

Transient changes in nucleus accumbens amino acid concentrations correlate with individual responsivity to the predator fox odor 2,5-dihydro-2,4,5-trimethylthiazoline

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Abstract

Predator odors elicit fear and defensive behavioral responses in rats, but a wide range of individual responsivity exists. The aim of this study was to examine whether individual differences in behavioral responsivity correlate with differences in amino acid neurotransmission to a predator fox odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT). We investigated the time course of behaviorally evoked amino acid neurotransmitter changes in the nucleus accumbens using on-line microdialysis coupled to capillary electrophoresis with 14-s temporal resolution. One subset of animals (high responders) showed a large, biphasic increase in amino acids, such as glutamate and GABA, which lasted about 3 min. These

neurochemical changes were highly correlated with increases in locomotion and burrowing, but lagged behind the behavioral changes by 2 min. A second subset of rats (low responders) showed neither behavioral activation nor changes in amino acid neurotransmission. As a positive control, rats were subjected to tail pinch, which evoked transient changes in amino acids in all animals. Cocaine (2 mg/kg, i.v.) increased locomotion but not amino acid levels. This work demonstrates that rapid and transient increases in amino acid neurotransmitters correlate with behavioral reactivity to salient stimuli.

Keywords: capillary electrophoresis, cocaine, fox odor, glutamate, tail pinch, taurine.

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Predator odors elicit defensive behavioral responses in rats that are characteristic of fear and stress (Blanchard *et al.* 2003). However, there is a wide range of individual responsivity, as with many other salient stimuli. Salient stimuli have been shown to activate neurobiological systems involved in reward, including the nucleus accumbens which is important in mediating the rewarding and incentive motivational effects of drugs of abuse (Redgrave *et al.* 1999; Sinha 2001). Understanding the neurochemical changes caused by predator odors may give insight into how brain reward circuits are activated and whether differences in neurotransmission are responsible for individual behavioral differences. Although many studies have focused on the neurotransmitter dopamine (Marinelli and Piazza 2002; Chefer *et al.* 2003), emerging evidence suggests that amino acid neurotransmission also plays an important role in the nucleus accumbens. For example, microdialysis studies have shown that glutamate increases in the accumbens during conditioned fear (Saulskaya and Marsden 1995b), forced swimming (Rada *et al.* 2003), tail pinch (Rada *et al.* 2003), restraint stress (Moghaddam 1993) and exposure to predator odors (Hotsenpiller and Wolf 2003). However, the response

is usually small and prolonged, so the physiological significance of the changes is difficult to ascertain. In addition, the time course of these glutamate changes sometimes appears delayed from the onset of the stressor (Saulskaya and Marsden 1995b). Higher-resolution temporal measurements would allow a better understanding of the temporal dynamics and interactions of neurotransmitters evoked by salient stimuli and how these changes correlate with behavior.

Microdialysis sampling is one of the most popular and versatile techniques for measuring chemical changes *in vivo* (Stiller *et al.* 2003). Multiple compounds can be monitored simultaneously, but the temporal resolution of these measurements is often poor, with sampling on a 5–20-min time scale (Olive *et al.* 2000). This level of temporal resolution gives an indication that changes in neurochemical levels

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Abbreviations used: OPA, *o*-phthalaldehyde; TMT, 2,5-dihydro-2,4,5-trimethylthiazoline.

occur, but it is not sufficient to resolve rapid changes in neurotransmitter levels that might correspond to specific behaviors. Coupling microdialysis sampling to analysis methods with high sensitivity, such as capillary electrophoresis, allows improvements in temporal resolution over the traditional liquid chromatography method (Paez and Hernandez 2001; Parrot *et al.* 2003). Use of on-line analysis, feasible because of the fast separations possible with capillary electrophoresis, allows convenient, nearly real-time chemical monitoring (Bowser and Kennedy 2001). The 14-s temporal resolution possible with on-line capillary electrophoresis analysis should allow measurement of neurotransmitters by microdialysis on a behaviorally relevant time scale.

We used rapid-sampling microdialysis with capillary electrophoresis to test the hypothesis that differences in amino acid neurotransmission correlate with differences in behavioral responsivity to a predator odor. Rapid and transient changes in extracellular concentrations of amino acids were identified after presentation of the fox odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a naturally occurring fear-evoking component of fox feces. Hotsenpiller and Wolf (2003) have demonstrated by microdialysis sampling at 10-min intervals that presenting TMT increases accumbens glutamate by 70% over basal levels for at least 30 min. With high-resolution temporal measurements, we detected large (400% over basal), rapid and transient (3–5 min duration) changes in glutamate levels in responsive rats, and the magnitude of the neurochemical changes correlated strongly with changes in locomotor and burrowing behavior. These experiments demonstrate that large, rapid changes in amino acid neurotransmitters in the nucleus accumbens are highly correlated with individual behavioral responsivity to a predator odor.

Materials and methods

Chemicals

TMT was purchased from Phero Tech, Inc. (Delta, BC, Canada). Sodium tetraborate, *o*-phthalaldehyde (OPA), β -mercaptoethanol, hydroxypropyl- β -cyclodextrin and all amino acid standards were purchased from Sigma (St Louis, MO, USA). Cocaine was obtained from RTI (Research Triangle Park, NC, USA). Sodium chloride, potassium chloride, sodium phosphate monobasic, sodium phosphate dibasic, magnesium sulfate, calcium chloride and formaldehyde were purchased from Fisher Scientific (Pittsburgh, PA, USA). All solutions were prepared with deionized water and filtered.

Microdialysis probes

Side-by-side microdialysis probes were constructed in-house as described previously (Petit and Justice 1991). Briefly, two capillaries (40 μ m inner diameter, 105 μ m outer diameter; Polymicro Technologies, Phoenix, AZ, USA) were ensheathed in a regenerated cellulose membrane (molecular weight cut-off 18 000 Da; Spectrum Laboratories, Rancho Dominguez, CA, USA). The working area of

the probe was 2 mm and the probe diameter was 200 μ m. The non-active areas of the probe were covered with polyimide coating (Alltech, Deerfield, IL, USA).

