cleared from the extracellular space. As expected for a photochemical key-and-lock mechanism, expression of $P2X_2$ and photolysis of caged ATP were required together for the successful reconstitution of GF-mediated behaviors (Figures 3C and 4, groups 3a–3d and 4).

Efficacy of Genetically Targeted Photostimulation

Photostimulation of the GF neurons or their synaptic targets in the thoracic ganglion elicited escape movements in 63% and 82% of trials, respectively (Figure 4D, groups 1a and 1b). These success rates were considerably higher than the frequencies of escape movements evoked by physiological stimuli in freely moving animals (34%–37%; Thomas and Wyman, 1984) but lower than those achieved by direct electrical stimulation, above threshold, of the giant fibers in restrained preparations (Tanouye and Wyman, 1980).

Occasional failures to respond to genetically targeted photostimulation could result from any number of causes that prevent the release of an effective dose of free ATP. For an optical uncaging reaction, the magnitude of the light-induced jump in agonist concentration is the product of two principal factors: the concentration of inci-

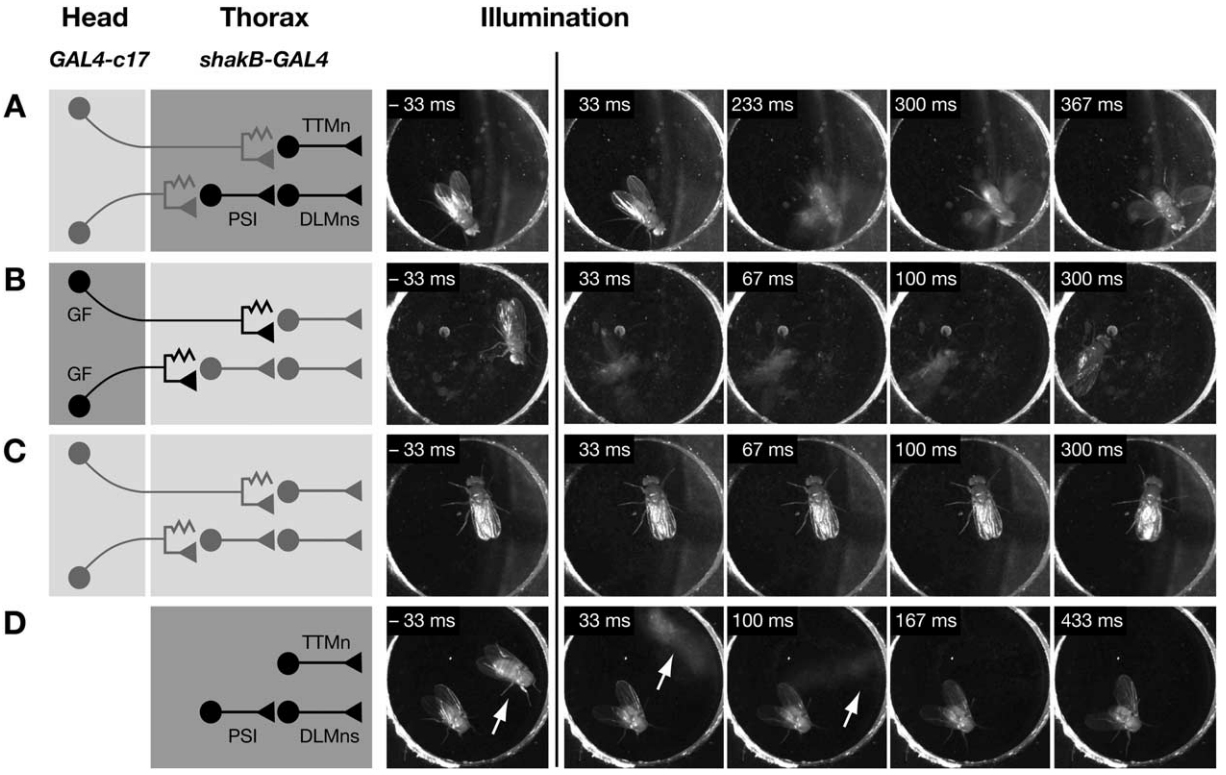

Figure 3. Genetically Targeted Photostimulation of the Giant Fiber System

Photostimulation experiments were performed in a cylindrical arena (diameter 8 mm, height 2 mm) that could be homogeneously illuminated with 355 nm laser light. The arena was covered by a glass ceiling in (A)–(C) but left open in (D), as decapitated flies do not spontaneously walk, jump, or fly, making their confinement unnecessary. Caged ATP was microinjected into the CNS in (A)–(C) and applied directly to the nerve cord in (D).

For each of the four experimental conditions (A–D), circuit diagrams on the left identify the neurons expressing $P2X_2$ in black on dark backgrounds. These simplified schemes of the bilaterally symmetric circuit depict the neuronal elements responsible for jumping on top and the elements responsible for flight at the bottom. The pair of giant fiber (GF) neurons in the brain (which are labeled selectively in the *GAL4-c17* enhancer trap line) project their axons to the thoracic ganglion, where they form mixed electrical and chemical synapses with the TTMn and PSI neurons. TTMn innervates the TTM muscle directly; PSI controls the DLM muscles indirectly via chemical synapses formed with the DLMns. The direct and indirect synaptic targets of the GF neurons in the thoracic ganglion, i.e., TTMn, PSI, and DLMns, are labeled in the *shakB-GAL4* line.