Animals and surgery

All animal experiments were approved by the University of Michigan animal care and use committee. Male Sprague–Dawley rats, 250–350 g, were purchased from Harlan (Indianapolis, IN, USA). For surgery, animals were anesthetized with ketamine (65 mg/kg i.p.) and dormitor (0.5 mg/kg i.p.). A guide cannula (Plastics One, Roanoke, VA, USA), extending 2 mm from the top of the skull, was surgically implanted above the nucleus accumbens (coordinates from bregma, anterior-posterior + 1.6 mm, medial-lateral + 1.1 mm) and cemented in place with dental cement. An extra cannula was also placed in the cement (not in the skull) to use as a tether. The animal was allowed to recover from surgery for at least 5 days before the experiment.

Behavioral experiments

All experiments were performed in a Ratum (BAS, Lafayette, IN, USA) in which the testing bowl automatically rotated in the opposite direction to the rat every time the animal turned more than 90°. This eliminated the need for a liquid swivel. Animals were tethered by a spring that was attached to the extra cannula post implanted in the dental cement. On the day of the experiment, the microdialysis probe was inserted via the guide cannula so the active area of the probe extended into the brain between 6 and 8 mm from the top of the skull. The probe was perfused with artificial CSF (145 mM NaCl, 2.68 mM KCl, 1.0 mM MgSO₄·7H₂O, 1.22 mM CaCl₂, 1.55 mM Na₂HPO₄, 0.45 mM NaH₂PO₄·H₂O, pH 7.4) at 0.8 μ L/min. Animals were habituated and probes equilibrated for at least 60 min until stable neurochemical levels were obtained. Neurochemical levels usually reached stable concentrations within 30 min of probe implantation. In previous work, similar probe implantation protocols allowed measurement of neuronally derived glutamate release (Lada *et al.* 1998). Baseline samples (130) were collected for 30 min and the experiment then started. For the fox odor experiment, 25 μ L TMT was placed on a 7-cm square piece of filter paper and hung in the top of the testing bowl for 12 min. Electropherograms were collected for at least 45 min following odor removal, until chemical levels returned to baseline. A tail pinch experiment was then performed by placing a small binder clip on the tail of the rat for 4 min. Chemical data were collected for at least 30 min after removal of the clip.

The animal's behavioral reactions were videotaped and later viewed for analysis by an independent observer. Time was divided into 15-s intervals, which approximates the sampling time for the microdialysis measurements. The amount of time an animal spent engaged in specific behaviors was recorded for each interval (so the maximum score was 15 s). In the fox odor experiment, the amount of time spent in locomotion (defined as the animal walking and moving its hind limbs) and burrowing (defined as the animal placing its head into the litter and pushing the litter) is reported. This burrowing behavior appeared similar to the defensive burying described in response to a shock probe (Pinel and Treit 1978), but it was not localized to a specific point in space. Only locomotion is reported for the tail pinch experiments.

Pilot experiments using almond odor were carried out in a separate group of rats according to a similar protocol ($n = 3$). After probe equilibration and collection of baseline samples for 30 min, 25 μL almond extract (McCormick brand, Baltimore, MD, USA) was applied to a piece of filter paper and hung in the top of the cage for 12 min.

A separate group of drug-naïve rats were surgically implanted with jugular catheters during the microdialysis surgery, as described previously (Crombag *et al.* 1996). During the microdialysis experiment, each rat was habituated for at least 1 h after probe implantation. Baseline samples were collected for 30 min, after which the rat received an injection of cocaine (2 mg/kg in 10 μL saline i.v.) over 5 s.

Microdialysis coupled to capillary electrophoresis with laser-induced fluorescence detection

Capillary electrophoresis instrumentation and the derivatization procedure have been described previously (Bowser and Kennedy 2001). Briefly, microdialysate was derivatized on-line with a solution of 10 mM OPA, 40 mM β -mercaptoethanol, 9 mM sodium tetraborate, 0.8 mM hydroxypropyl- β -cyclodextrin and 10% methanol (v/v) at pH 10.2. At a flow-gated interface, electrokinetic injections of the OPA-derivatized microdialysate samples were made on to the separation column (9.5 cm long, 10 μm inner diameter, 150 μm outer diameter capillary). When no injection was being made, a cross-flow buffer comprising 10 mM borate and 0.88 mM hydroxypropyl- β -cyclodextrin prevented leakage of the sample on to the separation column. The separation voltage was -20 kV, and the fluorescence was detected off-column in a sheath flow cuvette. Fluorescence was excited using a 351-nm diode-pumped solid-state laser (DPSS Lasers, San Jose, CA, USA) and 450 nm light was collected at a photomultiplier tube orthogonally from the beam. Electropherograms were collected and analyzed using in-house software written in LabVIEW (National Instruments, Austin, TX, USA) (Shackman *et al.* 2004).

On each test day, the system dead time (i.e. the time from sampling in the brain to detection of fluorescence) was calculated during calibration and the time course of the microdialysis data were corrected to account for this delay. The system dead time was also double-checked in some animals by testing the delay between probe implantation and the first observed signal. The dead time calculations by both methods always agreed.

Histology

After the experiment, animals were perfused with 0.9% saline and then 10% formaldehyde, and the brain was removed and stored refrigerated in 10% formaldehyde. Thirty-micron sections of each brain were sliced on a cryostat and mounted on to slides. The slices were stained with thionin and then compared with data in the atlas of Paxinos and Watson (1998) to verify probe placement. Probe placements are shown in Fig. 1.

Statistical analysis

Correlations of chemistry and behavioral data, and ANOVA were performed using GraphPad Prism software (San Diego, CA, USA). Cross-correlation analysis, performed in LabVIEW, was used to compare the peak positions of behavioral and neurochemical data. Values are reported as mean \pm SEM.