Selected individual frames of video recordings of photostimulation experiments are displayed on the right. The video frames are time-stamped in their upper left corner with respect to a 150-to-250 ms laser pulse ending at 0 ms. Complete video recordings of the experiments shown in (A), (B), and (D) are available online as Movies S1, S2, and S4, respectively.

(A) A fly expressing the *UAS-P2X*$_2$ transgene under the control of the *shakB-GAL4* driver in the TTMn-PSI-DLMns group of neurons in the thoracic ganglion responds to a 150 ms laser pulse with wing flapping.

(B) A blind *norpA*^{7} fly expressing the *UAS-P2X*$_2$ transgene under the control of the *GAL4-c17* driver in the pair of GF neurons responds to a 250 ms laser pulse with wing flapping.

(C) A fly lacking the *UAS-P2X*$_2$ transgene is unresponsive to a 250 ms laser pulse.

(D) Immobile, decapitated flies expressing the *UAS-P2X*$_2$ transgene under the control of the *shakB-GAL4* driver in the TTMn-PSI-DLMns group of neurons open their wings and fly (arrow) after photostimulation with a 150 ms laser pulse.

dent photons and the concentration of caged molecules at the stimulation site (McCray and Trentham, 1989). (Additional factors, such as the optical cross-section and the product quantum efficiency of the caged precursor, are clearly important but generally invariant between individuals or trials.) Differences in cuticle pigmentation, body size, or orientation of the fly with respect to the optical field are likely to affect the photon flux density in the vicinity of the neuronal targets; variations in the distribution volume of DMNPE-ATP, the diffusional distances of the injection and release sites from the neuronal targets, or the interval elapsed between injection and illumination are expected to alter the concentration of caged precursor. The efficacy of photostimulation declined with a measured half-life of ~75 min after the injection of caged ATP (Figure S1). Because behaviors indicative of "dark" uncaging (i.e., the spontaneous or enzymatic removal of the blocking group from DMNPE-ATP) were absent, these decay kinetics are likely to reflect the clearance of the caged compound from the CNS.

Specificity of Genetically Targeted Photostimulation

Three types of control experiments were performed to tie the light-evoked escape behaviors firmly to the selective optical stimulation of only the genetically desig-

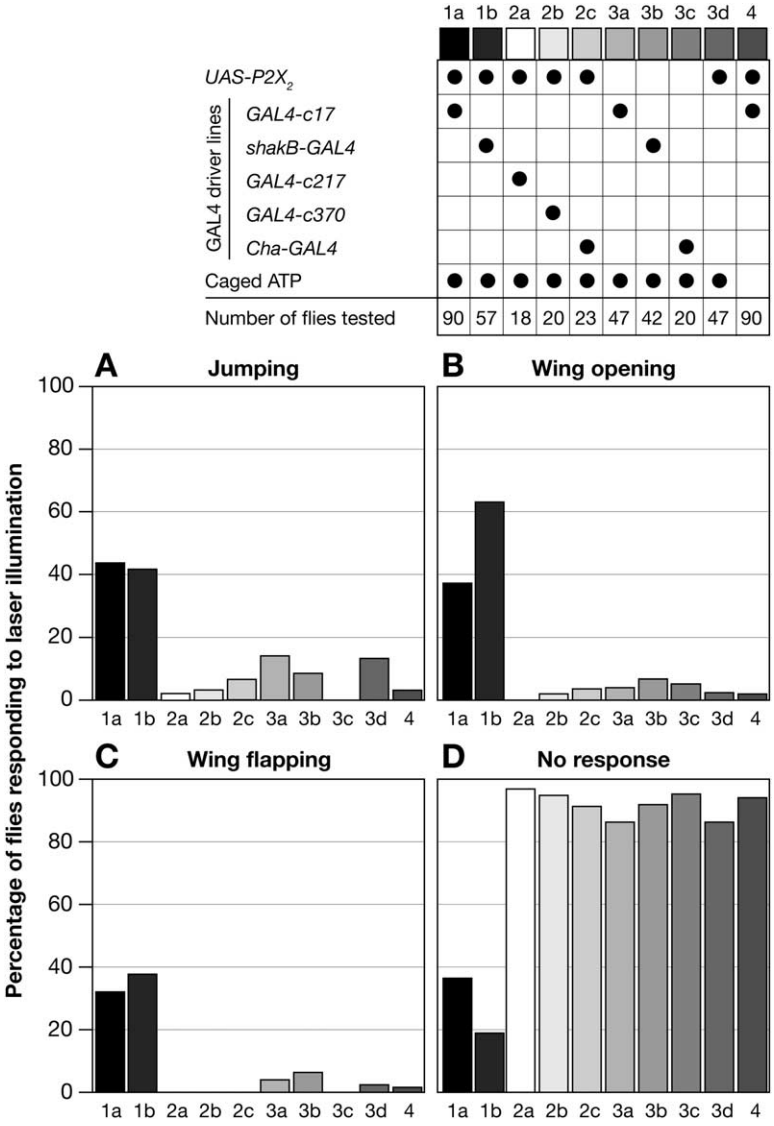

Figure 4. Specificity and Efficacy of Genetically Targeted Photostimulation of the Giant Fiber System

The frequencies of (A) jumping, (B) wing opening, (C) wing flapping, and (D) lack of a response to illumination were quantified in ten groups of blind *norpA*^{7} flies (groups 1a–4). Video recordings of photostimulation experiments were evaluated blindly, i.e., by an individual unfamiliar with the animals' experimental status. Flies exhibiting multiple forms of behavior (e.g., jumping followed by wing flapping) were scored in multiple categories.