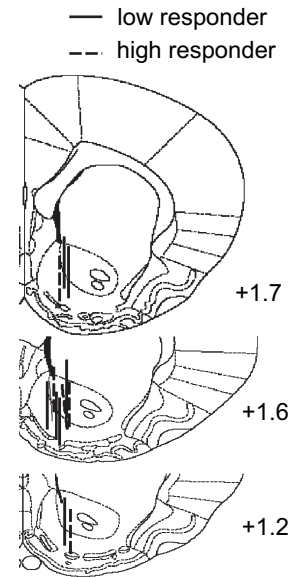


Fig. 1 Probe placements for fox odor experiment. The coordinates are given as millimetres from bregma.

Results

Fox odor experiment

Microdialysate samples were collected and analyzed on-line using capillary electrophoresis with laser-induced fluorescence detection. Overlapping injections were used such that a new sample was loaded on to the column before glutamate and aspartate had eluted from the previous injection (Bowser and Kennedy 2001). Glutamate, aspartate, GABA, taurine, glutamine, serine and glycine were separated and detected in 10.5 s (Fig. 2). The ultimate temporal resolution of this method was one sample every 14 s, because of the time needed to inject the sample on to the column.

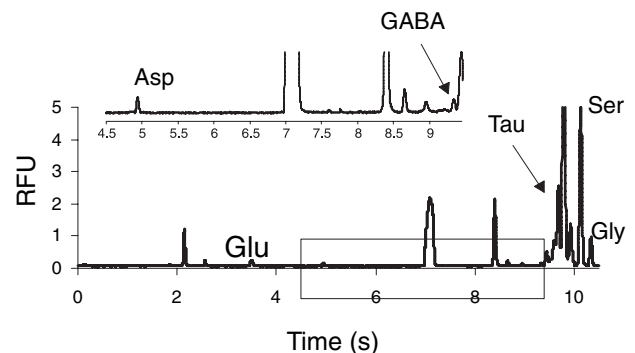


Fig. 2 Sample electropherogram of microdialysate. The inset is an enlargement of the boxed region from 4.5 to 9.5 s showing the aspartate and GABA peaks. RFU, relative fluorescence units.

Initially, the response of rats to the predator fox odor TMT was tested. For the neurochemical data, the peak height of each compound in each electropherogram was measured. Figure 3 shows examples of neurochemical (top) and behavioral (bottom) changes in three representative rats. The odor was placed in the cage at time 0 and removed after 12 min. Increases in glutamate, aspartate, GABA, taurine, serine and glycine were observed, but results for only two representative compounds are plotted for clarity. Individual rats are shown to emphasize the transient nature of the signals as well as the variation in neurochemical changes and behavior between rats.

The animals fell into two clearly distinguishable groups: those that showed sustained locomotor activity starting around 2 min after the fox odor (defined as 'high responders') and those that showed inactivity ('low responders'). Rats were divided into groups based on the cumulative locomotor behavior between 2 and 5 min after the fox odor presentation. Low responders spent less than 5 s in locomotion during this 3-min interval, whereas high responders all demonstrated over 25 s of locomotion. Rat A in Fig. 3 is an example of a high responder. In this animal, there was a period of hyperactivity and burrowing peaking around 3 min

after odor presentation. Rat A had a large, transient increase to over 2000% of basal levels (basal = 100%) in glutamate and taurine. The glutamate response peaked around 5 min after introduction of the fox odor and lasted for approximately 3 min. The taurine level changed more slowly and took approximately 8 min to return to baseline. In addition, a smaller, first phase of glutamate and taurine increase peaked around 2 min after TMT presentation (inset). The high responder illustrated in Fig. 3(b) clearly showed a sustained increase in locomotor and burrowing behavior. This animal also showed a first-phase increase in glutamate and taurine to about 200% of basal levels, followed by a larger, sustained increase. All high responders showed a biphasic neurochemical response. Rat C is a typical low responder. The animal showed only a short, immediate behavioral reaction to presentation of fox odor before settling back into inactivity. No changes were detected in the levels of any amino acids in this animal.

Figure 4 shows data averaged for all high ($n = 5$) and low ($n = 8$) responders. There was a clear difference in locomotor and burrowing behavior between the two groups between 2 and 5 min after odor presentation. The error for the locomotion and burrowing behavior was large beyond 5 min

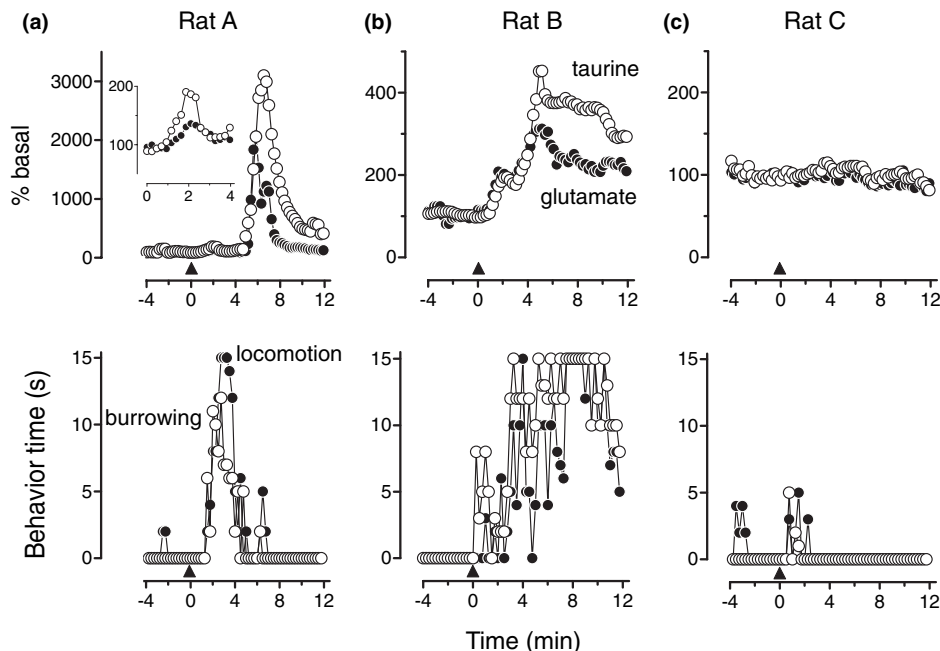


Fig. 3 Example of changes in neurochemistry (top graphs) and behavior (bottom graphs) after fox odor presentation to three different rats. The fox odor was placed in the cage at time 0 (arrow) and removed after 12 min. The neurochemical data were corrected to account for the measurement delay between the microdialysis sampling and on-line analysis with the capillary electrophoresis instrument, so the chemistry and behavior graphs are temporally aligned. (a) Rat A had a burst of locomotor (●) and burrowing (○) behavior starting 2 min after fox odor presentation, followed by a

large, transient change in glutamate (●) and taurine (○) levels. The inset shows the smaller first-phase change observed around 2 min. (b) Rat B had large, sustained changes in locomotor and burrowing behavior, and the accompanying neurochemical levels remained high throughout the behavior. A biphasic increase in glutamate and taurine was observed. (c) Rat C had only a small behavioral reaction to the fox odor immediately after presentation and showed no changes in amino acid neurotransmitter levels. Rats A and B were classified as high responders and rat C as a low responder.

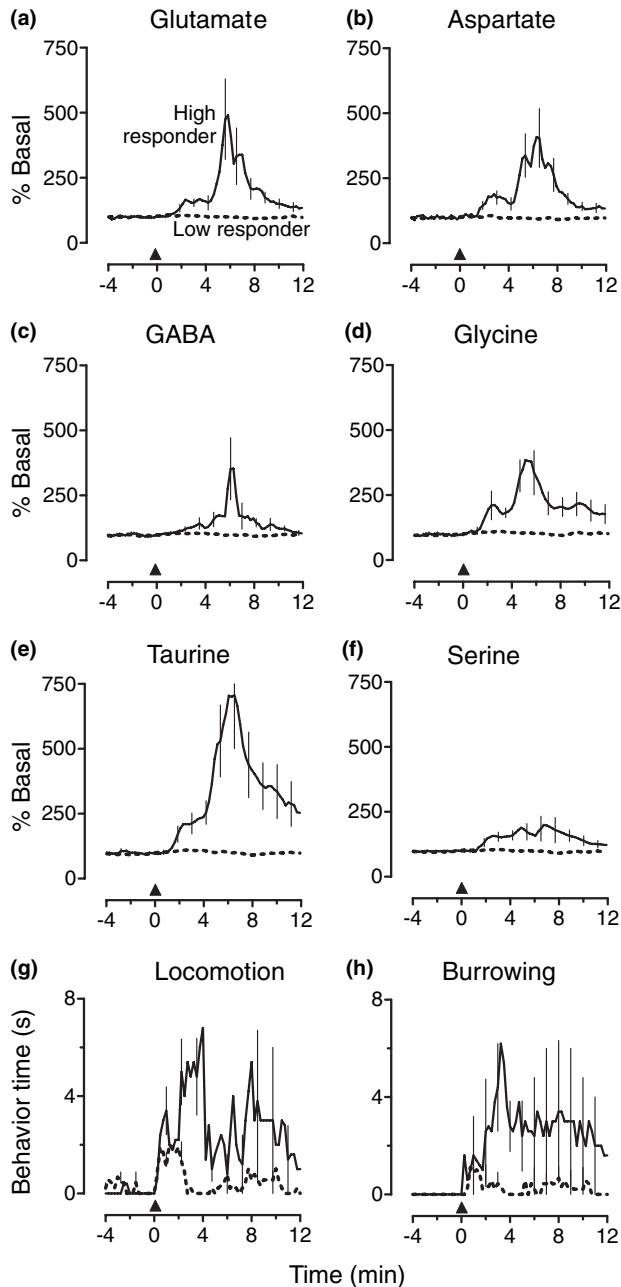


Fig. 4 Mean changes in neurochemistry and behavior after fox odor presentation. The arrow marks the beginning of odor presentation. Error bars shown every fifth point are SEM. Rats that showed any chemical changes after fox odor presentation were classified as high responders (solid lines; $n = 5$) and those that showed no neurochemical changes were classified as low responders (dashed lines; $n = 8$). There was an increase in (a) glutamate, (b) aspartate, (c) GABA, (d) glycine, (e) taurine and (f) serine in high responders. The behavioral reactions of (g) locomotion and (h) burrowing showed increases 2 min after odor presentation for high responders, whereas the low responders showed little activity after 2 min.

because a few animals showed a sustained response (as in Fig. 3(b)), but most high responders showed little locomotion beyond 5 min. Low responders initially explored and walked around after placement of the odor in the cage, but they tended to lose interest after a few minutes and returned to inactive states such as lying down and sleeping. The low responders did not show signs of freezing behavior normally associated with fear, such as a standing in a defensive posture. The neurochemical averages illustrate the biphasic nature of the increase in each amino acid in the high responders. Although levels of all six amino acids increased, the time course and magnitude of the changes varied with amino acid. In contrast, no changes from basal levels in the low responders were observed for any of the amino acids. Comparing the amino acid concentrations for low and high responders, there were significant main effects of group for each of the six amino acids ($F = 1.9\text{--}4.3$, $p < 0.01$) and time ($F = 1.8\text{--}3.9$, $p < 0.005$) and a significant interaction of group and time ($F = 11\text{--}24$, $p < 0.005$) (two-way repeated-measures ANOVA).