The characteristics of each experimental group are listed as bulleted entries in the legend on top. The column for group 1a, for example, indicates that flies in this group carry *UAS-P2X*$_2$ and *GAL4-c17* transgenes (to direct P2X$_2$ expression in the giant fiber [GF] neurons, see Figure 3), and that they have been microinjected with caged ATP.

The ten experimental groups fall into four broad categories: an experimental set (category 1) and three sets of control groups (categories 2–4). Flies in category 1 express the *UAS-P2X*$_2$ transgene in the giant fiber system, i.e., the GF neurons proper (*GAL4-c17*; subcategory 1a) or the TTMn-PSI-DLMns neurons (*shakB-GAL4*; subcategory 1b). Flies in category 2 express the *UAS-P2X*$_2$ transgene either in small groups of neurons outside the GF system (*GAL4-c217* and *GAL4-c370*; subcategories 2a and 2b, respectively) or in all cholinergic neurons (*Cha-GAL4*, subcategory 2c). Flies in category 3 do not express P2X$_2$ because they lack either the *UAS-P2X*$_2$ responder (subcategories 3a, 3b, and 3c) or a *GAL4* driver transgene (subcategory 3d). Flies in category 4 express the *UAS-P2X*$_2$ transgene in GF neurons (*GAL4-c17*) but have been injected with artificial hemolymph lacking caged ATP.

nated targets. To establish that the phototrigger had to be located within the GF system to activate the circuit, P2X$_2$ was expressed in two small groups of neurons outside the GF system, using driver lines *GAL4-c217* and *GAL4-c370*. These control strains (Manseau et al., 1997; Nakayama et al., 1997) were selected at random among the members of a collection of enhancer trap lines (http://www.fly-trap.org) that exhibited narrowly restricted expression patterns in brain structures other than the GF system and its principal input streams, the visual (Thomas and Wyman, 1984) and olfactory (McKenna et al., 1989) systems. Illumination of animals expressing the phototrigger in these neurons failed to elicit escape movements (Figure 4, groups 2a and 2b).

To demonstrate that the light-induced behaviors were due to the targeted activation of a specific circuit rather than indiscriminate neuronal excitation, the driver line *Cha-GAL4* (Yasuyama and Salvaterra, 1999) was used to place the phototrigger in all cholinergic neurons, the most abundant class of excitatory neurons in the *Drosophila* CNS (Buchner, 1991; Yasuyama and Salvaterra, 1999). Photostimulation of this extensive neuronal population caused convulsions that led to paralysis (Movie S3) rather than defined, coordinated behaviors such as wing opening or flight (Figure 4, group 2c).

Visual signals, in particular light-to-dark transitions that mimic the casting of shadows, are potent natural activators of the GF system (Thomas and Wyman, 1984; Trimarchi and Schneiderman, 1995). To exclude confounding visual input via UV-sensitive photoreceptors, flies were blinded with the help of the *norpA*^{7} allele (Hotta and Benzer, 1970; Pak et al., 1970), which eliminates an essential phototransduction component (Bloomquist et al., 1988). While *norpA*^{7} mutants rarely flew spontaneously, suggesting that the neural circuits for flight are visually gated, flight could be initiated effectively by direct photostimulation of the GF system (Figures 3B and 4, groups 1a and 1b, and Movie S2).

The successful reconstitution of flight in blind animals indicated that artificial neural signals could be

used to repair or bypass behavioral deficits. In an extreme demonstration of this principle, flies expressing $P2X_2$ in the TTMn-PSI-DLMns group of thoracic neurons were decapitated. The headless bodies stood characteristically motionless (Yellman et al., 1997) in the open arena until illuminated and then took flight on circuitous, collision-prone trajectories (Figure 3D and Movie S4).

Neuronal Control of Neural Circuits

Brief periods of artificially evoked activity in small sets of central neurons (encompassing only the two GF neurons in the limiting case; Figure 3B and Movie S2) suffice to elicit ordered sequences of behaviors in unrestrained flies: jumping followed by wing opening followed by wing beating and, where physically possible, flight (Figure 3D). The fact that episodes of wing beating and flight far outlast the GF stimulus (Figure 3 and Movie S4) implies a control mechanism in which the GF system, rather than issuing a continuous score of motor commands, sets autonomous thoracic oscillators (Wyman et al., 1984; Selverston and Moulins, 1985) in motion that generate the motor patterns necessary for wing movement independent of sustained GF input.

Rhythmic flight could also be activated by direct photostimulation of a neuronal element intrinsic to the thoracic oscillator, i.e., the DLMn motor neurons innervating the flight muscles (Figures 3A and 3D and Movie S1). The ability of the flight circuit to generate the same motor output in response to diverse natural or artificial triggers suggests the existence of at least two dynamically stable circuit attractors: quiescence (a node) and rhythmic wing movement (a limit cycle). Because transitions in behavior occur when the circuit is brought to an initial state feeding into a different attractor, a sparse code of command impulses can specify complex, lasting actions robustly and economically.

The transmission via fast chemical and/or electrical synapses of command impulses that switch a circuit between different attractor domains is by no means the only mechanism for neural control. Neuromodulators employ an entirely different strategy: they regulate circuits through G protein-mediated effects on voltage-gated conductances and synaptic transmission (Kaczmarek and Levitan, 1987). Dopamine, for instance, is thought to induce changes in striatal circuits of vertebrates that help enhance coincident synaptic inputs and suppress neuronal noise (Nicola et al., 2000). Clinical and experimental evidence suggests that dopaminergic function is important for planned movement and the coding of predictive reward in learning, the organization of exploratory behavior, and addiction.