Animals were defined as high responders or low responders based on behavior, but analysis of the relationship between behavior and neurochemistry showed that these two variables were highly predictive of one another (Fig. 5). The area under the large, second phase of the percentage basal versus time graph (as in Fig. 4) was calculated and plotted against the cumulative duration of locomotor activity 2–5 min after odor presentation. The r^2 values ranged from 0.7 to 0.9, indicating a strong correlation between the second-phase peak area and peak locomotor activity. The r^2 value of 0.92 for glutamate indicates, for example, that 92% of the variance in glutamate levels can be accounted for by variance in locomotion. These r^2 values showed that a statistically significant correlation existed between neurochemistry and behavior for all six amino acids ($t = 10\text{--}77$, $p < 0.001$). Similar r^2 values, of between 0.6 and 0.8, were obtained for correlations between neurochemistry and burrowing behavior ($t = 7\text{--}12$, $p < 0.01$). The low responders were all clustered at zero locomotion and burrowing, and showed no correlation between chemistry and behavior as a group. However, the high responders showed a gradient of responses, and r^2 values for just the high-responder group were similar to r^2 values obtained using data from all animals. There was no significant correlation between area under the first phase of the neurochemical response and locomotor activity for any of the amino acids (all $r^2 < 0.3$, $t = 0.5\text{--}2$, $p > 0.05$; data not shown).

Cross-correlation analysis was performed to determine how well the behavior and neurochemistry coincided in time for each individual. On average, the locomotor response coincided in time with the first phase of the glutamate response (they were separated by less than one 14-s time point). In all animals, the second phase of the neurochemical response lagged behind the peak behavior, by a mean \pm SEM

of 2.5 ± 0.5 min. However, as stated above, the behavioral response was highly correlated with the magnitude of the second phase of the neurochemical response, and not the first phase.

Pilot experiments were performed to determine whether the increased locomotor and neurochemical responses to TMT simply represented a reaction to any novel odor or placing the filter paper in the cage. A separate group of rats ($n = 3$) was presented an almond extract odor for 12 min as above. None of the rats showed hyperactivity or increased burrowing in response to the almond odor and no changes in the levels of any amino acids were detected. Furthermore, hanging a blank piece of filter paper in the top of the cage of two rats elicited no overt behavioral responses or changes in amino acids. These two rats later acted as high responders when a piece of

filter paper with fox odor was hung in the cage, demonstrating both hyperactivity and neurochemical changes.

Tail pinch experiment

After removal of TMT, and once amino acid levels had returned to baseline, all animals were exposed to a tail pinch lasting 4 min. All animals that failed to show any neurochemical response to fox odor (low responders) showed an increase in amino acid levels in response to the tail pinch. This experiment functioned as a positive control, proving that the probes were functioning in the low responders when they were presented with fox odor. Figure 6(a) shows an example of the neurochemical and behavioral reactions to tail

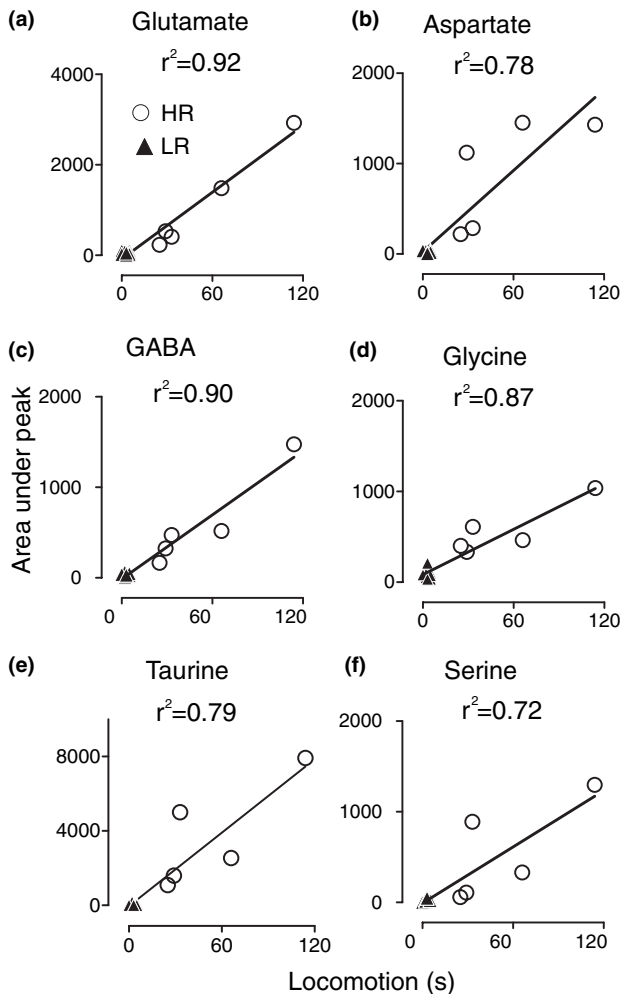


Fig. 5 Correlations between neurochemistry and behavior after fox odor presentation. For the neurochemical data, the area under the second, large peak was computed for percentage basal level versus time graphs (such as in Fig. 4). The cumulative locomotor time between 2 and 4 min was plotted for the behavioral data. (○) HR, high responder; (▲) LR, low responder. The best fit line and r^2 values are given.

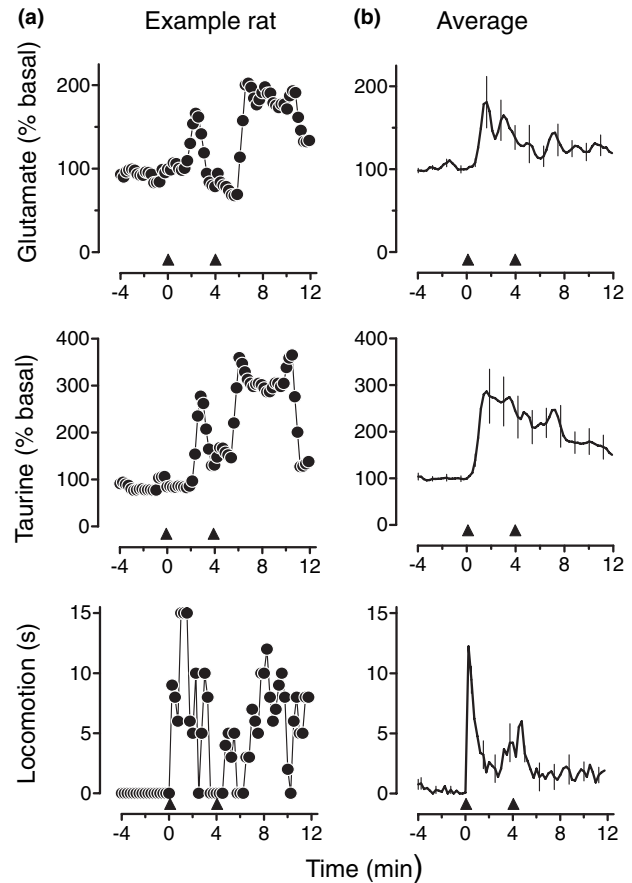


Fig. 6 Tail pinch data. Tail pinch was performed for 4 min starting at time 0 (arrows mark the beginning and end). (a) This rat was a low responder during the fox odor experiment. Locomotion increased during the tail pinch with a corresponding peak in neurochemical levels around 3 min. After the clip had been removed, the animal became excited, and exhibited a more sustained increase in neurochemical levels and locomotor activity. (b) Mean changes ($n = 13$) in glutamate (top panel) and taurine (middle panel) for all animals after the tail pinch. The error bars shown every fifth point represent SEM values. The peak locomotor activity occurred 15 s after the tail pinch was initiated but the peak neurochemical levels were delayed by about 1 min.

pinch of a rat that had no neurochemical response to the fox odor. This same rat showed an increase in glutamate and taurine during the tail pinch period that lasted less than 2 min. The amino acid peaks were slightly delayed relative to the increase in locomotor activity. After the clip had been removed from its tail, the rat had a sustained period of hyperactivity and corresponding neurochemical change.

Figure 6(b) shows the average response to the tail pinch for all animals. Glutamate and taurine levels increased, peaking about 2 min after initiation of the tail pinch. The average shows a more sustained increase rather than a sharp peak because the neurochemical peaks were of short duration and did not always align exactly in time between animals. In all animals, the glutamate peak lagged behind the peak

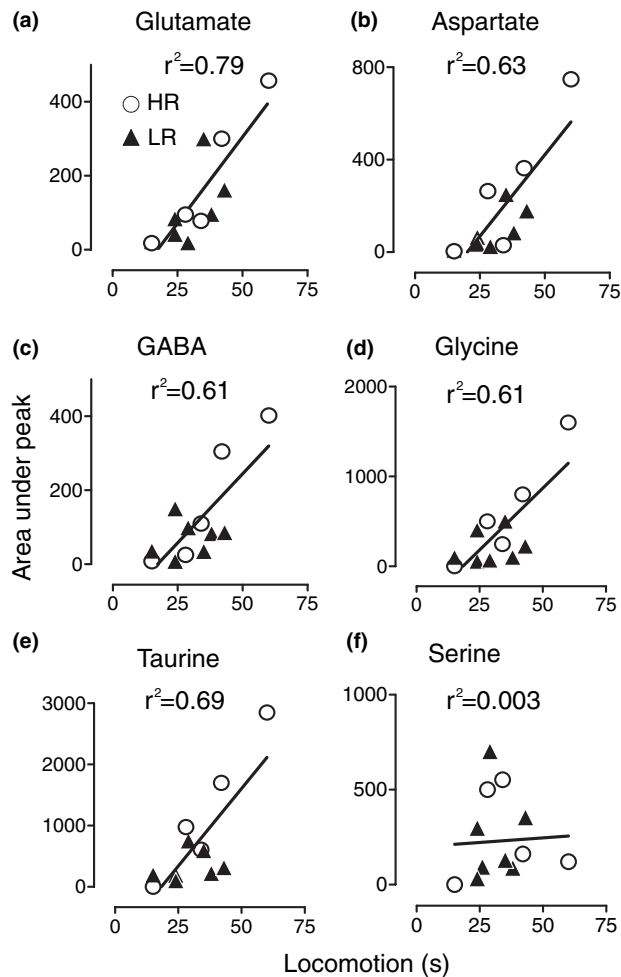


Fig. 7 Neurochemical and behavioral correlations for the tail pinch experiment. The area under the peak was calculated for the time during the tail pinch and plotted against cumulative locomotion in the first 2 min after the tail pinch. There was good correlation between neurochemistry and behavior for (a) glutamate, (b) aspartate, (c) GABA, (d) glycine and (e) taurine but not for (f) serine. (○) HR, high responder to fox odor; (▲) LR, low responder to fox odor. All low responders had some chemical changes in response to the tail pinch.

locomotion. Cross-correlation analysis of the individual animals reveals that the mean \pm SEM of this lag was 1.1 ± 0.2 min. Figure 7 shows correlation graphs for area under the first peak in amino acids (i.e. the peak that fell between 0 and 4 min) and locomotor activity in the first 2 min. The r^2 values were high and the correlation between amino acid changes and locomotion was significant for all amino acids but serine ($r^2 = 0.6-0.8$, $t = 7-11$, $p < 0.01$). The high and low responders from the fox odor experiment are delineated.

Relationship between chemical changes and locomotor activity

To determine whether any stimulus that increases locomotion is sufficient to evoke increased amino acid efflux, we administered cocaine (2 mg/kg, 5-s i.v. infusion) to a different group of drug-naïve animals ($n = 4$). Intravenous cocaine produced a rapid and large increase in locomotor activity (Fig. 8), but this was not accompanied by changes in dialysate concentration of any amino acid.