Dopaminergic Control of Movement

Flies possess a system of dopaminergic neurons (Budnik and White, 1988; Buchner, 1991) suspected to play similar roles. Loss of dopaminergic cells leads to a Parkinsonian syndrome of impaired movement (Feany and Bender, 2000); loss of the ability to synthesize dopamine (and serotonin) creates a learning phenotype (Tempel et al., 1984); drugs of abuse usurp dopaminergic signaling systems (Bainton et al., 2000).

The ~150 dopaminergic neurons in the CNS of adult flies are distributed among several clusters of 4–10 cells each (Budnik and White, 1988; Buchner, 1991). One unpaired and six paired clusters are located in the brain; several small clusters are scattered throughout the thoracic and abdominal ganglia (Budnik and White, 1988). The driver line *TH-GAL4* (Friggi-Grelin et al., 2003) provides selective genetic access to all but one of these clusters by capitalizing on regulatory sequences of the tyrosine hydroxylase gene, which encodes the rate-limiting enzyme in dopamine biosynthesis.

To examine the behavioral consequences of an acute increase in dopaminergic signaling, flies expressing $P2X_2$ in dopaminergic neurons were observed in a cylindrical quartz glass arena (diameter 25 mm, height 3 mm). Because the size of the arena prohibited whole-field illumination with adequate intensity, an automated video tracking system was designed that used the recorded coordinates of the fly as control signals for two galvanometric mirrors, creating a feedback loop that maintained a stable lock of the stimulating laser beam on its freely moving target. The beam was expanded to illuminate an elliptical spot of ~6 by 3 mm and attenuated to deliver 17 mW mm^{-2} of optical power.

Before stimulation of dopaminergic transmission, flies of genotype *TH-GAL4:UAS-P2X$_2$* behaved in a manner indistinguishable from that of the parental control strains. The majority of animals (68%, n = 40) traveled at average speeds of 12.6 mm s^{-1} during brief periods of activity (Figures 5A and 5B), which were interrupted by frequent pauses of considerable length. The flies' preferred trajectories circumscribed the perimeter of the arena and only rarely and briefly ventured into the open field at its center (Figures 5C, 6A, and 6C).

Exposure to four 150 ms pulses of UV light, spaced 1.5 s apart, caused marked and characteristic behavioral changes that lasted for periods of ~30–120 s. Optically stimulated dopamine release led to an instant increase in locomotor activity (Figure 5A). This increase was due to a reduced frequency of pausing and shorter average pause durations, as the average travel speed during periods of activity remained unchanged (Figure 5B). Strikingly, dopamine also affected the types of routes the flies elected to follow. Rather than staying close to the perimeter of the arena, as under preillumination conditions (Figures 6A and 6C), the trajectories after illumination frequently crisscrossed or looped through the center of the field (Figures 5C, 6B, and 6D). Occasionally, a fly moved in tightly wound circles (Figure 6D), a dopamine-induced stereotypie (McClung and Hirsh, 1998; Bainton et al., 2000) reminiscent of extrapyramidal asymmetries in vertebrates (Arbuthnott and Ungerstedt, 1975).

A minority of flies (32%, n = 40) exhibited high motor activity (Figure 7A) and centripetal locomotor patterns (Figures 6E, 6G, and 7C) before photostimulation, possibly because they were analyzed during a naturally occurring dopamine "high." Photostimulation of dopaminergic neurons in these animals caused transient locomotor arrest (Figures 6F, 6H, and 7A). The opposite effects of optically evoked dopamine release during periods of activity and quiescence recall the dose response of flies to cocaine (McClung and Hirsh, 1998; Bainton et al., 2000), a drug that increases dopamine