Effects of temporal resolution on data

Figure 9 shows the effects of improving temporal resolution on the measured signal. Using capillary electrophoresis to achieve temporal resolution of 14 s, a large, transient glutamate peak was observed in response to TMT. To determine what our data would have looked like if we had used the 10-min temporal resolution of traditional micro-

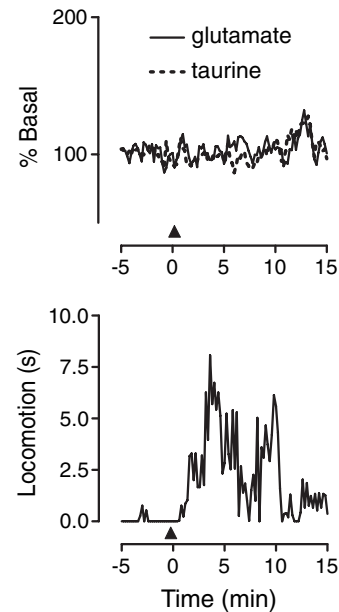


Fig. 8 Chemical and locomotor changes after cocaine administration. Cocaine (2 mg/kg, i.v.) was delivered at time 0 (arrow) by a 5-s infusion to drug-naïve rats ($n = 4$). Locomotor activity was increased for over 10 min but levels of glutamate or taurine were not significantly changed.

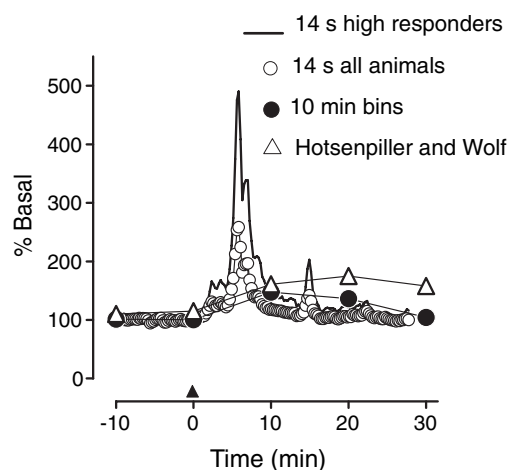


Fig. 9 Effect of temporal resolution of sampling on glutamate data. Capillary electrophoresis detection allows 14-s temporal resolution (solid line, high responders; ○, all animals). When our data for all animals was binned into 10-min samples, the temporal profile became typical of that observed with traditional liquid chromatography detection (●). △, Data from Hotsenpiller and Wolf (2003), who used traditional liquid chromatography detection to measure glutamate after presenting rats with a fox odor (data replotted with authors' permission).

dialysis, the glutamate peak heights were averaged from 40 electropherograms and plotted. For comparison, data are plotted from a previous published study that also used fox odor as a stimulus (Hotsenpiller and Wolf 2003), but with 10-min sampling.

Discussion

The goal of this study was to examine whether differences in amino acid neurotransmission correlate with individual differences in responsivity to a predator odor. Presentation of fox odor produced rapid and transient increases in amino acid neurotransmitters in the nucleus accumbens that were much larger than previously reported. For example, glutamate increased by a mean \pm SEM of $400 \pm 120\%$ after presentation of fox odor. Furthermore, these increases in glutamate peaked and returned to baseline within 3–5 min. Only a subset of animals showed increased extracellular amino acid levels in response to fox odor, and these same animals were the only ones to show behavioral hyperactivity in response to this stimulus. Indeed, an almost linear relationship existed between the magnitude of the change in glutamate and the behavioral reaction to fox odor. However, the peak neurochemical changes lagged the behavioral response by about 2 min. Although the magnitude of the increases in amino acid concentrations correlated highly with locomotor activity ($r^2 = 0.7\text{--}0.9$), behavioral hyperactivity alone was not sufficient to induce changes in amino acids, because

cocaine greatly increased locomotor activity without evoking any neurochemical changes. This work demonstrates that rapid, transient increases in amino acid neurotransmitters in the nucleus accumbens correlate with individual responsivity to a salient stimulus.

Time course of amino acid changes

The changes in amino acid levels observed after presentation of fox odor were much larger and faster than had previously been reported using traditional microdialysis with 10-min sampling (Hotsenpiller and Wolf 2003). To facilitate comparison of our results with previous studies, we averaged our 14-s samples to show what our results would have looked like with 10-min sampling (Fig. 9). This produced a temporal profile of changes in glutamate that was highly consistent with that reported by Hotsenpiller and Wolf (2003), demonstrating that the difference in our results is due to temporal resolution and not other experimental differences. In addition to the greater magnitude of change, the higher temporal resolution revealed a biphasic change in amino acid levels after fox odor that was not apparent with slower sampling. Tail pinch also rapidly increased amino acid concentrations. Our results are similar to those in the only other report of glutamate changes during a tail pinch in the nucleus accumbens, which was performed using capillary electrophoresis with 30-s microdialysate sampling (Rada *et al.* 2003). We suspect that this phenomenon had not been reported earlier because the effect is small and would be difficult to detect when averaging samples over 10 min.

Capillary electrophoresis allowed the quantification of multiple amino acids simultaneously, revealing that levels of glutamate, aspartate, GABA, taurine, serine and glycine all increased in response to fox odor or tail pinch. This indicates that the brain's response to these stimuli is complex, resulting in many neurotransmitters being activated. Amino acids in the brain extracellular fluid can be derived from neurons and glial cells. Without knowledge of the cellular source of the changes observed here, it is not possible to reach firm conclusions about the mechanisms underlying these changes. It is possible that the change in glutamate represents a primary response, perhaps from activation of corticostriatal pathways, that causes the changes in other amino acids; however, the temporal resolution of our measurements (14 s) is not sufficient to ascertain whether the increase in glutamate preceded that of the other neurotransmitters. In support of this hypothesis, previous microdialysis studies have shown that raising the endogenous glutamate level can also increase GABA and taurine concentrations (Del Arco and Mora 1999; Del Arco *et al.* 2000). Furthermore, glutamate neurons synapse on to GABA interneurons and the primary source of GABA in the accumbens is local. Although all of the neuroactive amino acids showed dynamic concentration changes, the selectivity of the response is reflected in the differences in magnitude and time course for the amino acids

(Fig. 4). For example, the magnitude of serine changes was smaller than that of glutamate, and serine levels did not correlate with behavior following tail pinch. In addition, glutamate and GABA levels rose and fell quickly, whereas taurine concentrations took much longer to return to basal levels. These differences in time course might result from tighter regulation of glutamate and GABA levels or may be due to the role of taurine in regulating osmolarity (Pasantes-Morales and Schousboe 1997). Similarly, *in vitro* studies have shown that GABA and aspartate release and clearance are much faster than those of taurine when a brain slice is exposed to a hypo-osmotic solution (Pasantes-Morales *et al.* 2004).

Correlation between neurochemistry and behavior

Our studies indicate a strong correlation between extracellular amino acid levels and behavioral activation after salient stimuli such as TMT. However, by administering cocaine to some animals we showed that increased locomotion itself is not sufficient to cause a change in amino acid levels. These results are consistent with other studies that found no changes in glutamate in the nucleus accumbens following the acute administration of moderate doses of cocaine (Smith *et al.* 1995; Reid and Berger 1996). Therefore, it is likely that a psychological response causes both the behavioral hyperactivity and the neurochemical changes. Whether this emotional response after TMT is fear is debated. Some believe that TMT is simply an aversive noxious odor, because a rat's response to TMT does not respond to anxiolytic drugs or support conditioning (Blanchard *et al.* 2003; Dielenberg and McGregor 2001). Others have reported that TMT increases corticosterone production (Day *et al.* 2004) and dopamine metabolism (Morrow *et al.* 2000), similar to responses evoked by conditioned fear. Pilot studies using an almond odor indicated that the response we observed is not universal to all novel odors.

Responses to salient stimuli have wide, natural variation and understanding the neural substrates underlying these individual differences might aid in the treatment of anxiety (Prasad *et al.* 1996). Rodents can be screened for anxiety using a number of behavioral tests, such as an elevated maze and response to novelty. High and low responses to TMT have been observed in CD-1 mice (Hebb *et al.* 2004) and the high responders to TMT also exhibited a high level of anxiety during a light-dark test. In these high-responder mice, significantly more fos-related antigen cell counts were detected in the accumbens shell after exposure to fox odor. Our study extends these observations by showing differential neurochemical changes in the accumbens of rats with a high level of response to TMT that accompanies high-anxiety behaviors.

One interesting finding from this study is that the peak in amino acid levels lagged the behavioral changes produced by the fox odor by 2 min. We have shown previously by microdialysis coupled to capillary electrophoresis that phar-

macologically and electrically stimulated increases in extracellular amino acids occur immediately upon stimulation (Lada *et al.* 1998; Bowser *et al.* 2001); therefore, the delay is not a measurement artifact of the technique. Although the first phase of the biphasic amino acid change occurred around the time that high responders began to exhibit hyperactivity, there was no correlation between the magnitude of this increase (i.e. the area under the first phase of the amino acid response) and the peak in locomotion. Instead, there was a high degree of correlation between locomotor activity and the area under the curve of the second phase of the neurochemical response.

With the temporal resolution of conventional microdialysis, the chemical and behavioral changes would appear to occur simultaneously. However, the different temporal profile for neurochemical and behavioral changes revealed by rapid sampling indicates that the neurochemical changes are secondary to the behavioral changes and might be caused by the behavioral changes. Indeed, the temporal resolution of our measurements demonstrates that the neurochemical increases clearly occurred while the stressor was present (i.e. during the odor presentation or tail pinch), but were delayed with respect to peak behavioral activity. In addition, animals that had prolonged behavioral activation also demonstrated a prolonged neurochemical response that started before the hyperactivity subsided (see Fig. 3b). It is therefore unlikely that the initial increase in amino acids is attributable to relief from danger, as suggested by Saulskaya and Marsden (1995b). Instead, the amino acid changes probably represent a response to a psychological state, such as stress or fear, caused by the stimuli. Glucocorticoids are also released during stress and glucocorticoids can rapidly (within minutes) cause release of excitatory amino acids (Makara and Haller 2001). It is possible, therefore, that the delayed increase in glutamate is a secondary effect, triggered by release of stress hormones such as corticosterone.

The dynamic extracellular amino acid changes could have many functions. Extracellular glutamate acts as a neuromodulator, activating receptors and modulating synaptic transmission, even if the release is not of neuronal origin (Baker *et al.* 2002). Indeed, the rapid increases in glutamate levels we observed may contribute to a delayed dopamine release after salient stimuli. Previous studies have shown a delayed, prolonged (tens of minutes) increase in dopamine in the accumbens after a conditioned emotional response (Saulskaya and Marsden 1995a), exposure to red fox urine (Bassareo *et al.* 2002), or amphetamine (Xue *et al.* 1996) that is inhibited by NMDA receptor antagonists. The glutamate release in response to salient stimuli may also be responsible for local memory processes in associative fear learning because NMDA receptor antagonists inhibit dopamine release prevent acquisition of a conditioned emotional response (Saulskaya and Marsden 1995b). In addition, extracellular glutamate changes after stressful stimuli activate

reward circuits similar to drugs of abuse. For example, McFarland *et al.* (2004) have shown an increase in glutamate in the nucleus accumbens core during a footshock used to reinstate cocaine-seeking behavior, and that inactivating the accumbens core or shell blocks footshock-induced reinstatement. Thus, the increases in glutamate observed after salient stimuli are consistent with a role for glutamate in stress-related drug relapse.

Conclusions

We found that fox odor and tail pinch produced rapid and transient changes in amino acid neurotransmitters in the nucleus accumbens. These results show that extracellular amino acid changes in response to external, salient stimuli are larger and more dynamic than previously observed. In addition, these neurochemical changes were highly correlated with individual differences in behavioral responsivity to these stimuli. The timing of the peak amino acid responses suggests that they are secondary to behavioral activation and are evoked by a psychological state. The improved temporal resolution of microdialysis measurements allows a better correlation of specific behavioral and neurotransmitter changes, and might aid our understanding of the neurotransmitter control of a variety of behaviors.

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