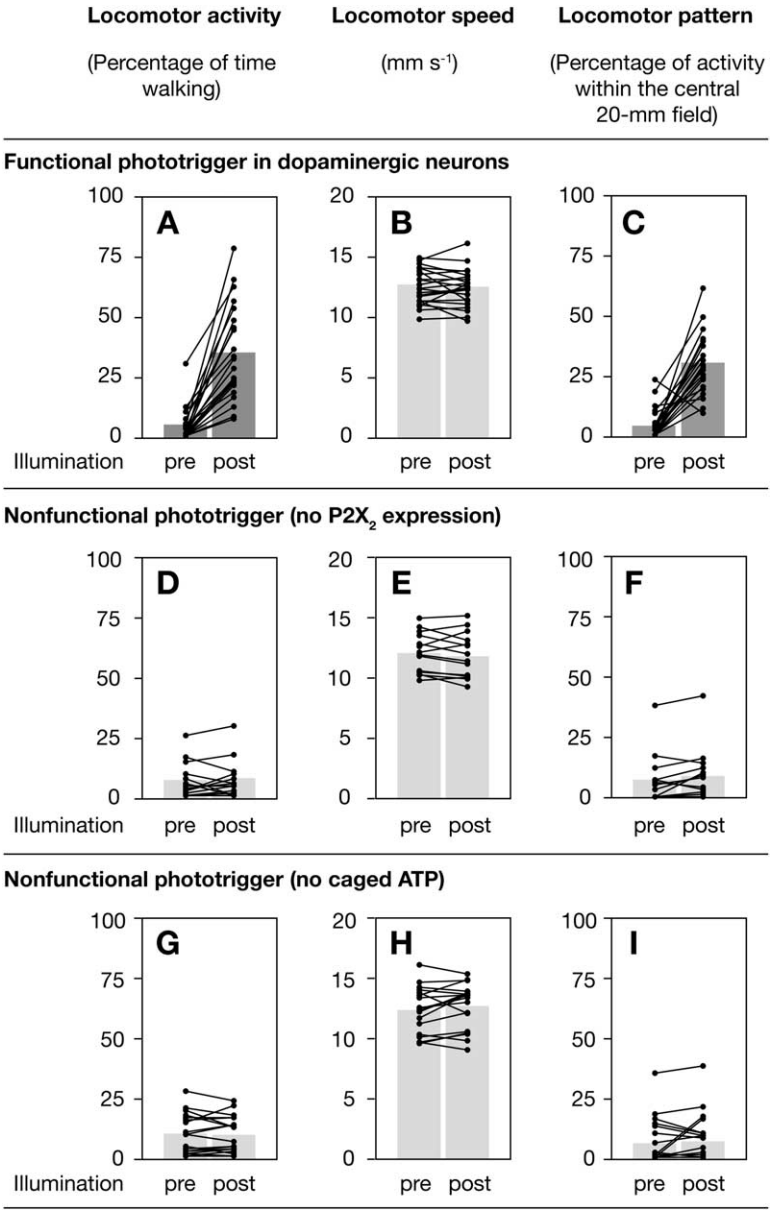

Figure 5. Genetically Targeted Photostimulation of Dopaminergic Neurons in Flies with Low Basal Locomotor Activity

Photostimulation experiments were performed with the help of an automated video tracking system in a cylindrical quartz glass arena (diameter 25 mm, height 3 mm). Flies were allowed to accustom to the arena for 5 min and were then observed under preillumination conditions for 2 min; they were classified as having low basal locomotor activity (and included in the present data set) if they spent $\leq$30% of the preillumination period walking. (Data on animals with basal locomotor activity >30% are presented in Figure 7.) The flies were subsequently exposed to four 150 ms pulses of 355 nm laser light and observed under postillumination conditions for another 2 min. Position coordinates were recorded every 33 ms under pre- and postillumination conditions and used to compute three locomotor variables: Locomotor activity (left column; A, D, and G) quantifies the percentage of time a fly spent walking. Locomotor speed (center column; B, E, and H) quantifies the average travel speed during periods of activity. Locomotor pattern (right column; C, F, and I) quantifies the percentage of activity taking place in the central 20 mm field of the 25 mm arena; this variable measures the tendency of a fly to venture from the perimeter of the arena into the open center.

Three categories of flies were analyzed: The top row (A–C) displays data from flies microinjected with caged ATP and expressing the *UAS-P2X*$_2$ transgene under the control of the *TH-GAL4* driver in dopaminergic neurons (n = 22). The center row (D–F) displays data from flies microinjected with caged ATP and carrying the *UAS-P2X*$_2$ responder transgene but lacking expression (owing to a lack of the *TH-GAL4* driver transgene) (n = 14). The bottom row (G–I) displays data from flies expressing the *UAS-P2X*$_2$ transgene under the control of the *TH-GAL4* driver in dopaminergic neurons but lacking caged ATP (n = 19). Data points corresponding to the same individual under pre- and postillumination conditions are connected by solid black lines. Shaded bars indicate group averages; significant differences between pre- and postillumination conditions ($p < 0.005$, as determined by applying the Bonferroni correction for multiple comparisons to independently performed paired t tests) are symbolized by dark gray shading (A and C). Consistent with the failure rate of genetically targeted photostimulation in experiments on the GF system (Figure 4D), 10 of 40 flies that expressed P2X$_2$ in dopaminergic neurons and had been microinjected with caged ATP lacked detectable responses to laser light. Data from these flies were excluded from Figures 5 and 7.

levels by inhibiting synaptic reuptake (Ritz et al., 1987): low cocaine doses tend to stimulate movement, whereas high doses tend to suppress it, often to the point of akinesis (McClung and Hirsh, 1998; Bainton et al., 2000). Several mechanisms could account for these observations, among them state-dependent effects of dopamine on its postsynaptic targets (Nicola et al., 2000), nonlinearities in dopaminergic signal transduction, or depression of dopaminergic synapses following massive stimulation.

Regardless of their basal activity level, and irrespective of whether they lacked P2X$_2$ expression in dopaminergic neurons or the injection of caged ATP, control flies were insensitive to illumination (Figures 5 and 7). Neither their locomotor activity (Figures 5D, 5G, 7D, and 7G), nor their average travel speed (Figures 5E, 5H, 7E, and 7H), nor the patterns of trajectories traced by moving animals (Figures 5F, 5I, 7F, and 7I) changed significantly after exposures to light.

Studies in primates have suggested a functional partition of dopaminergic neurons into subsystems serving different purposes and operating at different timescales (Schultz, 2002). These subsystems are thought to comprise a tonic component responsible for behavioral facilitation (which includes the facilitation of movement) and a phasic component that encodes predictive re-

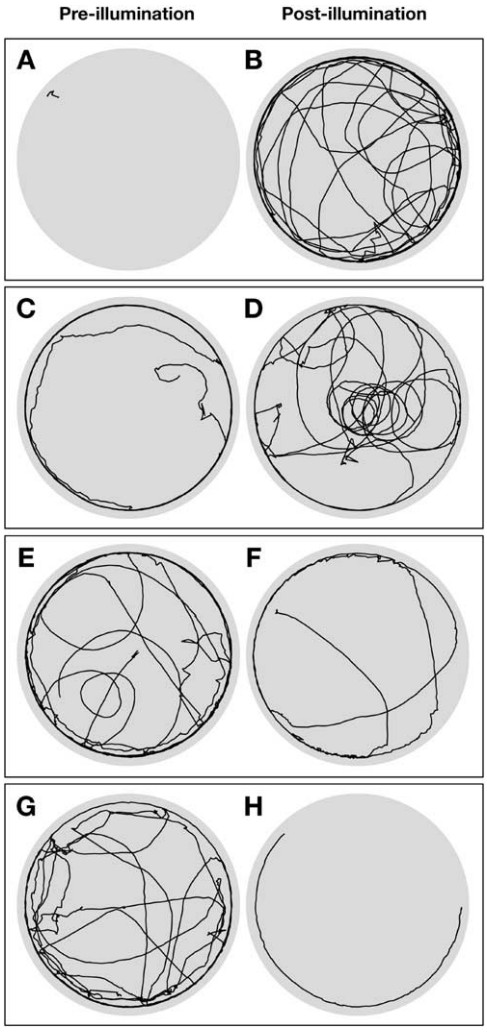

Figure 6. Movement Trajectories of Individual Flies before and after Genetically Targeted Photostimulation of Dopaminergic Neurons

Photostimulation experiments were performed with the help of an automated video tracking system in a cylindrical quartz glass arena (shaded circles; diameter 25 mm). Flies microinjected with caged ATP and expressing the *UAS-P2X*$_2$ transgene under the control of the *TH-GAL4* driver in dopaminergic neurons were allowed to accustom to the arena for 5 min, observed under preillumination conditions for 2 min, exposed to four 150 ms pulses of 355 nm laser light, and observed under postillumination conditions for another 2 min. Each row contains plots of position coordinates, recorded every 33 ms, of the same individual before and after laser illumination. The four examples are arranged, from top to bottom, in the order of increasing preillumination locomotor activity.

ward. It is tempting to view the two principal behavioral changes induced by dopaminergic stimulation in the fly in an analogous light: the increase in locomotor activity (Figure 5A) would then reflect facilitation of movement, while the more adventurous exploration of the central arena (Figures 5C, 6B, and 6D) might be motivated by a dopamine signal predicting an altered expectation of reward and punishment. Future experiments with genetic mosaics (Hotta and Benzer, 1970) in which subsets of dopaminergic neurons are light addressable should help clarify whether such a division of labor indeed exists and delineate the anatomical boundaries between subsystems.

Reconstitution of Function versus Loss of Function

Reconstitution of function, the ultimate test of causality and specificity in biology, has been applied sparingly in neuroscience because identifying and stimulating functionally circumscribed but anatomically dispersed populations of neurons in moving animals has been difficult. The capacity to remote control genetically delineated sets of neuronal targets promises to resolve this difficulty and will open many new possibilities for the analysis of neural circuits and the search for the cellular substrates of behavior. The strategy developed here for two systems of neurons and their associated behaviors, i.e., the GF system and escape movements and the dopaminergic system and locomotion, can be extended immediately to screens of existing collections of enhancer trap lines (or mosaic offspring in which expression of the phototrigger is restricted to smaller subsets of neurons) and other behaviors. Examples include searches for the neuronal signals guiding different forms of movement (Burrows, 1996), courtship (Quinn and Greenspan, 1984; Broughton et al., 2004), mating, aggression (Chen et al., 2002), feeding, grooming (Yellman et al., 1997), learning (Quinn and Greenspan, 1984), and sleep and wakefulness (Hendricks et al., 2000; Shaw et al., 2000), as well as attempts to identify the neural symbols representing reward and punishment (Schwaerzel et al., 2003), expectation, and categories of generalization (Liu et al., 1999).

As progress in the molecular taxonomy of neuronal cell types grants genetic access to an ever-increasing number of circuit elements in many species, genetically targeted stimulation is likely to play a key role in elucidating the functions of these diverse classes of neurons, in vitro and in vivo (Miesenböck, 2004). In either situation, the temporally and spatially controlled induction of spikes should prove more practical and informative than loss-of-function approaches (Sweeney et al., 1995; Johns et al., 1999; Kitamoto, 2001; Lechner et al., 2002; Slimko et al., 2002; Banghart et al., 2004) that depend on the occurrence of spontaneous activity (and its subsequent disruption) to produce phenotypes. Importantly, while loss-of-function strategies can establish necessity—is activity in a specific group of neurons required for a specific behavior?— reconstitution alone can demonstrate sufficiency (Kupfermann and Weiss, 1978; Miesenböck, 2004) and separate the information-carrying features of neuronal activity patterns from secondary or incidental ones.

Experimental Procedures

S2 Cell Imaging

cDNAs encoding rat TRPV1 (Caterina et al., 1997) or a covalent trimer of rat P2X$_2$ (Brake et al., 1994; Valera et al., 1994; Zemelman et al., 2003) in pAc5.1/V5-HisA (Invitrogen) were transfected into *Drosophila* Schneider S2 cells on coverslips. Three days after transfection, the cells were loaded with 10 μM Calcium Green 1-acetoxymethyl ester (Molecular Probes) for 30 min and visualized on a Zeiss Axioskop FS microscope equipped with a 40×/0.8 W Achroplan objective. Fluorescence was excited at 450–490 nm (HQ 470/40, Chroma); emitted light in the 500–550 nm band (HQ 525/50, Chroma) was detected by a PentaMax-512EFT CCD camera (Roper Scientific). The cells were superfused continuously at ~6

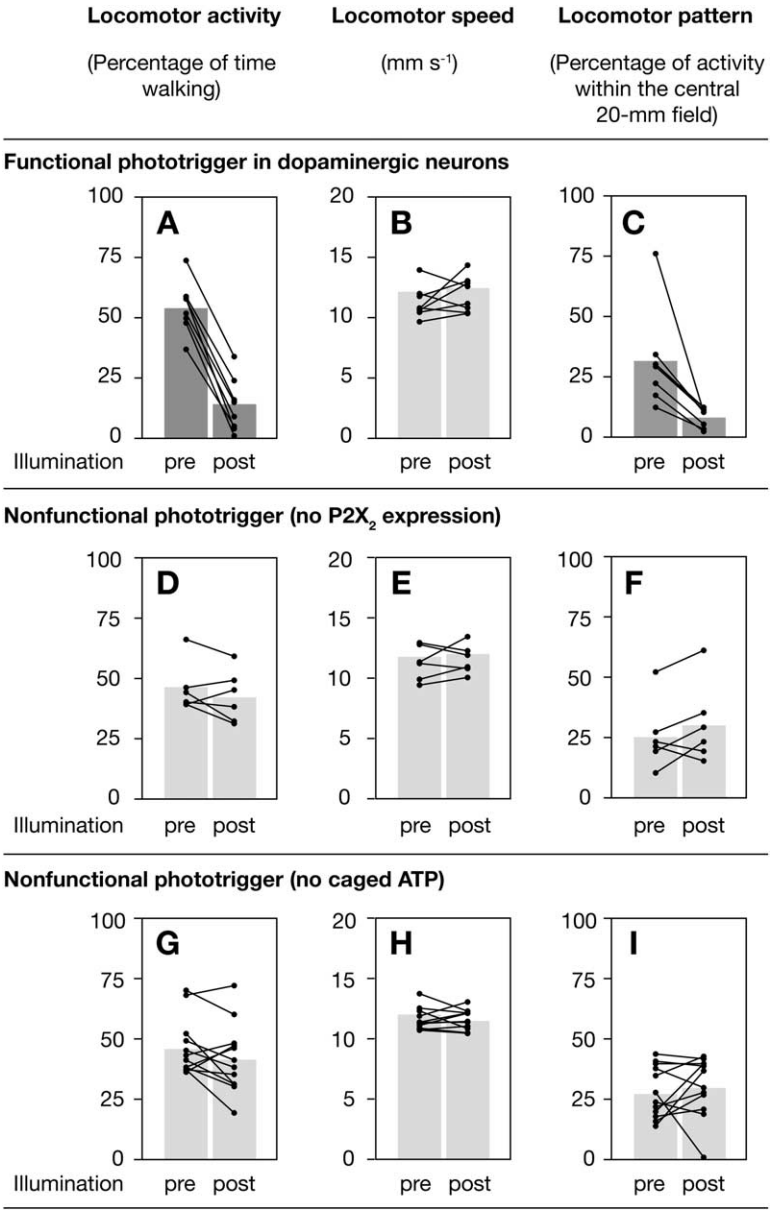

Figure 7. Genetically Targeted Photostimulation of Dopaminergic Neurons in Flies with High Basal Locomotor Activity

Photostimulation experiments were performed with the help of an automated video tracking system in a cylindrical quartz glass arena (diameter 25 mm, height 3 mm). Flies were allowed to accustom to the arena for 5 min and were then observed under preillumination conditions for 2 min; they were classified as having high basal locomotor activity (and included in the present data set) if they spent >30% of the preillumination period walking. (Data on animals with basal locomotor activity $\leq$30% are presented in Figure 5.) The flies were subsequently exposed to four 150 ms pulses of 355 nm laser light and observed under postillumination conditions for another 2 min. Position coordinates were recorded every 33 ms under pre- and postillumination conditions and used to compute three locomotor variables: Locomotor activity (left column; A, D, and G) quantifies the percentage of time a fly spent walking. Locomotor speed (center column; B, E, and H) quantifies the average travel speed during periods of activity. Locomotor pattern (right column; C, F, and I) quantifies the percentage of activity taking place in the central 20 mm field of the 25 mm arena; this variable measures the tendency of a fly to venture from the perimeter of the arena into the open center.

Three categories of flies were analyzed: The top row (A–C) displays data from flies microinjected with caged ATP and expressing the *UAS-P2X$_2$* transgene under the control of the *TH-GAL4* driver in dopaminergic neurons (n = 8). The center row (D–F) displays data from flies microinjected with caged ATP and carrying the *UAS-P2X$_2$* responder transgene but lacking expression (owing to a lack of the *TH-GAL4* driver transgene) (n = 6). The bottom row (G–I) displays data from flies expressing the *UAS-P2X$_2$* transgene under the control of the *TH-GAL4* driver in dopaminergic neurons but lacking caged ATP (n = 12). Data points corresponding to the same individual under pre- and postillumination conditions are connected by solid black lines. Shaded bars indicate group averages; significant differences between pre- and postillumination conditions ($p < 0.005$, as determined by applying the Bonferroni correction for multiple comparisons to independently performed paired t tests) are symbolized by dark gray shading (A and C). Consistent with the failure rate of genetically targeted photostimulation in experiments on the GF system (Figure 4D), 10 of 40 flies that expressed P2X$_2$ in dopaminergic neurons and had been microinjected with caged ATP lacked detectable responses to laser light. Data from these flies were excluded from Figures 5 and 7.

ml min^{-1} with imaging buffer (10 mM Na-HEPES, pH 7.3, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM glucose) and stimulated by perfusing a 1 ml bolus of 10 μM capsaicin (Fluka) or 100 μM ATP (Amersham Biosciences) into the recording chamber.

Drosophila Strains

Strains *yw; P{w$^+$;;UAS-P2X$_2$}III* and *norpA^7;; P{w$^+$;UAS-P2X$_2$}III* carry GAL4-responsive transgenes encoding trimeric rat P2X$_2$ (Zemelman et al., 2003). Transgene expression was activated in defined sets of neurons by crossing the *UAS-P2X$_2$* responder strains to a series of GAL4 driver lines. Pan-neuronal expression of P2X$_2$ was controlled by driver strains *P{Nrv2-GAL4}* (Sun et al., 1999) and *P{GawB}elav^{C155}* ("*elav-GAL4*"; Lin and Goodman, 1994), expression in cholinergic neurons by strain *P{Cha-GAL4.7.4}19B* ("*Cha-GAL4*"; Yasuyama and Salvaterra, 1999), and expression in dopaminergic neurons by strain *TH-GAL4* (Friggi-Grelin et al., 2003). Two lines with highly restricted expression patterns in the CNS were used to target specific neuronal elements of the GF system: line *P{GAL4}-c17* was used to drive the expression of P2X$_2$ in the GF neurons proper (Allen et al., 1999; Trimarchi et al., 1999), line *P{shakB-GAL4}II* to drive expression in the TTMn-PSI-DLMns group of neurons in the thoracic ganglion (Jacobs et al., 2000). Control strains *P{GAL4}-c217* (Manseau et al., 1997) and *P{GAL4}-c370* (Nakayama et al., 1997) were selected from a collection of enhancer trap lines (http://www.fly-trap.org) with restricted neuronal expression outside the GF pathway and the visual (Thomas and Wyman, 1984) and olfactory (McKenna et al., 1989) systems.

Larval Electrophysiology

Wandering third-instar larvae were pinned to a Sylgard-coated recording chamber and dissected in Schneider's insect medium (Sigma), leaving all segmental nerves intact. The larval filets were

superfused with HL3 solution (Broadie, 2000) at ~6 ml min^{-1} and stimulated by perfusing a 1 ml bolus of 200 μM purine nucleotide (Amersham Biosciences) in HL3 into the recording chamber. The membrane potentials of muscle fibers 6 or 7, segments 2–5, were recorded in bridge mode (Axoclamp-2B, Axon Instruments) with sharp intracellular electrodes filled with 3 M KCl (15–20 MΩ). Signals were low-pass filtered at 0.5 kHz (CyberAmp 380, Axon Instruments) and digitized at 1 kHz (Digidata 1200, Axon Instruments).

Photostimulation

Males doubly heterozygous for driver and responder transgenes and, where indicated, hemizygous for *norpA*^{7} were studied. All experiments were performed under yellow safelight conditions (Roscolux 10 Medium Yellow).

To remove contaminating free ATP, 5 mg DMNPE-ATP (Molecular Probes) in 100 μl artificial hemolymph (5 mM Na-HEPES, pH 7.3, 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO4, 5 mM trehalose, 10 mM sucrose) was incubated with 20 units of apyrase (Sigma) at room temperature for 1 hr. The reaction was passed through a Microcon YM-10 centrifugal filter (Millipore; 14,000 g, 30 min) to remove the enzyme and assayed for the absence of ATP (ATP Bioluminescence Assay Kit CLS II, Roche). Estimates of DMNPE-ATP concentrations in the filtrate are based on its 351 nm absorbance and an extinction coefficient of 4,400 $M^{-1}cm^{-1}$.

Adult males aged 2–3 days were anesthetized on ice, immobilized in a custom-fabricated microchannel plexiglass plate, and microinjected (Nanoject II, Drummond) through the ocellus with 13–41 nl of 40–70 mM DMNPE-ATP in artificial hemolymph, which was supplemented with 5% (v/v) green food color (McCormick) as a tracer. Successful injections led to homogeneous, sharply confined dye fills of the CNS and rapid, complete recoveries.

Behavioral experiments were conducted in cylindrical quartz glass arenas (diameters 8 and 25 mm) that could be illuminated by Q-switching a frequency-tripled Nd:YVO_4 laser generating pulses of 355 nm light at 100 kHz (DPSS Lasers, model 3507-100). The laser beam was shuttered and/or attenuated with the help of an acousto-optic deflector (IntraAction model ASN-802832 with ME-802 driver). For whole-field stimulation experiments in the 8 mm arena, the laser beam was expanded to deliver an average power of 8 mW mm^{-2} homogeneously across the arena, which was viewed by a Hamamatsu C-2400 CCD camera through a Zeiss Stemi 2000-C dissection microscope. For video tracking experiments in the 25 mm arena, the CCD camera was equipped with a Navitar NAV-2514 objective. The position of the fly in the arena was analyzed online by subtracting a stored image of the empty arena from the current video frame, defining the contour of the fly by thresholding the difference image, and computing the centroid of the area above threshold. The resulting x-y coordinates were fed back to a pair of galvanometric mirrors (GSI Lumonics VM-500S x-y scan unit with MiniSAX servo controllers) that automatically positioned the laser beam to track the movements of the fly. In this experimental configuration, the laser illuminated an elliptical area of 6 × 3 mm with an average power of 17 mW mm^{-2}. A virtual instrument written in LabVIEW 7.1 (National Instruments) controlled the Q-switch, the acousto-optic deflector, the galvanometric mirrors, and the acquisition of images through National Instruments analog output (PXI-6713) and video acquisition boards (PXI-1411).

Supplemental Data

Supplemental Data include one figure and four movies and can be found with this article online at http://www.cell.com/cgi/content/full/121/1/141/DC1/.

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